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ORIGINAL ARTICLE

L-Tetrahydropalmatine enhances the sensitivity of human ovarian cancer cells to cisplatin via microRNA-93/PTEN/Akt cascade

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Summary

Purpose: *To evaluate the effect of L-Tetrahydropalmatine (L-THP) on the sensitivity of a cisplatin resistant ovarian cancer (OC) cell line. As miR-93 is reported to be overexpressed in OC and cisplatin resistance, we also evaluated its pathway in OC.*

Methods: *The levels of miR-93 were evaluated using RT-PCR and Luciferase assay was performed to confirm the target of miR-93. The extent of apoptosis was evaluated by Annexin V and propidium iodide (PI) staining, whereas Hoechst 33258 staining was done for identifying the number of apoptotic cells.*

Results: *The cisplatin-resistant A2780/DDP cell line showed lower survival rate compared to control when incubated with L-THP along with cisplatin. L-THP caused G0/G1 cell cycle arrest and increased the sensitivity to cisplatin. Furthermore, we found that the levels of miR-93 in cisplatin-resistant cells were highly expressed compared to parental cells. L-THP suppressed the expression of miR-93 and increased the levels of PTEN, a crucial tumor suppressor in OC. It was further observed that the cells transfected with PTEN siRNA showed increased survival compared with the control group and this phenomenon could be reversed by the AKT inhibitor Tricirbine. The A2780 cells treated with PTEN siRNA showed similar survival rate to the cells with miR-93 overexpression.*

Conclusion: *The findings of this study suggested L-THP increased the sensitivity of ovarian cancer cells to cisplatin via modulating miR-93/PTEN/AKT pathway in A2780/DDP ovarian cancer cell line.*

Key words: *cisplatin resistant cells, L-Tetrahydropalmatine, ovarian cancer*

Introduction

Ovarian cancer (OC) is considered as the deadliest cancer among all other gynecological malignancies [1]. It is reported to affect approximately 0.2 million women yearly all over the world and alone accounts for about 0.125 million deaths annually [2]. OC produces fewer symptoms in early stages and progresses easily to advanced stage; the patients usually undergo surgery for the removal of tumor and receive chemotherapy which includes platinum derivatives. Most of the OCs are initially sensitive to chemotherapy; however, recurring tumors are reported to be more aggressive, metastatic and acquire resistance to chemotherapy [3]. Hence there is an urgent need to explore newer molecules that can combat resistance and increase the sensitivity towards conventional chemotherapeutic agents.

L-Tetrahydropalmatine (L-THP) is a naturally occurring tetrahydro protoberberine isoquinoline alkaloid obtained mainly from genera *Stephania*

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and Corydalis [4]. Clinically, L-THP is important in Chinese clinical practice for more than 40 years [5]. The compound has multiple pharmacological activities which also include anticancer effects. A number of published reports suggests that L-THP exerts anticancer effect against breast cancer MCF-7 cell line by altering the uptake of $^{99}Tc^{m}$-MIBI [6]. L-THP enhances the sensitivity of doxorubicin in leukemia EU-4 cell line by down-regulating X-linked inhibitor of apoptosis protein (XIAP) [7]. L-THP has been reported to exert antiproliferative activity in combination with berberine against MDA-MB-231 breast cancer cells [8].

The class of isoquinoline alkaloids is reported to show potential effect against a number of human cancer cell lines [9-12]. The alkaloid interacts mainly with DNA [13] and causes also apoptosis and cell cycle arrest in some cancer cell lines [12-14]. The isoquinoline alkaloids also cause G2/M cell cycle arrest and apoptosis *in vivo* [15]. In a study reported earlier, the isoquinoline alkaloid berberine induced cell cycle arrest in G1 phase and caused caspase-3-dependent apoptosis [16].

Based on these studies, we hypothesized the potential anti-tumor effect of L-THP against OC. In the current study, we evaluated this hypothesis against an OC cell line and the outcomes revealed that L-THP, when combined with cisplatin, increased the antitumor effect of this drug via miR-93/PTEN/AKT pathway.

Methods

Culture conditions and cell lines

A2780 human ovarian cancer cell line was obtained from the Linyi People's Hospital, Shandong Province, China. The cisplatin-resistant cell line A2780/DDP was developed as discussed earlier [17] in which the cells were subjected to progressive higher concentrations of cisplatin. The cells were cultured in RPMI medium with 10% fetal bovine serum (FBS), streptomycin (100 µg/ml) and penicillin (100 units/ml) and were then incubated in humid conditions along with 5% CO_2 at 37°C.

Assay for cell viability

For the study of cell viability, both cisplatin-sensitive and -resistant cell lines were seeded in 96-well plates at 1×10^5 cells per well. When the cells reached 80% confluence, they were exposed to DMSO (serving as control, 0.1 % v/v), various concentrations of L-THP alone, cisplatin (1-16 µg/ml), or L-THP and cisplatin in combination. The number of viable cells was determined after 72 hrs of these treatments using MTT assay as described earlier [18]. Briefly, the cells were incubated with MTT solution (20 µl) for 4 hrs, followed by addition of DMSO (150 µl) for dissolving the formed crystals of formazan. The optical density was measured at 490 nm wavelength using a microplate reader.

RT-PCR study

RT stem-loop PCR analysis was performed to detect the levels of miR-93. The RT-PCR primers were obtained from Sangon Bio. Co. Shanghai, China. One-Step SYBR Prime script miRNA RT-PCR kit (Takara, Bio, Shiga, Japan) was used for RT-PCR. A RT-PCR Detection System CFX-96 (Bio-Rad, USA) was utilized to analyze the expression of mature miR-93 in cancer cells and the expression of U6 was used as control.

Transfection of A2780/DDP cells with miR-93 inhibitor and mimic

About 5×10^5 A2780/DDP cancer cells in 6-well plates were cultured and allowed to reach a confluence of 70-80%. The A2780/DDP cells were then transfected with human miR-93 inhibitors or its negative control (NC) at 50 nmol/l with Lipofectamine™ 2000 Transfection Reagent (40 nmol/l) (Thermo Fischer, USA) in serum free medium. The sequences of both miR-93 mimic and inhibitor or negative control are presented in Table 1.

Apoptosis studies by annexin V and propidium iodide staining

After treating with L-THP, cisplatin and their combination, the cells were trypsinized and washed with phosphate buffer saline (PBS) and resuspended in 10mM Hepes binding buffer. The cells were then stained using Annexin V antibody (5 µl) conjugated with FITC and PI (5 µl) for 25 min in the dark at room temperature. Then, the cells were analyzed by FACS caliber flow cytometer (Becton Dickson, USA) using CellQuest Research software. Early apoptosis was identified as Annexin V-positive and PI negative cells. The extent of apoptosis was calculated as the ratio of the number of apoptotic cells to the total number of cells for each kind of treatment (L-THP, cisplatin and their combination).

Luciferase assay

To construct luciferase reporter vector, the binding sites for miR-93 in the phosphatase and tensin homolog

Table 1. Sequences of both miR-93 mimic and inhibitor or negative control

Sr. no.	*Target*	*Sequence*
1	miR-93 inhibitor	CUACCUGCACGAACAGCACUUUG
2	Negative control inhibitor	CAGUACUUUUGUGUAGUACAA
3	miR-93 mimic	CAAAGUGCUGUUCGUGCAGGUAG
4	Negative control mimic	UUCUCCGAACGUGUCACGUTT

(PTEN) 3-UTR fragment were amplified by PCR using the reverse primers. The amplified proteins were then inserted in the luciferase gene psiCHECK2 vector, named as PTEN 3'-UTR-wild. Phusion Site-Directed Mutagenesis Kit (ThermoFisher) was used to mutate the miR-93 target site in 3'-UTR of PTEN, and the resulting construct was named as PTEN 3'-UTR-mut. Cells were transfected with miR-93 mimics and with either PTEN 3'-UTR-mut or wild. The Luciferase assay was done 48 hrs post transfection using Luciferase Reporter Gene Detection Kit (Sigma, USA).

Immunoblotting studies

The A2780/DDP cisplatin-resistant cancer cells were washed with ice-cold PBS twice, followed by lysis using lysis buffer (Sigma, USA). The SDS-PAGE gels (8%) were loaded with cell lysates and were transferred to polyvinylidene difluoride (PVDF) membranes. The blots were incubated with I^{ry} antibodies PTEN, phosphorylated AKT1, AKT1 and the GAPDH (Santa Cruz, USA) for 12 hrs at 4°C. Enhanced chemiluminescence was used for detecting bands in accordance to manufacturer's instructions (Bio-Rad, California, USA). The bands were normalized to GAPDH.

Transfection of A2780 cells with siRNA

A2780 cells were transfected with PTEN siRNA and negative control siRNA by PTEN siRNA kit (Cell Signaling Techno, Denvers, USA) in accordance to the manufacturer's protocol. Fluorescence microscopy was used to count the percentage of fluorescein-labeled cells. The cells were evaluated for viability and the expression of PTEN and pAKT using western blot.

Statistics

The statistical analysis was carried out using GraphPad Prism 6 software. All the data are presented as mean±SD. Student's t-test was performed for comparisons between groups and Scheffe test was used for multiple comparisons. Statistical significance was set at $p<0.05$.

Results

Antiproliferative effect of L-THP against A2780/DDP ovarian cancer cells

MTT assay was performed to confirm the cisplatin-resistant behavior of A2780/DDP cell line by comparing cisplatin-sensitive A2780 parental cell line. The results of MTT assay showed that the IC50 values (50% inhibitory concentration) of cisplatin were at least four times higher in A2780/DDP versus parental A2780 cells (Figure 1A). A dose-dependent growth inhibitory effect was shown by L-THP alone (0,50,100,150 and 200 µM) on the selected ovarian cell line (Figure 1B). L-THP at 50 µM did not exhibit any notable effects on cell growth and exhibited antiproliferative effect at concentra-

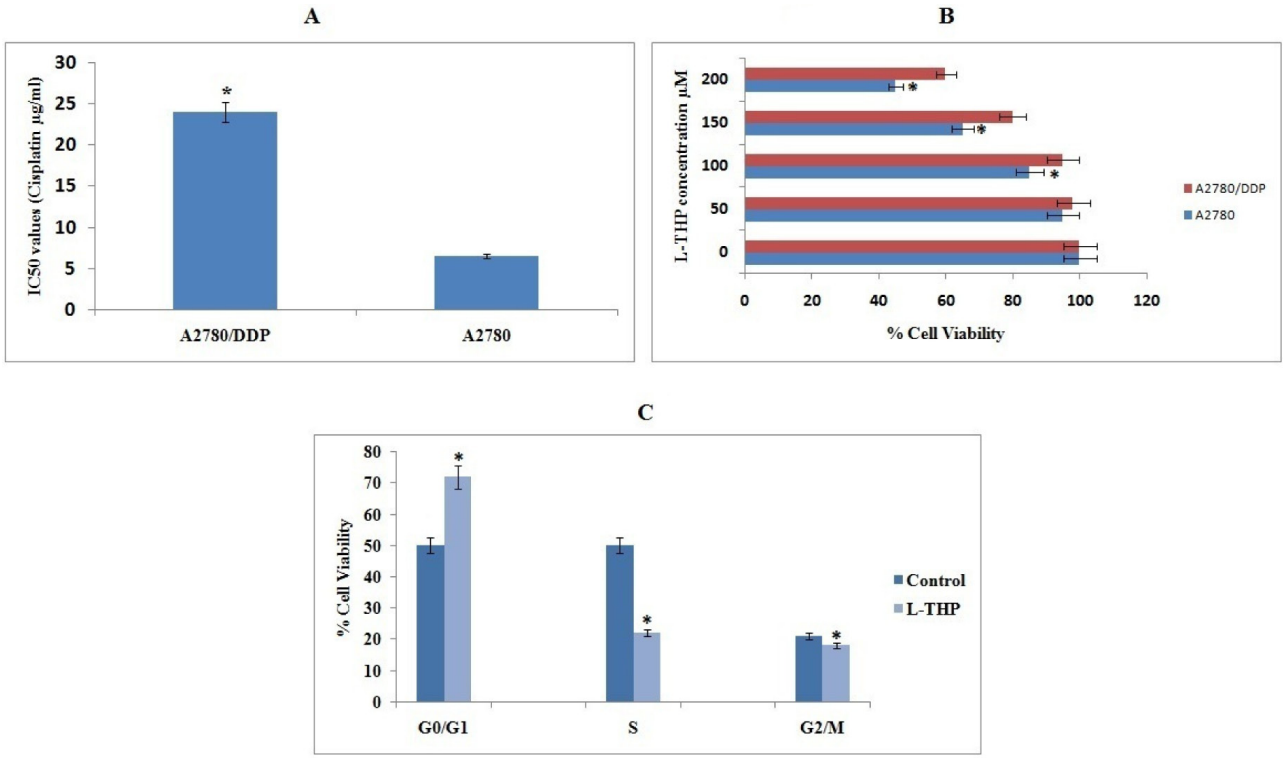

Figure 1. Antiproliferative effect of L-THP on ovarian cancer cells. **A:** For IC50 values by MTT assay, the cisplatin resistant A2780/DDP cells were treated with different concentrations of cisplatin for 48h (*$p<0.05$ compared to A2780 parental cells). **B:** MTT assay was done for cell viability, and the cells were exposed to L-THP at various doses. **C:** Flow cytometer study was done for cell cycle analysis (*$p<0.05$ compared to control).

tions 100 μM or above. Cell cycle analysis showed that L-THP increased the numbers of cells at G0/G1 phase versus the negative control (Figure 1C).

L-THP increases the sensitivity of ovarian cancer cells towards cisplatin

A non-cytotoxic concentration of L-THP (50 μM) which would exert minimal effect on cell growth was chosen for the sensitization studies. In order to study the effect of L-THP, the cancer cells were incubated with cisplatin at different concentrations (2, 4, 6, 8 and 10 μg/ml) in the absence or presence of L-THP (50 μM). The results suggested that at 6 μg/ml concentration the cisplatin-resistant A2780/DDP cells which were incubated along with L-THP showed significant reduction in survival compared to cells receiving only cisplatin ($p<0.05$) (Figure 2A). This suggested that L-THP increased the sensitivity of A2780/DDP cells to cisplatin. The activity was almost doubled as shown by the mean apoptotic population of A2780/DDP cells, when comparing the cells treated in combination and cisplatin (6 μg/ml) alone (Figure 2B;Figure 3A). The results of Hoechst 33258 staining also showed that combining L-THP with cisplatin resulted in increased cisplatin-induced apoptosis in cisplatin resistant cell lines (Figure 3 B). The results clearly indicated the role of L-THP in increased the inhibitory effect when combined with cisplatin on cisplatin resistant cells.

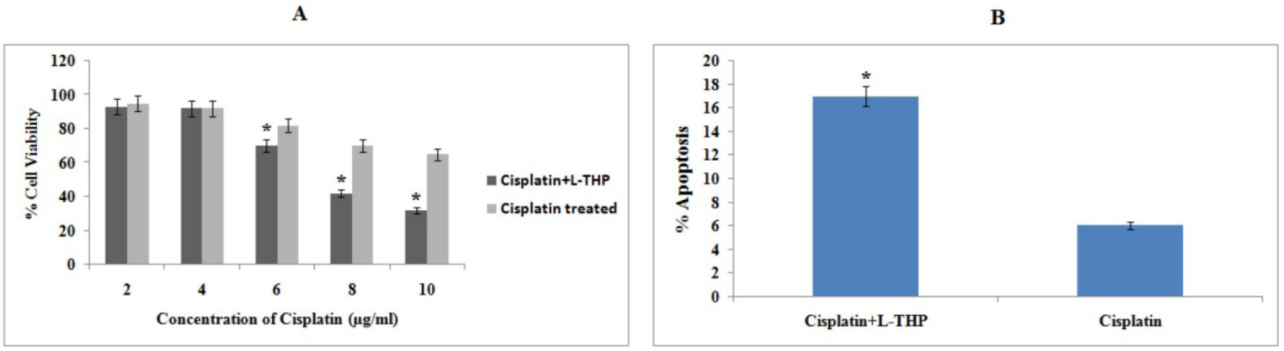

Figure 2. L-THP enhances sensitivity of ovarian cancer cells to cisplatin. **A:** effect of L-THP treatment on cisplatin mediated cytotoxicity. **B:** Cell apoptosis in A2780/DDP cells exposed to cisplatin alone or in combination with L-THP. *P<0.05 compared to cells treated with cisplatin only.

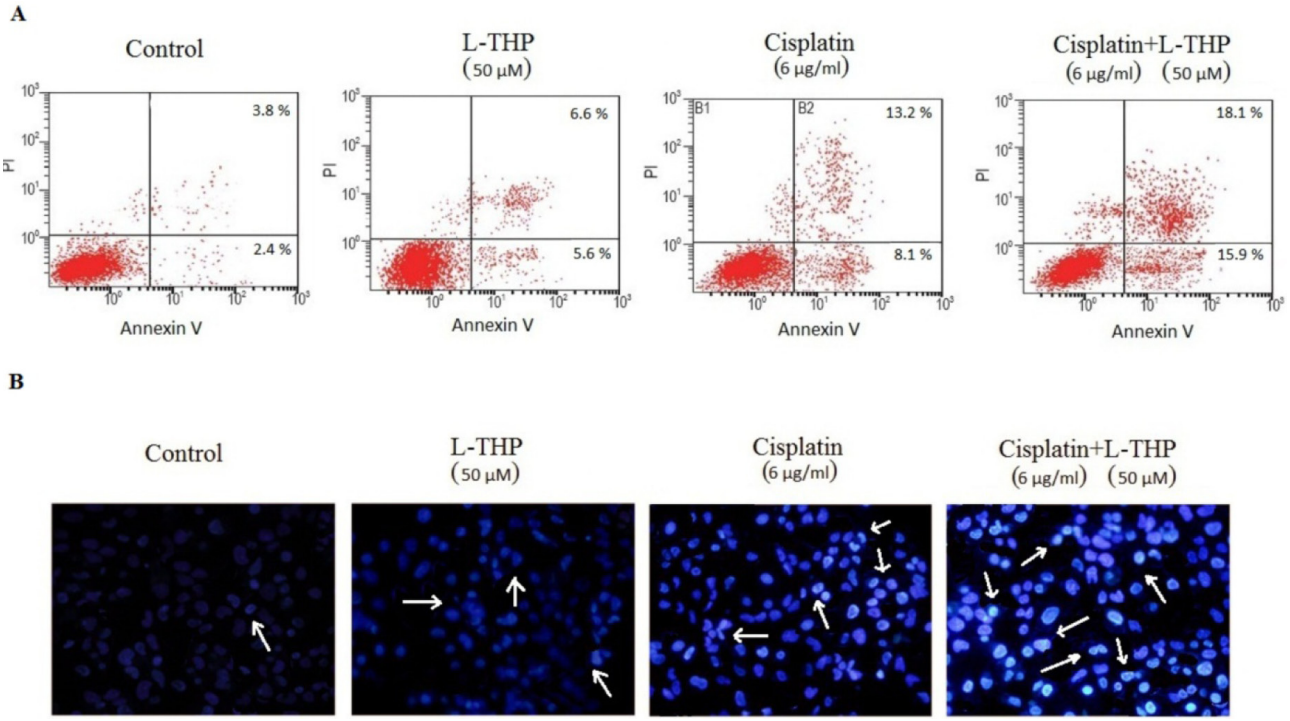

Figure 3. Cell apoptosis studies. **A:** Annexin V and PI assay for A2780/DDP cells treated with cisplatin alone and in combination with L-THP. **B:** apoptosis studies Hoechst 33258 staining. The arrows show Hoechst positive cells.

Inhibitory effect of L-THP on the levels of miR-93 in A2780/DDP

We further investigated the involvement of miR-93 in L-THP mediated increase in cisplatin-mediated cytotoxicity. The results showed that miR-93 levels were higher in cisplatin-resistant cells compared to parental A2780 cells. The levels of miR-93 decreased remarkably followed by L-THP treatment (Figure 4A). The study was further extended to investigate the effects of miR-93 in miR-93 mimics transfected A2780 cells for cisplatin mediated cytotoxicity. The results demonstrated significant elevation in the expression of miR-93 ($p<0.05$) (Figure 4B) in cells transfected with miR-93 mimics. When the miR-93 mimics transfected parental A2780 cells a prominent survival advantage was shown compared to negative control (Figure 4C, $p<0.05$), which suggested that elevated expression of miR-93 modifies the sensitivity of A2780 cells against cisplatin.

PTEN is regulated by miR-93

Online software TargetScanHuman was utilized for verifying the exact molecular mechanism of miR-93 in the sensitization against cisplatin. The 3'-UTR of PTEN mRNA showed the site for miR-93, hence we speculated that PTEN is a direct target of miR-93 in ovarian cancer. To confirm our hypothesis, we transfected A2780 cells with miR-93 mimics and PTEN 3'-UTR-wild or mut Luciferase reporter construct. A significant decrease ($p<0.05$) in the activity of PTEN 3'-UTR-wild reporter by miR-93 was observed (Figure 5A), however the miR-93 did not affect the Luciferase reporter vector with mutation in PTEN 3'-UTR. The outcomes of immunoblotting and RT-PCR studies confirmed that miR-93 mimics suppressed the levels of mRNA and protein of PTEN compared to NC group (Figures 5 B, C and D). All the experiments suggested that miR-93 regulates PTEN directly.

Confirmation of PTEN/AKT pathway as mechanism for cisplatin resistance

In order to confirm the involvement of PTEN in the mechanism of resistance, the A2780 cells were transfected with PTEN siRNA after the cells were treated with different doses of cisplatin. The results suggested that PTEN siRNA decreased PTEN levels (Figure 5B) and enhanced the phosphorylated AKT/total AKT ratio, which could be reversed by

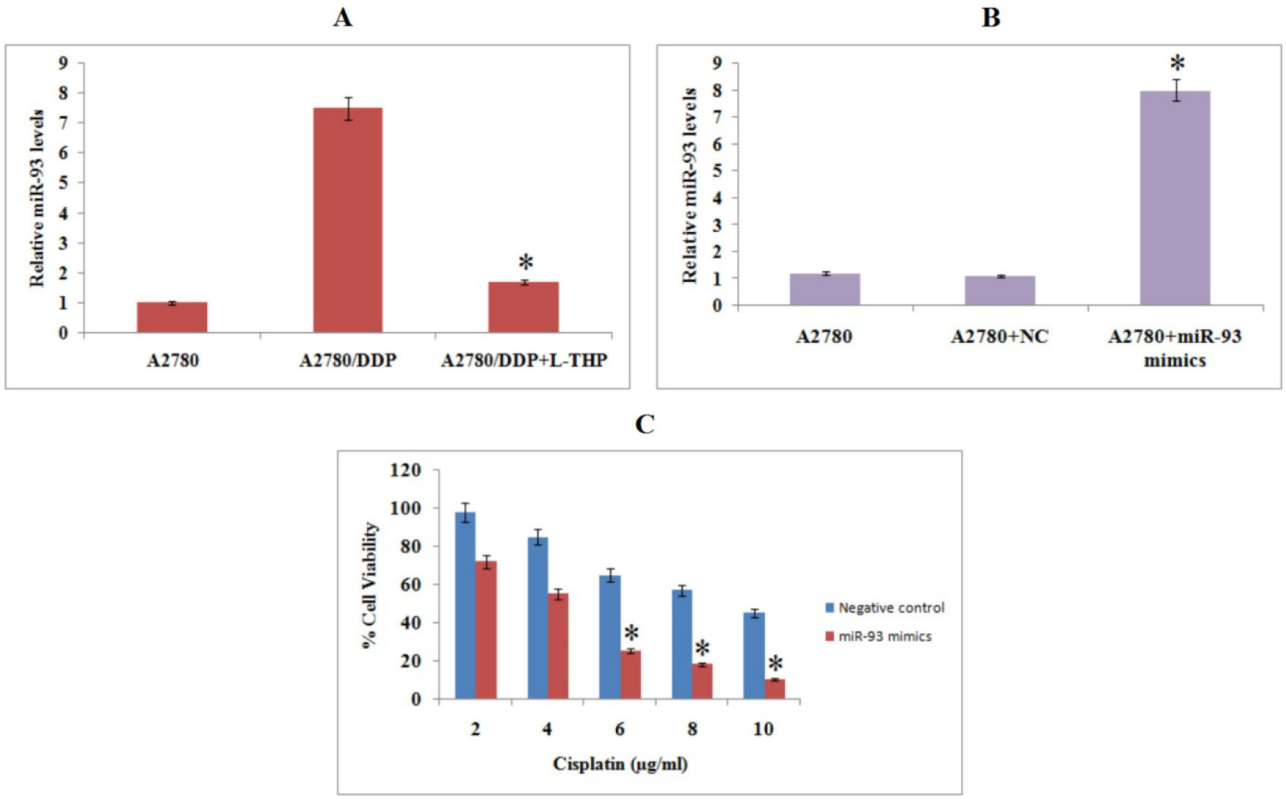

Figure 4. Expression of miR-93 is responsible for resistance in A2780/DDP cells. **A:** The relative miR-93 levels were highly expressed in A2780/DDP cells ($p<0.05$ compared to A2780/DDP L-THP untreated cells). **B:** The expression of miR-93 increased in A2780 cells transfected with miR-93 mimics (*$p<0.05$ compared to A2780 NC group). **C:** The cells after transfection with miR-93 mimics were exposed to various doses of cisplatin (*$p<0.05$ compared to negative control).

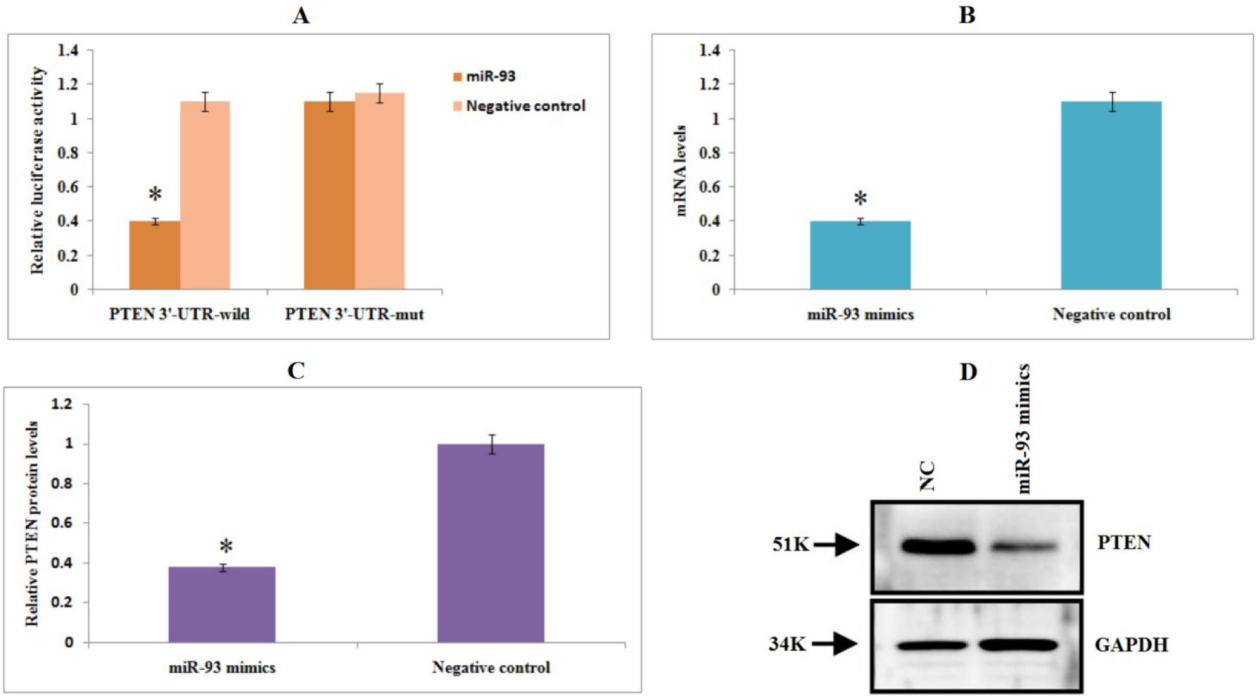

Figure 5. PTEN was found to target of miR-93. **A:** Decrease in luciferase activity of PTEN 3'-UTR-wild reporter was mediated by miR-93 (*p<0.05 compared to PTEN 3'-UTR-mut miR-93 transfected cells). **B:** The PTEN mRNA levels decreased by overexpression of miR-93 (*p<0.05 compared to negative control). **C,D:** The western blot suggested PTEN protein levels decreased after overexpression of miR-93 (*p<0.05 compared to negative control).

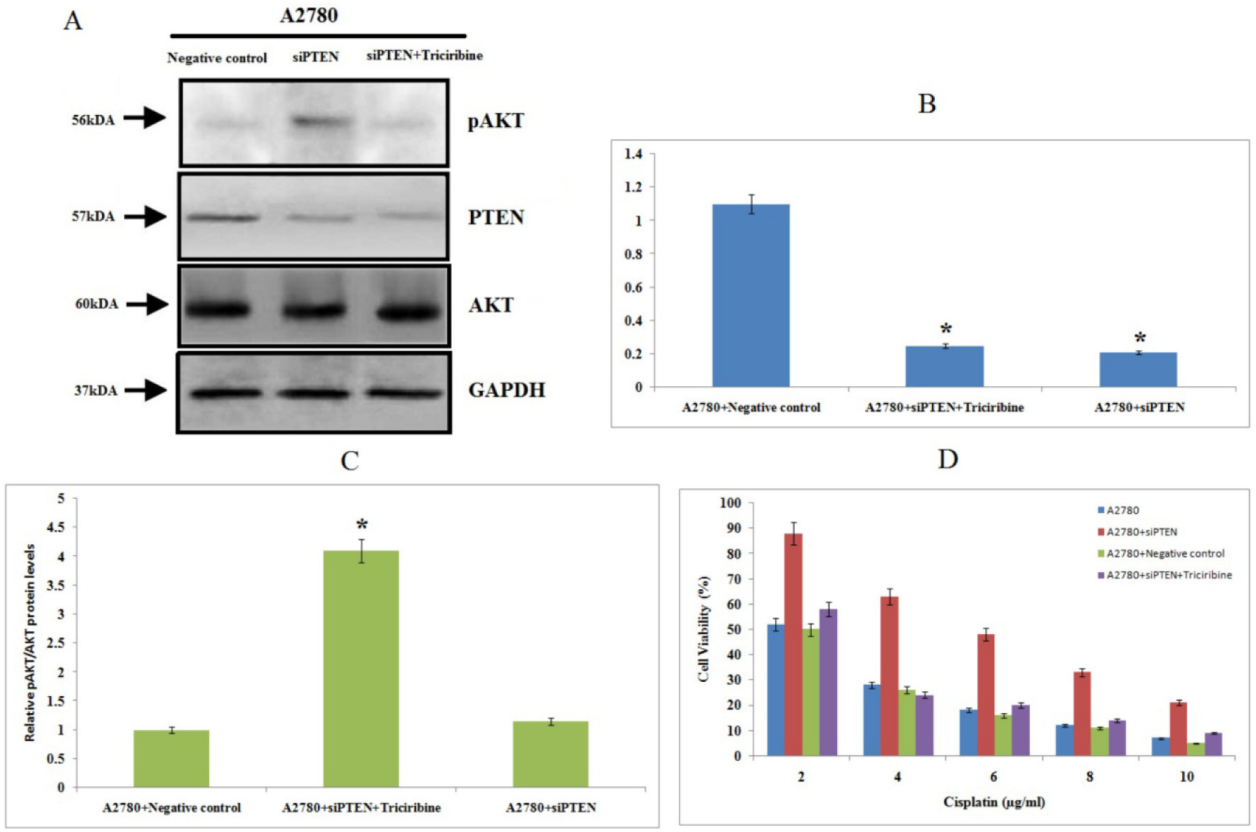

Figure 6. PTEN/AKT is an important pathway responsible for cisplatin resistance in A2780/DDP cells. **A,B:** The protein levels of PTEN reduced after transfecting with PTEN siRNA. **C:** The ratio of phosphorylated AKT/total AKT increased in cells transfected with PTEN siRNA, and the effect was reversed by Triciribine (*p<0.05). **D:** Cell survival increased significantly in cells transfected with PTEN siRNA versus the negative control group, and the effect was party reversed by Triciribine. P<0.05 compared to negative control group.

Triciribine treatment (Figures 6 A,B,C). The studies further showed increased survival of A2780 cells as compared to NC after treatment with PTEN siRNA which could be reversed by the AKT inhibitor Triciribine (Figure 6D). Overall, it was observed that the cells treated with PTEN siRNA exhibited similar survival rate to the cells with overexpression of miR-93. This finding confirmed miR-93 conveys resistance by PTEN/AKT pathway.

Discussion

Studies have suggested that miRs modulate the chemoresistance of cancer cells partially via inducing defective apoptosis [19-22]. In the present study, we discovered that L-THP increased the sensitivity of A2780 parent and resistant OC cell lines by decreasing the levels of miR-93.

Platinum derivatives are the drugs of choice for treating OC because they show high efficacy, but this efficacy in many cases does not last and tumor cells develop resistance which leads to disease progression [23]. This necessitates understanding the mechanism of resistance for treating the cancer successfully. MiRs are reported to act as regulators in a number of cellular biological processes, such as apoptosis, proliferation, metastasis, differentiation and drug resistance [24,25]. Different types of miRs (miR-141, miR-451, miR-214, miR-21 and miR-23a) are reported to be associated with resistance to platinum derivatives in various cancers [26-28]. Accumulating evidence shows overexpression of miR-93 in OC cell lines resistant to cisplatin, such as OVCR3 and SKOV3 cell lines [17]. The results of this study were more or less similar to previous studies; miR-93 was overexpressed in A2780/DDP cancer cell line, but not in its parental strain, suggesting the involvement of miR-93 in cisplatin resistance. L-THP increased the sensitivity of A2780/DDP cells to cisplatin via decreasing expression of miR-93.

Studies have been suggesting the involvement of phosphatase and tensin homolog (PTEN) in drug resistance. Earlier researches involving the chemoresistant cell lines HL60AR and EU21 suggested that transfection with PTEN resulted in increased chemosensitivity [29-31]. Working on the same line, our study found that PTEN was a preferable target of miR-93 which is involved in cisplatin resistance in A2780/DDP cancer cells. The experiments suggested that decrease in PTEN causes increase in cell survival.

This study confirmed the role of miR-93 in obstructing the translation of PTEN and inducing activation of AKT. We found that miR-93 played a driving role in increasing the chemosensitivity of A2780/DDP cisplatin resistant cell line. Our findings were in accordance with earlier reports which supported the role of miR-214, a member of miR family proteins responsible for cisplatin resistance in OC cell lines [32,33].

In conclusion, our experiments confirmed that L-THP plays an inhibitory role on the expression of miR-93 and could increase the expression of PTEN in cisplatin-resistant A2780/DDP cell line. We demonstrated a driving role of miR-93 in conveying cisplatin resistance. According to our study, the PTEN/AKT pathway may be downstream of miR-93 mediated resistance against A2780/DDP cells and may provide a new target for effective treatment of resistant cases of OC.

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Conflict of interests

The authors declare no conflict of interests.

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