

Research Article

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microRNA-1236-3p regulates DDP resistance in lung cancer cells

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Keywords: Drug resistance; Lung cancer; microRNA-1236-3p; TPT1 gene; Pim-3 signaling pathway

Abstract: Lung cancer is a malignant tumor leading to the most cancer-related deaths worldwide. The treatment efficiency of lung cancer remains poor mainly due to chemotherapy drug resistance, including cisplatin. MicroRNAs (miRNAs) are closely related to chemotherapy resistance of tumor cells. Here, we illustrated the underlying mechanism of miR-1236-3p on the DDP resistance in lung cancer cells. In this study, we found that the expression level of miR-1236-3p was significantly decreased in lung cancer tissues and A549 cell line. In addition, the half maximal inhibitory concentration (IC₅₀) of DDP in A549 cells was significantly lower than that in A549/DDP cells, while the expression level of miR-1236-3p was prominently down-regulated in A549/DDP cells. Combining the online tool TargetScan and a dual-luciferase reporter assay, tumor protein, translationally-controlled 1 (TPT1) was proved to be the direct target gene of miR-1236-3p. The MTT and flow cytometry assays demonstrated that up-regulation of miR-1236-3p could markedly inhibit A549/DDP cell proliferation but promote apoptosis, which could be significantly reversed by pcDNA3.1-TPT1 plasmids. Finally, we further demonstrated that miR-1236-3p could restrain the expression levels of TPT1, Pim-3, phosphate-Bcl-2-associated death promoter (p-BAD) and B-cell lymphoma-extra large (Bcl-XL) in A549/DDP cells, while the inhibition could be reversed by pcDNA3.1-TPT1 as well. In a word, our study demonstrated that miR-1236-3p could reverse DDP resistance by modulation of TPT1 gene and inhibition of Pim-3 signaling pathway in lung cancer cells.

1 Introduction

Lung cancer is one of the most common malignant cancers leading the most cancer-related deaths around the world [1]. In China, lung cancer causes about 23% of deaths annually among men, and the overall five-year survival of lung cancer patients remains under 17% [2]. Besides surgical excision, platinum-based chemotherapy such as cisplatin (DDP) is one of the most effective ways concerning various cancer treatments, including lung cancer [3]. However, the long-term and continuous infusion of DDP frequently results in the drug resistance, causing treatment interruption or failure [4]. Therefore, it is urging to discover the underlying mechanisms of DDP resistance in lung cancer cells in order to improve chemotherapy efficiency.

MicroRNAs (miRNAs) are small, non-coding regulatory RNAs containing 21-25 nucleotides, functioning as critical regulators of post-transcription gene expression [5,6]. Accumulating evidences proved that dysregulation of miRNAs was involved in regulating cancer cell development and drug resistance in various tumors, such as lung cancer [7], breast cancer [8], gastric cancer [9] and et al. Previous studies reported that up-regulation of miR-1236-3p could significantly inhibit lung cancer cell proliferation, migration and invasion [10]. In consequence, miR-1236-3p may function as a tumor suppressor and participate in modulating the tumor development process in lung cancer. However, whether or not miR-1236-3p is related to drug resistance in lung cancer still remains to be explored.

In the current study, the underlying mechanism of miR-1236-3p in DDP-resistant lung cancer cells was illustrated. The results suggested that up-regulation of miR-1236-3p could reverse DDP resistance in lung cancer cells through targeting TPT1 and inhibition of the Pim-3 signaling pathway.

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2 Materials and methods

2.1 Specimens

30 pairs of lung cancer tumor tissues (Table. 1) and non-tumor adjacent tissues were obtained from Weifang Traditional Chinese Hospital between July 2014 and April 2016 from patients who had undergone surgical resection. All the patients were pathologically diagnosed with lung cancer without receiving any radiotherapy or chemotherapy. After resection, all of the tissues were preserved in liquid nitrogen at -80° . This project was approved by the Ethical Committee of Weifang Traditional Chinese Hospital and the written consent was obtained from each patient or relative.

2.2 Cell culture

Human normal lung epithelial cells (BESA-2B) and lung cancer cell line (A549) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

DDP resistant A549 sub-line A549/DDP was purchased from the Cancer Hospital of Peking Union Medical College, Chinese Academy of Medical Sciences. All the cell lines were cultivated in Dulbecco's Modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing penicillin-streptomycin. All the cell lines were cultivated in a humid atmosphere with 5% CO_2 at 37° .

2.3 Dual-luciferase reporter assay

TPT1 3'UTR was amplified from cDNA of 293 cells and inserted into pGL-3 (Promega, USA). The 293 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cotransfected with the wild-type 3'UTR of TPT1 containing the two putative miR-1236-3p binding sites (Site 1: 473-480 and Site 2: 1085-1091) and mutant TPT1 3'UTR (TPT1-3'UTR) with either NC mimics or miR-1236-3p mimics via Lipofectamine[®]2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). After transfection,

Table 1: Clinicopathological features in 30 lung cancer tumor specimens

Clinicopathological variable	Cases	Expression level		P value (*P<0.05)
		miR-1236-3p high (n=4)	miR-1236-3p low (n=26)	
Age				
≤60 years	17	1	16	0.170
>60 years	13	3	10	
Gender				
Female	22	2	6	0.260
Male	8	2	20	
Tumor size				
≥2cm	25	1	24	0.000
<2cm	5	3	2	
Smoking				
Yes	20	2	18	0.448
No	10	2	8	
Weight (Kg)				
≥65	16	3	13	0.351
<65	14	1	13	
TNM stage				
I/II	26	2	24	0.020
III/IV	4	2	2	
BMI				
≥22	19	1	18	0.875
<22	11	3	8	

the cells were cultivated at 37°, 5% CO₂ for 4 h. Then, the luciferase activities were confirmed using a dual-luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.4 Cell transfection

The NC mimics and miR-1236-3p mimics were purchased from Sigma-Aldrich (Shanghai, China). According to the project, A549/DDP cells were divided into three different groups in order to measure the transfection efficiency of miR-1236-3p: i) the control group (un-treated cells); ii) the negative control (NC) group (to eliminate non-sequence specific effects) and iii) the mimics group (cells were transfected with miR-1236-3p mimics). The A549/DDP cells were seeded into six-well plates, with the density of 4×10⁵ cells/well, then the miR-1236-3p or the NC mimics were transfected into cells with Lipofectamine[®]2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's protocol.

The pcDNA3.1 plasmids were purchased from Gene-Chem (Shanghai, China). The pcDNA3.1-TPT1 plasmids were constructed by inserting the TPT1 CDS expression into the pcDNA3.1 vector. According to the project, A549/DDP cells were divided into three different groups in order to confirm the expression of TPT1 gene in different groups: i) the control group (un-treated cells); ii) pcDNA3.1 group (cells transfected with blank plasmid) and iii) pcDNA3.1-TPT1 (cells transfected with pcDNA3.1-TPT1 plasmids). The A549/DDP cells were seeded into six-well plates, with the density of 4×10⁵ cells/well, then the pcDNA3.1 or the pcDNA3.1-TPT1 were transfected into cells using Lipofectamine[®]2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the instructions.

2.5 RNA extraction and PR-qPCR assay were conducted

Total RNA from tissues or cells were isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Meanwhile, miRNAs were isolated from tissues or cells via the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). Afterward, TaqMan[™] MicroRNA Reverse Transcription Kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was applied to synthesize cDNA. The RNA quantity was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). Then, 2 µl RNA were reverse transcribed into

cDNA using TaqMan[™] Reverse Transcription Reagents (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, RT-qPCR (Real-time Quantitative polymerase chain reaction) analysis was performed as followed. Briefly, 2 µl-cDNA, 10 µl-SYBR Green RT-qPCR Master Mix (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1 µl-primers and 7 µl ddH₂O were mixed together during the whole PCR reaction process. The PCR reaction conditions were described as followed: i) initially denatured at 94° for 3min; ii) denatured at 94° for 30s, annealed at 55° for 30s, extended at 72° for 30s, this procedure was repeated 30 cycles and iii) finally extended at 72° for 10min. In data processing, the relative expression level of miR-1236-3p was normalized to U6 small nuclear RNA (Thermo Fisher Scientific, Inc., Waltham, MA, USA), while the expression levels of TPT1 and related proteins were normalized to β-actin (R&D Systems China, Co. Ltd, Shanghai, China) according to the 2^{-ΔΔCt} method. The primer sequences were described as followed: miR-1236-3p forward, 5'-CCAATCAGCCTCTCCCTT-3' and reverse, 5'-TATGGTTGTTACGACTCTTCAC-3'; U6 forward, 5'-ATTGGAACGATACAGAGAAGATT-3' and reverse, 5'-GGAACGCTTCACGAATTTG-3'; TPT1 forward, 5'-TATTGGACTACCGTGAGG-3' and reverse, 5'-CAAGCAGAAGCCAGTTATG-3'; β-actin forward, 5'-AGATGTGGATCAGCAAGCAG-3' and reverse, 5'-GCGCAAGTTAGTTTTGTCA-3'.

2.6 The IC₅₀ of DDP determined by MTT assay

The cytotoxic activity of DDP was determined by the colorimetric MTT assay. In brief, cells with the density of 4×10³ cells/ml were seeded on a 96-well plate with 100 µl culture medium per well. Cells were incubated at 37° in a humid atmosphere with 5% CO₂ for 24 h and treated with cisplatin subsequently. Refer to previous study and experiment [11], a concentration of 20 µg/ml-DDP was prepared and added to the 96-well plate. After incubating for 24 h, 20 µl-3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) was added to the cells in the dark and incubated for 4 h at 37°, 5% CO₂, covered with aluminum foil. Afterward, 100 µl DMSO (Solarbio[®], Beijing, China) was added to each well to dissolve the formazan crystals formed, and the 490/570 nm double-wave-length measurement optical density was measured via a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The potency of cell growth inhibition for the test agent was expressed as the half maximal (50%) inhibitory

concentration, IC50. All the procedures were conducted in triplicate.

2.7 The proliferation rate of A549/DDP cells confirmed by MTT assay

According to the experimental design, all the following experiments were divided into four different groups: i) the control group (un-treated cells); ii) the negative control (NC) group (to eliminate non-sequence specific effects); iii) the miR-1236-3p mimics group (cells were transfected with miR-1236-3p mimics), and iv) the miR-1236-3p mimics+pcDNA3.1-TPT1 group (cells were transfected with miR-1236-3p mimics and pcDNA3.1-TPT1).

Cells from different groups were seeded on a 96-well plated, with the density of 5×10^3 cells/well. After incubation for 24 h, 20 μ l-3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) was added to the cells in the dark and incubated for 4 h at 37°, 5% CO₂, covered with aluminum foil. Afterward, 100 μ l DMSO (Solarbio®, Beijing, China) was added to each well to dissolve the formazan crystals formed, and the absorbance was read at 490 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). All the procedures were performed in triplicate.

2.8 Apoptosis rate of A549/DDP cells confirmed by a flow cytometry

The apoptosis rate of cultured A549/DDP cells was determined using Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI; BD Pharmigen, San Diego, CA, USA). In brief, cells from different groups with the density of 3×10^5 cells/well were seeded in a six-well plate and cultured for 24 h. After being washed with phosphate-buffered saline (PBS; Solarbio®, Beijing, China) three times, cells were stained with eBioscience™ flow cytometry staining buffer (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 15 min in the dark. The fluorescence was assessed by CytoFLEX flow cytometry (Beckman Coulter, Inc., CA, USA).

2.9 Effect of miR-1236-3p on the expression levels of TPT1, Pim-3, BAD and Bcl-XL

After washed with phosphate-buffered saline (PBS; Solarbio®, Beijing, China), cells from different groups were lysed by the RIPA buffer (Solarbio®, Beijing, China) sup-

plemented with a protease inhibitor cocktail (MedChem Express, Shanghai, China). All the protein was quantified using a Bradford protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Equal amounts (20 μ g) of the cell lysates were separated by 8% SDS-PAGE (Detai-Bio, Nanjing, China) and transferred onto polyvinylidene difluoride membranes (PVDF; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Then, the blots were blocked in 50 ml 5% bovine serum albumin (BSA; Haoran Biological Technology Co., Ltd, Shanghai, China) for 1 h. Later on, the primary antibodies were added on the membrane at 37° overnight: mouse anti-TPT1 (1:1000; A5442; ABclonal, Wuhan, China), rabbit anti-Pim-3 (1:500; ab71321; Abcam, Cambridge, MA, USA); rabbit anti-phospho-BAD (1:5000; ab129192; Abcam, Cambridge, MA, USA); rabbit anti-Bcl-XL (1:1000; ab32370; Abcam, Cambridge, MA, USA) and mouse anti- β -actin (1:1000; ab8226; Abcam, Cambridge, MA, USA). After washed with phosphate-buffered saline (PBS; Solarbio®, Beijing, China) three times, the secondary antibodies IgG H&L (HRP; 1:1000; ab7090; Abcam, Cambridge, MA, USA) and IgG H&L (Cy3®; 1:2000; ab97035; Abcam, Cambridge, MA, USA). Subsequently, the membranes were washed four times with phosphate-buffered saline (PBS; Solarbio®, Beijing, China), containing 0.1% Tween 20 (Sigma-Aldrich, Shanghai, China). The results were visualized using the X-ray films after adding 200 μ l chemiluminescent system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A molecular Imager ChemiDox XRS system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to quantify the relative expression levels of TPT1 and related-proteins. β -actin functioned as the reference protein.

2.10 Statistical analysis

SPSS 17.0 statistical software (IBM Corporation, Armonk, NY, USA) was used to analyze the data. The data were presented as the mean \pm deviation. Student's t-test was used to determine differences between two groups, while one-way analysis of variation (ANOVA) followed by NewmanKeuls analysis was applied to verify differences among three or four groups. A P value <0.05 was considered to be statistically significant.

3 Results

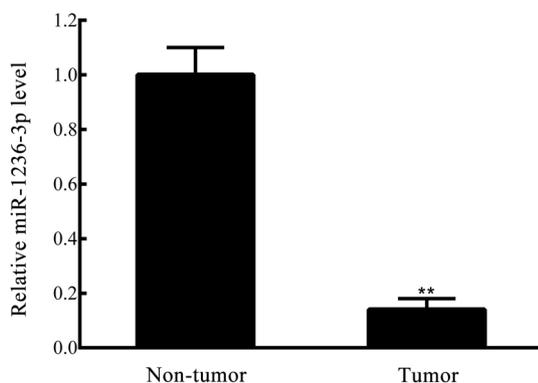
3.1 The lower expression level of miR-1236-3p in tumor and cancer cells

As shown in Fig. 1A, the expression level of miR-1236-3p was significantly lower in the lung cancer tumor tissues as compared with non-tumor adjacent tissues ($P < 0.01$). Meanwhile, Fig. 1B further demonstrated that the expression level of miR-1236-3p was remarkably restrained in the A549 lung cancer cell line, compared to the human normal lung epithelial cells BEAS-2B ($P < 0.01$). Collectively, these results demonstrated that the expression level of miR-1236-3p was lower in lung cancer tumor tissues and cell lines than in non-tumor adjacent tissues and normal cells, respectively.

3.2 The IC₅₀ of DDP and miR-1236-3p expression level in lung cancer cells

To detect the half maximal inhibitory concentration (IC₅₀) of DDP for chemotherapy agent, two lung cancer cell lines were exposed to 20 $\mu\text{g/ml}$ DDP for 24 h. After that, the MTT data revealed that the IC₅₀ of DDP in A549 cells was lower than that in A549/DDP cells (Fig. 2A; $P < 0.01$). Subsequently, the expression level of miR-1236-3p in two cell line was observed via RT-qPCR assay. As the results presented, the expression level of miR-1236-3p was markedly decreased in the A549/DDP cells compared with the A549 cells (Fig. 2B; $P < 0.01$). Hence, our data suggested that DDP-resistant cell line A549/DDP was presented with a down-regulated expression level of miR-1236-3p.

A



3.3 miR-1236-3p regulates the DDP drug resistance in lung cancer cells

After the transfection of cells with NC mimics or miR-1236-3p mimics, the transfection efficiency of miR-1236-3p was detected by RT-qPCR assay. As shown in Fig. 3A, the expression level of miR-1236-3p was prominently increased in the miR-1236-3p mimics group as compared to NC mimics group ($P < 0.01$). Afterward, the IC₅₀ of DDP in different groups was measured by MTT assay. The data in Fig. 3B demonstrated that the IC₅₀ value of DDP was significantly decreased in the miR-1236-3p mimics group in contrast with NC mimics group ($P < 0.01$).

3.4 TPT1 gene is the direct target of miR-1236-3p

Combining the online bioinformatics tool TargetScan, TPT1 was predicted to be a possible target gene of miR-1236-3p. In order to verify this prediction, a dual-luciferase reporter assay was conducted subsequently. As shown in Fig. 4A and B, the 3'-UTR of the gene TPT1 were proved to contain the binding sequences for the miR-1236-3p in both 473-480 and 1085-1091 positions, suggesting that TPT1 may be a downstream target gene of miR-1236-3p. Meanwhile, as shown in Fig. 4C and D, the luciferase activities in the wild-type TPT1 3'UTR (TPT1-3'UTR) plasmid-transfected cells were significantly restrained by the transfection of miR-1236-3p-mimics, whereas there were no variation in the mutant-type TPT1 3'UTR (TPT1-3'UTR) plasmid-transfected cells ($P < 0.01$).

B

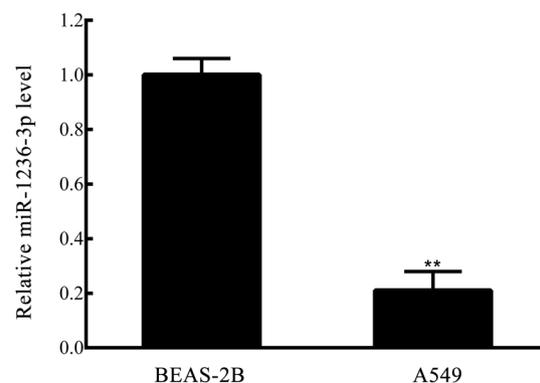


Figure 1: The lower expression level of miR-1236-3p in tumor tissues and cancer cells. (A) The expression level of miR-1236-3p in 30 pairs of lung cancer tumor tissues and non-tumor adjacent tissues was measured by RT-qPCR assay. (B) The same method was applied to detect the expression level of miR-1236-3p in BEAS-2B and A549 cells. ** $P < 0.01$, tumor vs non-tumor group; and A549 vs BEAS-2B group. Tumor, lung cancer tumor tissues; non-tumor, non-tumor adjacent tissues; BEAS-2bB, human normal lung epithelial cell line; A549, lung cancer cell line; miR, microRNA.

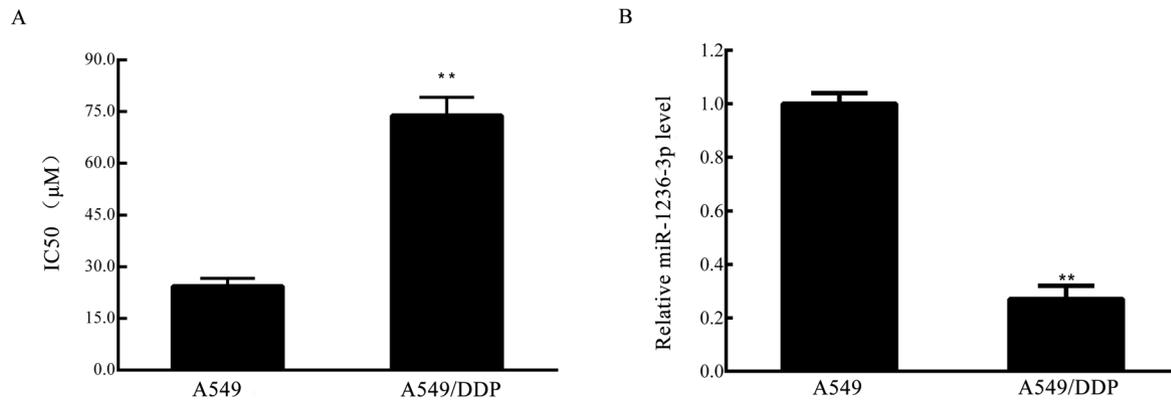


Figure 2: IC50 of DDP and the expression level of miR-1236-3p in A549 and A549/DDP cells. (A) The concentration of 20 μg/ml-DDP was added to verify the IC50 values in A549 and A549/DDP cells after 24 h exposure. (B) The expression level of miR-1236-3p in A549 and A549/DDP cells was measured by RT-qPCR assay. ** $P < 0.01$, A549/DDP vs A549 group. A549, lung cancer cell line; A549/DDP, human lung adenocarcinoma drug-resistant cell line; miR-1236-3p, microRNA-1236-3p mimics-transfected cells; IC50, the half maximal inhibitory concentration.

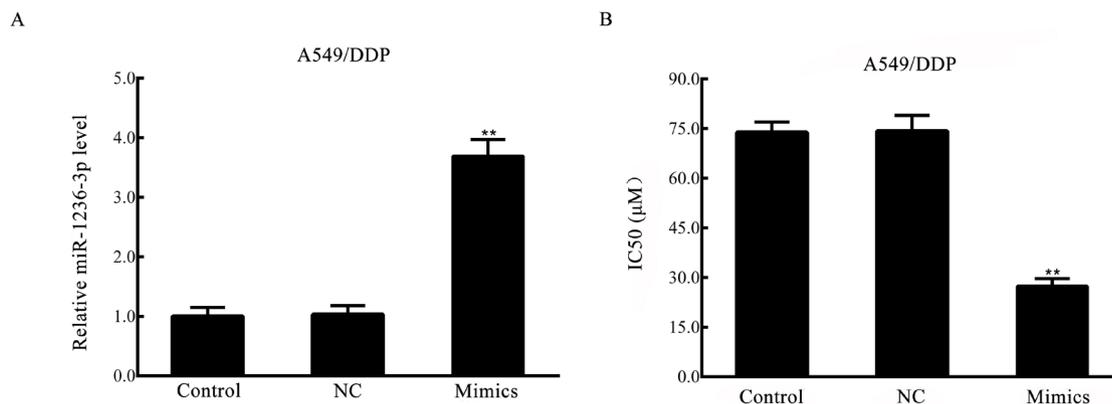


Figure 3: miR-1236-3p regulates the DDP resistance in lung cancer cells. (A) The transfection efficiency of miR-1236-3p was detected by RT-qPCR assay. (B) The IC50 values of DDP were analyzed by MTT assay. ** $P < 0.01$, mimics vs NC group. Control, un-treated cells; NC, miR-1236-3p mimics negative control-transfected cells; miR-1236-3p, cells transfected with miR-1236-3p mimics group; A549, lung cancer cell line; A549/DDP, human lung adenocarcinoma drug-resistant cell line; IC50, the half maximal inhibitory concentration; miR, microRNA.

3.5 The expression level of TPT1 gene is confirmed after pcDNA3.1-TPT1 transfection

Since TPT1 was proved to be the direct target gene of miR-1236-3p, cells were transfected with pcDNA3.1 or pcDNA3.1-TPT1 in order to confirm the expression level of TPT1 in different groups prior to the identification of the effects of TPT1 overexpression on miR-1236-3p mimics induced changes. The RT-qPCR assay results illustrated that the mRNA expression level of TPT1 was higher in the pcDNA3.1-TPT1 group, in contrast with the pcDNA3.1 group (Fig. 5; $P < 0.01$). Moreover, the protein expression of TPT1 by western blot assay presented the same trend of variation as well (Fig. 5B and C; $P < 0.01$).

3.6 Up-regulation of miR-1236-3p markedly decreases the proliferation rate of A549/DDP cells by targeting TPT1

For the sake of further studying the effect of miR-1236-3p on lung cancer cells, A549/DDP cells were transfected with NC mimics, miR-1236-3p mimics, and miR-1236-3p mimics plus pcDNA3.1-TPT1. Analysis of the cell proliferation rate of A549/DDP cells demonstrated that miR-1236-3p mimics may restrain the proliferation rate ($P < 0.01$) and this phenomenon could be reversed by pcDNA3.1-TPT1 (Fig. 6; $P < 0.01$).

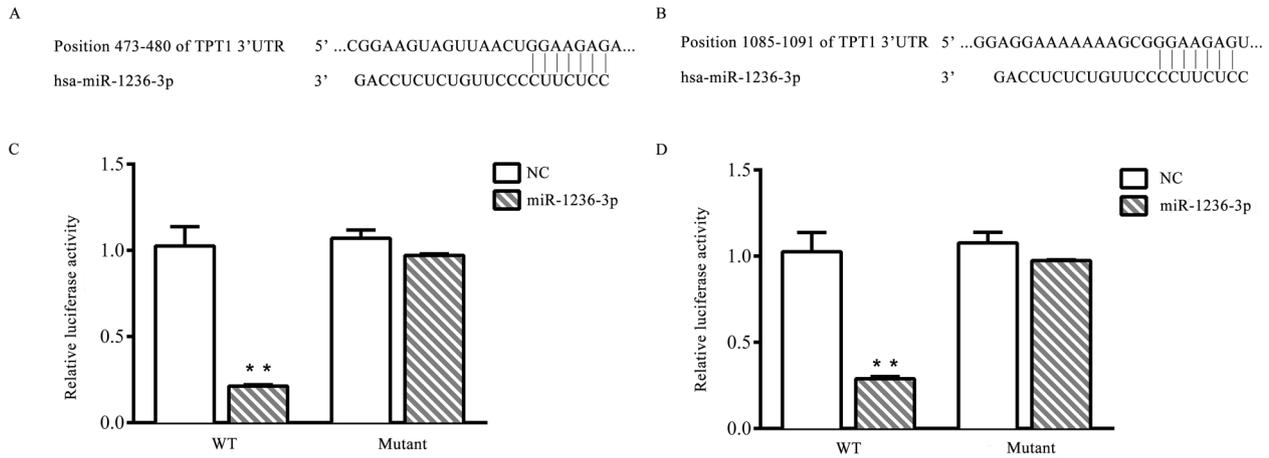


Figure 4: miR-1236-3p directly targets TPT1. (A) Sequence alignment of the paired site of the 3'-UTR of miR-1236-3p and TPT1 (position 473-480); (B) Sequence alignment of the paired site of the 3'-UTR of miR-1236-3p and TPT1 (position 1085-1091); (C-D) The activity of the luciferases in different groups. ** $P < 0.01$, miR-1236-3p vs NC group. WT, wild-type; NC, miR-1236-3p mimics negative control-transfected cells; miR-1236-3p, microRNA-1236-3p mimics-transfected cells; TPT1, tumor protein, translationally-controlled 1; UTR, untranslated region.

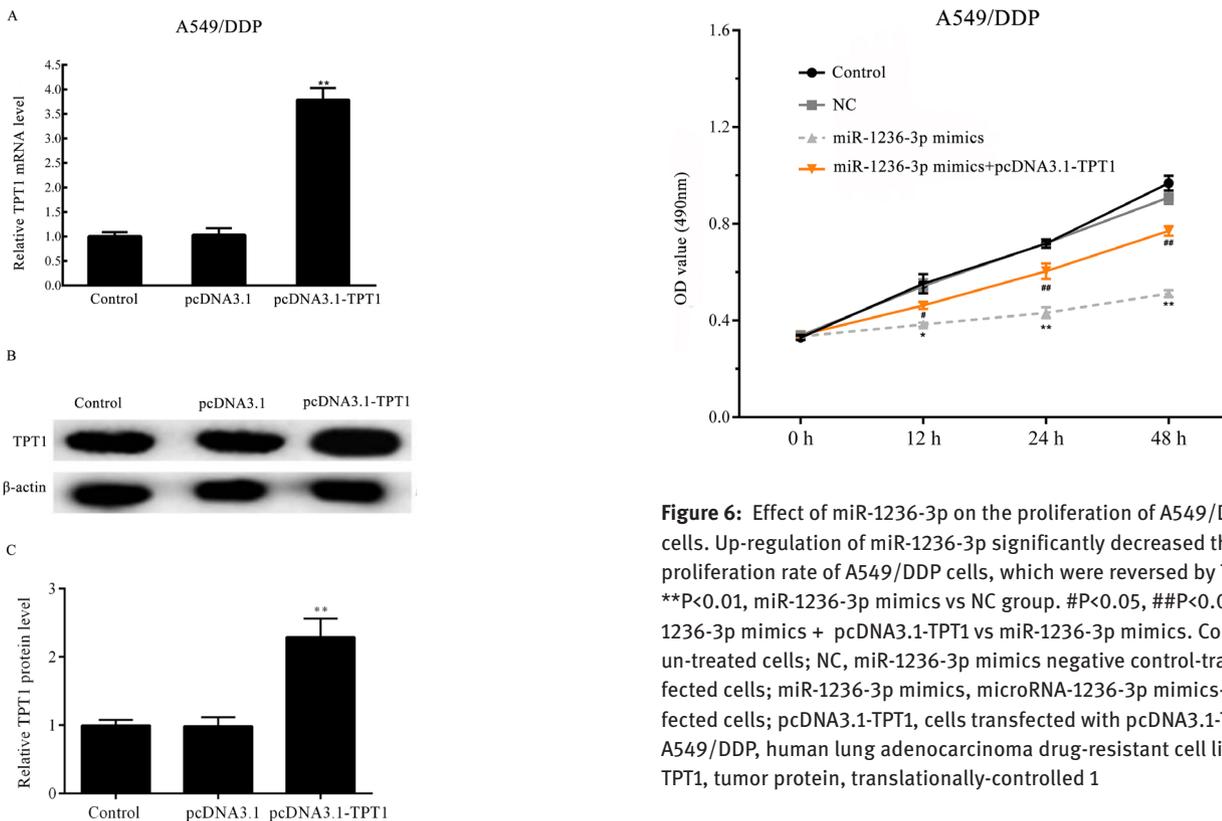


Figure 5: The expression level of TPT1 mRNA and protein in different groups. Relative TPT1 mRNA expression level in different groups measured by RT-qPCR assay. (B and C) Relative protein expression level of TPT1 in different groups measured by western blot assay. ** $P < 0.01$, pcDNA3.1-TPT1 vs pcDNA3.1 group. Control, un-treated cells; pcDNA3.1, cells transfected with pcDNA3.1 plasmid; pcDNA3.1-TPT1, cells transfected with pcDNA3.1-TPT1; A549/DDP, human lung adenocarcinoma drug-resistant cell line; TPT1, tumor protein, translationally-controlled 1; β-actin, human gene and protein symbol ACTB.

Figure 6: Effect of miR-1236-3p on the proliferation of A549/DDP cells. Up-regulation of miR-1236-3p significantly decreased the proliferation rate of A549/DDP cells, which were reversed by TPT1. ** $P < 0.01$, miR-1236-3p mimics vs NC group. # $P < 0.05$, ## $P < 0.01$, miR-1236-3p mimics + pcDNA3.1-TPT1 vs miR-1236-3p mimics. Control, un-treated cells; NC, miR-1236-3p mimics negative control-transfected cells; miR-1236-3p mimics, microRNA-1236-3p mimics-transfected cells; pcDNA3.1-TPT1, cells transfected with pcDNA3.1-TPT1; A549/DDP, human lung adenocarcinoma drug-resistant cell line; TPT1, tumor protein, translationally-controlled 1

3.7 Up-regulation of miR-1236-3p markedly increases the apoptosis rate of A549/DDP cells by targeting TPT1

In order to confirm the effect of miR-1236-3p on lung cancer cells, a flow cytometry method was performed accordingly. The Fig. 7A-D represented the apoptosis rate among four different groups respectively, while the Fig. 7E was the corresponding quantified results of the flow

cytometry assay. As the data exhibited, the apoptosis rate in cells transfected with miR-1236-3p mimics was significantly promoted ($P < 0.01$), whereas the variation could be reversed by pcDNA3.1-TPT1 ($P < 0.05$).

3.8 Effect of miR-1236-3p on the expression levels of TPT1, Pim-3, BAD and Bcl-XL

As to confirm whether miR-1236-3p and TPT1 participated in the regulation of apoptosis, the expression levels of Pim-3, BAD and Bcl-XL in different groups were measured by western blot assay. As shown in Fig. 8A and B, the expression levels of TPT1, Pim-3, p-BAD and Bcl-XL were prominently decreased in the miR-1236-3p mimics group ($P < 0.01$), whereas the variation was reversed by pcDNA3.1-TPT1 ($P < 0.01$).

4 Discussion

According to previous studies, miR-1236-3p was reported to be engaged in regulating the tumor development process. For instance, miR-1236-3p could inhibit invasion and migration in gastric cancer [12], bladder cancer [13], ovarian carcinoma [14] and lung adenocarcinoma [15]. Consistent with previous studies, our data demonstrated that miR-1236-3p was down-regulated in lung cancer tumor tissues and A549 cell lines, suggesting that miR-1236-3p functioned as a tumor suppressor in lung cancer. Furthermore, according to the IC50 value determined by MTT assay, the DDP-resistant cell line A549/DDP was presented with the down-regulated expression level of miR-1236-3p, indicating that miR-1236-3p was involved in regulating DDP resistance in lung cancer.

As the results exhibited, TPT1 was proved to be the direct target gene of miR-1236-3p by a dual-luciferase reporter assay. Recently, TPT1 was associated with cancer progression and was highly expressed in various tumors [16]. As known, knockdown of TPT1 was proved to inhibit

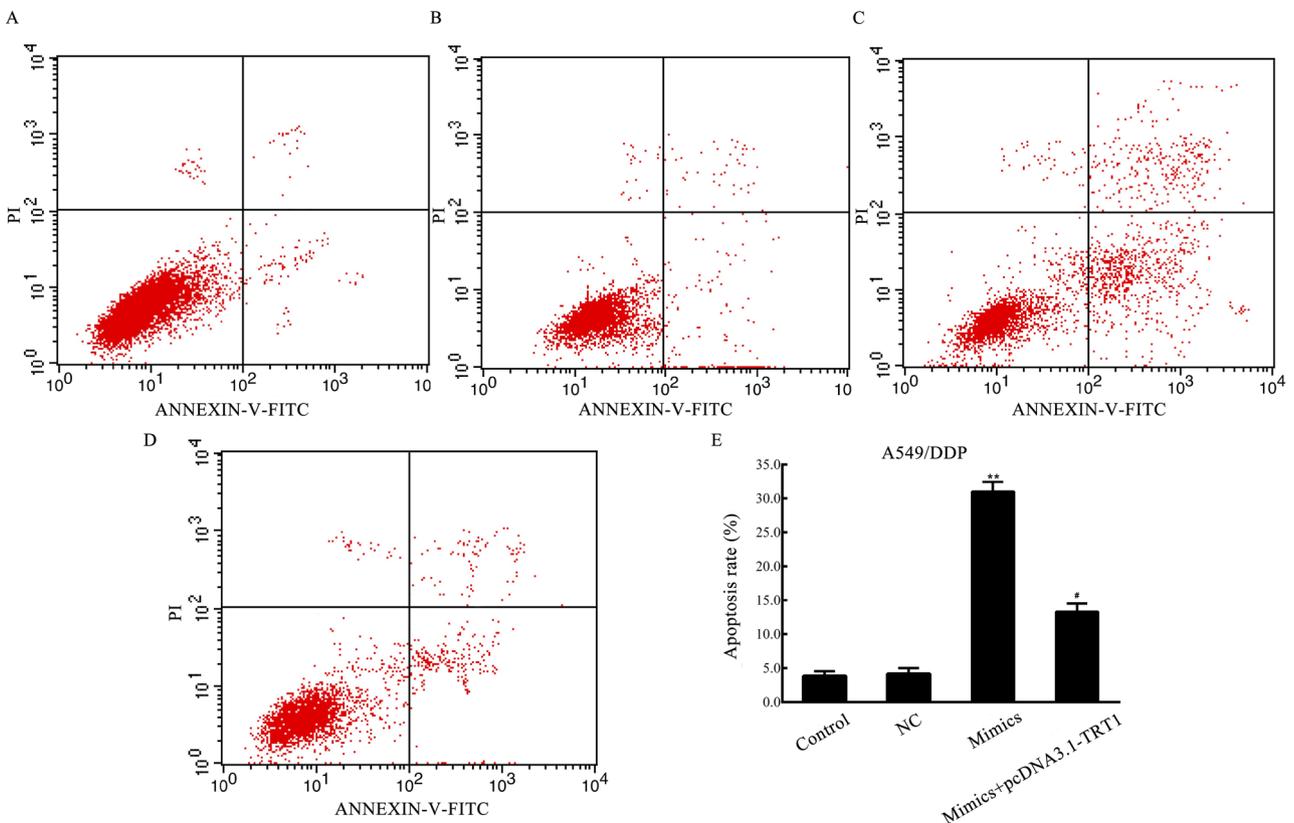


Figure 7: Effect of miR-1236-3p on the apoptosis of A549/DDP cells. (A) Control group; (B) NC group; (C) miR-1236-3p mimics group; (D) miR-1236-3p mimics+pcDNA3.1-TPT1 group; (E) Quantified results of the apoptosis rate of A-D was exhibited. ** $P < 0.01$, mimics vs NC group. # $P < 0.05$, mimics + pcDNA3.1-TPT1 vs mimics. Control, un-treated cells; NC, miR-1236-3p mimics negative control-transfected cells; miR-1236-3p mimics, microRNA-1236-3p mimics-transfected cells; pcDNA3.1-TPT1, cells transfected with pcDNA3.1-TPT1; A549/DDP, human lung adenocarcinoma drug-resistant cell line; TPT1, tumor protein, translationally-controlled 1.

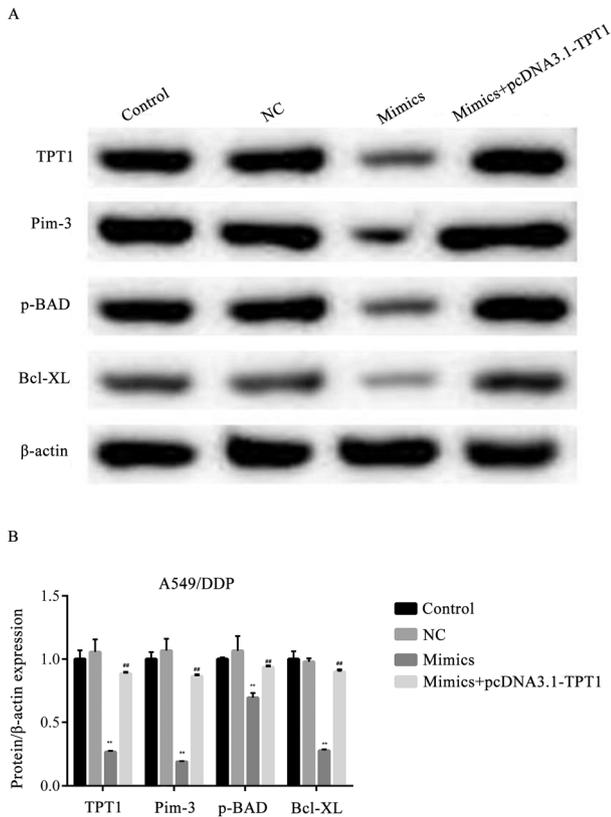


Figure 8: Effect of miR-1236-3p on the protein expression levels of TPT1, Pim-3, p-BAD and Bcl-XL. (A) The protein expression levels of TPT1, Pim-3, p-BAD and Bcl-XL were exhibited; (B) The corresponding data of A was presented. ** $P < 0.01$, mimics vs NC group. ### $P < 0.01$, mimics + pcDNA3.1-TPT1 vs mimics. Control, un-treated cells; NC, miR-1236-3p mimics negative control-transfected cells; miR-1236-3p mimics, microRNA-1236-3p mimics-transfected cells; pcDNA3.1-TPT1, cells transfected with pcDNA3.1-TPT1; A549/DDP, human lung adenocarcinoma drug-resistant cell line; TPT1, tumor protein, translationally-controlled 1; BAD, Bcl-2-associated death promoter; Bcl-xL, B-cell lymphoma-extra large; β -actin, human gene and protein symbol ACTB.

cell proliferation or metastasis in colon cancer [17] and prostate cancer [16]. Moreover, the TPT1 gene was reported to be up-regulated in A549 lung cancer cells as well [18]. Thus, we suggested that TPT1 exerted the function as an oncogene taking part in modulating tumor development and evolution, however, whether TPT1 was involved in modulating drug resistance remained uncertain. Accordingly, our study illustrated the function that TPT1 exerted concerning the DDP resistance in lung cancer cells. As stated in MTT and flow cytometry, up-regulated miR-1236-3p significantly inhibited A549/DDP cell proliferation but promoted apoptosis by down-regulating the expression level of TPT1. That is to say, miR-1236-3p could reverse DDP resistance in lung cancer cells by restraining the expression level of TPT1. Collectively, our study firstly

discovered the potential mechanism of miR-1236-3p and its target gene TPT1 in regards to the DDP resistance in lung cancer, which may contribute to the clinical chemotherapy treatment.

In order to further illustrate the mechanism of how miR-1236-3p mediated the DDP resistance in lung cancer cell by targeting TPT1 gene, the expression levels of related proteins were measured by western blot assay. Pim-3 is a member of the proto-oncogene Pim family [19], which could prevent apoptosis and promote cell proliferation [20]. In addition, TPT1 was considered to be able to interact with Pim-3 and enhance Pim-3 protein stability [21]. According to our results, miR-1236-3p down-regulated the expression levels of TPT1 and Pim-3, which were corresponded to previous studies. Meanwhile, a previous study pointed out that Pim-3 could inactivate BAD in human pancreas and colon carcinoma by phosphorylating BAD (p-BAD), that is Pim-3 may enhance the expression level of p-BAD [22,23]. BAD is known as a member of Bcl-2 family, binding to the Bcl-2/Bcl-XL complex and promoting apoptosis [24,25]. However, p-BAD dissociates from this complex, leading to the release of Bcl-2 and Bcl-XL, exerting the function of suppressing cell apoptosis [26,27]. Bcl-XL, a member of the Bcl-2 family as well, was found to be highly increased in A549/DDP cells [28]. Meanwhile, Bcl-XL was also reported to be up-regulated in A549 cell lines, inhibiting lung cancer cell apoptosis as well [29]. That is, TPT1, Pim-3, p-BAD and Bcl-XL functioned as oncogenes suppressing cell apoptosis in lung cancer cells. Another study had been reported that downregulation of TPT1 could restrain the expression levels of Pim3, p-BAD and Bcl-XL, promoting cell apoptosis [21]. Results of western blot exhibited that miR-1236-3p may inhibit the expression levels of TPT1, Pim-3, p-BAD and Bcl-XL, the known tumor promoter genes in lung cancer, by targeting TPT1.

To sum up, up-regulating of miR-1236-3p could reverse DDP resistance in lung cancer cells by targeting TPT1 through Pim-3 signaling pathway.

Conflicts of interest: The authors have no conflicts of interest to declare.

References

- [1] Parkin D.M., Bray F., Ferlay J., Pisani P., Global cancer statistics, 2002, *A Cancer Journal for Clinicians.*, 2005, 55, 74-108; DOI: 10.3322/canjclin.55.2.74

- [2] Chen W., Zheng R., Baade P.D., Zhang S., Zeng H., Bray F., *et al.*, *Cancer Statistics in China, 2015*, *A Cancer Journal for Clinicians.*, 2016, 2, 115-132; DOI: 10.3322/caac.21338
- [3] D'Addario G., Pintillie M., Leigh N.B., Feld R., Cerny T., Shepherd F.A., *Platinum-based versus non-platinum based chemotherapy in advanced non-small-cell lung cancer: a meta-analysis of the published literature*, *Journal of Clinical Oncology.*, 2015, 23, 2926-2936; DOI: 10.1200/JCO.2005.03.045
- [4] Wallner K E., Li G.C., *Effect of drug exposure duration and sequencing on hyperthermic potentiation of mitomycin-C and cisplatin*, *Cancer Research.*, 1987, 47, 493-495; DOI:
- [5] He L., Hannon, G.J., *MicroRNAs: small RNAs with a big role in gene regulation*, *Nature Reviews Genetics.*, 2004, 5, 522-531; DOI: 10.1038/nrg1379
- [6] Eddy S.R., *Non-coding RNA genes and the modern RNA world*, *Nature Reviews Genetics.*, 2001, 2, 919-929; DOI: 10.1038/35103511
- [7] Qiu T.Z., Zhou L., Wang T.S., Xu J., Wang J., Chen W.J., Zhou X., *et al.*, *miR-503 regulates the resistance of non-small cell lung cancer cells to cisplatin by targeting Bcl-2*, *International Journal of Molecular Medicine.*, 2013, 32, 593-598; DOI: 10.3892/ijmm.2013.1439
- [8] Olga K., Jody F., James M., Yaroslav I., Volodymyr P.T., Vasyly' F.C., *et al.*, *Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin*, *Molecular Cancer Therapeutics.*, 2008, 7, 2152-2159; DOI: 10.1158/1535-7163.MCT-08-0021
- [9] Xia L., Zhang D.X., Du R., Pan Y.L., Zhao L.N., Sun S.R., *et al.*, *miR-15b and miR-16 modulate multidrug resistance by targeting BCL2 in human gastric cancer cells*, *International Journal of Cancer.*, 2008, 123, 372-379; DOI: 10.1002/ijc.23501
- [10] Li C.C., Ge Q.Q., Liu J.X., Zhang Q.S., Wang C.H., Cui K., *et al.*, *Effects of miR-1236-3p and miR-370-5p on activation of p21 in various tumors and its inhibition on the growth of lung cancer cells*, *Tumor Biology.*, 2017, 39, 101042831771082; DOI: 10.1177/1010428317710824
- [11] Xie C.Y., Pan Y.Q., Hao F.X., Gao Y., Liu Z., Zhang X.W., *et al.*, *C-Myc participates in β -catenin-mediated drug resistance in A549/DDP lung adenocarcinoma cells*, *APMIS.*, 2015, 122, 1251-1258; DOI: 10.1111/apm.12296
- [12] An J.X., Ma M.H., Zhang C.D., Shao S., Zhou N.M., Dai D.Q., *miR-1236-3p inhibits invasion and metastasis in gastric cancer by targeting MTA2*, *Cancer Cell International.*, 2018, 18, 66; DOI: 10.1186/s12935-018-0560-9
- [13] Zhang Q.S., Miao S., Li C.C., Cui K., Ge Q.Q., Chen Z., *S-phase kinase-associated protein 2 impairs the inhibitory effects of miR-1236-3p on bladder tumors*, *American Journal of Translational Research.*, 2018, 10, 731-743; ISSN: 1943-8141/AJTR0070260
- [14] Wang Y., Yan S., Liu X.L., Zhang W.J., Li Y.W., Dong R.F., *et al.*, *miR-1236-3p represses the cell migration and invasion abilities by targeting ZEB1 in high-grade serous ovarian carcinoma*, *Oncology Reports.*, 2014, 31, 1905-1910; DOI: 10.3892/or.2014.3046
- [15] Bian T.T., Jiang D.S., Liu J., Yuan X.P., Feng J., Li Q., *et al.*, *miR-1236-3p suppresses the migration and invasion by targeting KLF8 in lung adenocarcinoma A549 cells*, *Biochemical and Biophysical Research Communications.*, 2017, 492, 461-467; DOI: 10.1016/j.bbrc.2017.08.074
- [16] Gnanasekar M., Thirugnanam S., Zheng G.X., Chen A.S., Ramaswamy K., *Gene silencing of translationally controlled tumor protein (TCTP) by siRNA inhibits cell growth and induces apoptosis of human prostate cancer cells*, *International Journal of Oncology.*, 2009, 34, 1241-1249; DOI: 10.3892/ijo.00000252
- [17] Ma Q., Geng Y., Xu W.W., Wu Y.S., He F.L., Shu W., *et al.*, *The role of translationally controlled tumor protein in tumor growth and metastasis of colon adenocarcinoma cells*, *Journal of Proteome Research.*, 2010, 9, 40-49; DOI: 10.1021/pr9001367
- [18] Marco G., Valeria M., Laura G., Marzia N., Simona D., Barbara A., *et al.*, *Proteomic investigation in A549 lung cell line stably infected by HPV16E6/E7 oncogenes*, *Oncogenes.*, 2009, 77, 427-439; DOI: 10.1159/000176209
- [19] Boryana K.P., Li Y.Y., Zheng H.C., Omura K., Fujii C., Tsuneyama K., *et al.*, *Proto-oncogene, Pim-3 with serine/threonine kinase activity, is aberrantly expressed in human colon cancer cells and can prevent BAD-mediated apoptosis*, *Cancer Science.*, 2007, 98, 321-328; DOI: 10.1111/i.1349-7006.2007.00390.x
- [20] Fujii C., Nakamoto Y., Lu P.R., Tsuneyama K., Boryana K.P., Kaneko S., *et al.*, *Aberrant expression of serine/threonine kinase Pim-3 in hepatocellular carcinoma development and its role in the proliferation of human hepatoma cell lines*, *International Journal of Cancer.*, 2005, 114, 209-218; DOI: 10.1002/ijc.20719
- [21] You Y., Tan J.X., Gong L., Dai H.S., Chen H.W., Xu X.J., *et al.*, *MicroRNA-216b-5p functions as a tumor-suppressive RNA by targeting TPT1 in pancreatic cancer cells*, *Journal of Cancer.*, 2017, 8, 2854-2865; DOI: 10.7150/jca.18931
- [22] Zhang F., Liu B., Wang Z., Yu X.J., Ni Q.X., Yang W.T., *et al.*, *A Novel Regulatory Mechanism of Pim-3 Kinase Stability and its Involvement in Pancreatic Cancer Progression*, *Molecular Cancer Research.*, 2013, 11, 1508-1520; DOI: 10.1158/1541-7786.MCR-13-0389
- [23] Li Y.Y., Boryana K.P., Yuichiro N., Hiroshi I., Chifumi F., Naofumi, M., *Pim-3, a proto-oncogene with serine/threonine kinase activity, is aberrantly expressed in human pancreatic cancer and phosphorylates bad to block bad-mediated apoptosis in human pancreatic cancer cell lines*, *Cancer Research.*, 2006, 66, 6741-6747; doi: 10.1158/0008-5472.CAN-05-4272
- [24] Gross A., McDonnell J.M., Korsmeyer S.J., *BCL-2 family members and the mitochondria in apoptosis*, *Genes and Development.*, 1999, 13, 1899-1911; DOI: 10.1101/GAD.13.15.1899
- [25] KSR S., MA A., Li P., MK A., Wang E., Chouchane A.I., *et al.*, *Targeting proapoptotic protein BAD inhibits survival and self-renewal of cancer stem cells*, *Cell Death and Differentiation.*, 2014, 21, 1936-1949; DOI: 10.1038/CDD.2014.140
- [26] Xia R.Y., Zhang R.R., Jiang Z., Sun Y.J., Liu J., Chen F.H., *K9(C4H4FN2O2)2Nd(PW11O39)2·25H2O induces apoptosis in human lung cancer A549 cells*, *Oncology Letters.*, 2016, 13, 1348-1352; DOI: 10.3892/OL.2016.5543
- [27] Zhang H.Y., Yang W., Lu J.B., *Knockdown of GluA2 induces apoptosis in non-small-cell lung cancer A549 cells through*

the p53 signaling pathway, *Oncology Letters.*, 2017, 14, 1005-1010; DOI: 10.3892/OL.2017.6234

- [28] Zhang J., Liu J., Li H., Wang J., β -catenin signaling pathway regulates cisplatin resistance in lung adenocarcinoma cells by upregulating Bcl-xl, *Molecular Medicine Reports.*, 2016, 13, 2543-2551; DOI: 10.3892/MMR.2016.4882
- [29] Zhang Y., Wang J.H., Lu Q., Wang Y.J., Bag3 promotes resistance to apoptosis through Bcl-2 family members in non-small cell lung cancer, *Oncology Letters.*, 2011, 27, 109-113; DOI: 10.3892/OR.2011.1486