EFFECT OF TIGAR KNOCKOUT ON THE APOPTOSIS OF A549 CELLS AND EXPRESSION OF EPITHELIAL MESENCHYMAL MARKERS IN DOX-RESISTANT NON-SMALL CELL LUNG CANCER

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ABSTRACT

Objective*: To investigate the effect of TIGAR knockout on the apoptosis of A549 cells and expression of epitheli-al/mesenchymal markers in doxorubicin (DOX)-resistant non-small cell lung cancer (NSCLC).*

Methods: *A DOX-resistant A549 cell line (A549/DOX) was established in this study, and the gene was knocked out through TIGAR-targeted siRNA reverse transfection. The TIGAR protein, cell viability, colony survival, ROS, NADPH and expression levels of apoptosis-related markers and epitheli-al/mesenchymal markers of the control group and siRNA transfection group were compared.*

Results: *After DOX treatment, the cell viability of A549/DOX cell line was significantly higher than that of the original A549 cell line (P<0.05). The TIGAR protein, cell viability, colony survival, levels of NADPH, Bcl-2, PARP and vimentin in the siTIGAR transfection group were significantly lower than those in the control group (P<0.05). The levels of ROS, Bax, Caspase-3, Caspase-9, PARP degradation products and E-caderin in the siTIGAR transfection group were significantly higher than those in the control group (P<0.05).*

Conclusion: *TIGAR knockout can inhibit the EMT of DOX-resistant NSCLC and be used as a potential therapeutic target for patients with chemotherapy resistance.*

Keywords: *TIGAR, lung cancer, targeted therapy, apoptosis, epithelial-mesenchymal transition.*

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Introduction

In recent years, the incidence rate of lung cancer has grown year by year, and it has become a leading cause of can-cer-related death. The number of patients with non-small cell lung cancer (NSCLC) accounts for about 80-85% of the total number of patients with lung cancer, and the 5-year survival rate is less than 15%[(1)]. Chemotherapy is a commonly-used therapeutic means for NSCLC, which can effectively reduce the risk of recurrence after surgery and enhance long-term survival benefits. However, acquired chemotherapy resistance seriously affects the clinical prognosis[(2)]. Doxorubicin (DOX) is a chemotherapeutic drug targeting topoisomerase II, which effectively inhibits protein synthesis and DNA replication, but the efficacy for advanced NSCLC is only 30-50%. An overwhelming majority of patients develop drug resistance or cardiotoxicity after treatment[(3)]. TIGAR is TP53-induced glycolysis and apoptosis regulator en-coded by the C12orf5 gene, which can get involved in a variety of pathophysiological functions, such as cell cycle maintenance, apoptosis and autophagy, by blocking cell glycolysis and promoting cell metabolism.

In addition, the activation of TIGAR also promotes the production of NADPH in cells, improves the scavenging of ROS by invigorating reduced glutathione, and inhibits apoptosis induced by oxidative stress[(4)]. Some scholars argue that glucose utilization

disorder is closely related to resistance to chemotherapy using antitumor drugs[5]. In the process of epithelial-mesenchymal transition (EMT), differentiated epithelial cells lose adhe-sion and apical-basal polarity and then turn to fibroblast-like mesenchymal phenotype[6]. Some scholars confirm that tumor metastasis is one of the leading causes of cancer-related death and it is closely related to the mesenchymal properties of tumor cells[7].

The role that TIGAR plays in apoptosis and autophagy has been explored in depth. However, whether TIGAR gets involved in the EMT regulation of lung cancer cells and its specific role are rarely reported. Mean-while, the role that TIGAR plays in the DOX resistance of lung cancer cells is also yet to be clarified[8, 9]. This study intends to investigate the effect of TIGAR knockout on the apoptosis of A549 cells and expression of epithelial/mesenchymal markers in doxorubicin (DOX) resistant NSCLC. Below, the process will be reported.

Materials and methods

Reagents and instruments

The RPMI-F12 medium is com-posed of fetal bovine serum (FBS) and penicillin-streptomycin. And the 0.25% trypsin-EDTA and phosphate buffered saline DPBS were supplied by Gibco; The transfection reagents RNAiMax and Lipofectamine®RNIMAX were supplied by Invitrogen. siRNA was purchased from Dharmacon.

Doxorubicin hydro-chloride, acrylamide, phenylmethyl-sulfonyl fluoride (PMSF), dithiothreitol (DTT), carmine S, TEMED, Tris-HCl, dimethyl sulfoxide (DMSO), β-mercaptoethanol and sodium dodecyl sulfate (SDS) were supplied by Sig-ma-Aldrich. Tween-20, glycine, trichloroacetic acid (TCA), β-mercaptoethanol, NP-40, EDTA, EGTA, β-glycerophosphate and H2O2 were supplied by Merck. The monoclonal an-tibodies against TIGAR, Bcl-2, Casperin-9 and GAPDH were provided by Abcam. The polyclonal antibodies against vimentin and E-caderin were provided by Santa Cruz. The Cell Proliferation Kit I (MTT) and DCFDA were provided by Sigma-Aldrich. The colorimetric NADPH assay kit was sourced from Abcam.

Cell Culture and establishment of a DOX-resistant A549 cell line (A549/DOX)

10% FBS and 1% penicil-lin-streptomycin were added to RPMI-F12 medium to culture A549 lung cancer cells and A549/DOX. The incubation conditions were a 5% CO_2 hu-midified incubator at 37°C. The A549/DOX cell line was established by treating the A549 cell line stepwise by increasing the DOX concentration progressively (from 0.1μM to 1.1μM). A549 lung cancer cells were cultured in DOX medium with an initial concentration for 2~3d, washed with PBS and then treated by a moderate dose of DOX. This process was repeated until the concentration was up to 1.1μM. The drug resistance was evaluated by a MTT test. In order to guarantee DOX resistance, the author added A549/DOX cells to DOX at an appropriate concentration.

SiRNA Transfection

The gene was knocked out through TIGAR-targeted siRNA reverse transfection. The reagents used included transfection medium (Opti-mem), transfection reagents (Lipo RNAiMax, Lipofectamine®RNAiMax, Invitrogen TM), related siRNA (siControl or siTIGAR target sequences in TIGAR coding sequences: 5'-GAAUAACGGUAAAGUAUG-3') and serum-free basal medium (RPMI-F12).

After the mixture of cell suspension, transfection reagents and related siRNA, the cells were resuspended, coincubated for 1h, and then blended with cell suspension to form siRNA compound. After 6h of transfection, the whole RPMI-F12 medium was added for culture. 48h later, cells were collected and western blot was conducted to verify whether the transfection succeeded.

Preparation of Protein Sample and Western Blotting

The cells collected were centrifuged in a frozen PBS buffer, and then RIPA lysis buffer+ protease and phosphatase inhibitor [1 mM β-glycerophosphate, 1μM leucine protease, 1 mM EDTA, 1% (v/v) Triton-X 100, 0.1% (w/v)SDS] were added for the purpose of cell lysis. 0.3μM aprotinin and phenylmethylsulfonyl flu-oride (PMSF) were added and incubated for 45min.

The total protein concentration was measured and standardized using a Bradford protein assay. Through PAGE, the protein samples were separated elec-trophoresis and then transferred to a PVDF membrane. 5% skimmed milk powder (SMP) and Tris-buffered saline with 0.1% Tween 20 (TBST) were added to block for 2h at room temperature, and then the primary antibody was added for incubation at 4°C overnight.

The membrane was washed with TBST, and then incubated using the secondary antibody con-

taining horseradish peroxidase for 1h at room temperature. The membrane was washed by TBST for 5 times, 5min at a time. Quantitative analysis was done with enhanced chemiluminescence (ECL), X-ray film and Image-J software.

Determination of colony survival

After transfection, the A549/DOX cells were seeded in a 6-well plate, and the cell medium was refreshed every 3 days, over a period of 12-15 days. After that, methanol/acid (3:1) solution was added successively to fix for 5min. Methanol was used to prepare 0.5% crystal violet and stain for 15min.

The plate was flushed gently with distilled water, and then dried at room temperature. The number of colonies was counted manually, with a colony with more than 50 cells being considered as a viable colony. This process was repeated for three times. Western blot assay was conducted to determine the effectiveness of siRNA transfection.

Cell viability test

Through a MTT test, the cell activity was measured. After transfection, the cells were seeded in a 96-well plate. After 24h of culture, 10μl MTT reagent was added to each well.

The cells were cul-tured with a mixture of MTT solution/medium for 4 hours. After incubation, 100μl solubilizing solution was added to each well for incubation overnight.

The MTT results were evaluated by the absorbance at the wavelength of 490 nm, and the results were verified by a western blot assay.

ROS and NADPH in the cells

The cells were inoculated in a 24-well plate and cultured overnight, with 2×104 cells in each well. After the re-moval of medium, the cells were washed with phosphate buffered saline (D-PBS) twice.

After trypsin digestion, the cells were centrifuged at 5000 rpm for 4min, washed with cold D-PBS, resuspended in PBS containing 20 mM glucose and 2μM DCF-DA, and then cultured at 37°C for 25min. The absorbance was measured at a wavelength of 490nm and 520nm with a spectrophotometer (Perkin Elmer LS-55, USA).

Statistical treatment

SPSS24.0 software was selected to analyze data. The measurement data were compared by a t-test and ANOVA and expressed as ($\bar{x}\pm s$). The significance level was $\alpha=0.05$.

Results

Establishment of A549/DOX

After DOX treatment, the cell viability of A549/DOX cell line was significantly higher than that of the original A549 cell line (P<0.05). See Table 1.

Group	DOX0.5μM	DOX1.0μM	DOX1.5μM	DOX2.0μM
Original A549 cell line	90.27±16.74	70.68±7.31	44.68±14.08	17.62±6.45
A549/DOX cell line	103.39±20.80	92.26±15.70	68.26±20.40	64.84±17.42

Table 1: Comparison of cell viability between A549 cell line and A549/DOX cell line (%).

Effect of TIGAR knockout on cell viability and colony survival

The TIGAR protein in the siTIGAR transfection group was significantly lower than that in the control group (P<0.05).

At the same time, the cell viability and colony survival of A549/DOX cell line in siRNA transfection group were signifi-cantly lower than those in the control group (P<0.05). See Table 2.

Group	Relative density (TIGAR/GADPH)	Colony survival	Cell viability (%)	DCF-DA	Relative level of NADPH
Control group	1.04±0.16	61.59±17.08	1.03±0.21	53.27±7.46	0.82±0.25
siTIGAR transfection group	0.33±0.09	27.90±8.71	0.67±0.13	77.32±10.20	0.58±0.13

Table 2: Effect of TIGAR knockout on cell viability and colony survival of A549/DOX cell line.

Effect of TIGAR knockout on the levels of ROS and NADPH

The level of ROS in the siTIGAR transfection group was significantly higher than that in the control group (P<0.05).

The level of NADPH in the siTIGAR transfection group was significantly lower than that in the control group (P<0.05). See Table 3.

Group	DCF-DA	Relative level of NADPH
Control group	53.27±7.46	0.82±0.25
siTIGAR transfection group	77.32±10.20	0.58±0.13

Table 3: Effect of TIGAR expression on the levels of ROS and NADPH.

Effect of TIGAR knockout on the expressionS of apoptosis-related markers

The levels of Bcl-2 and PARP in the siTIGAR transfection group were significantly lower than those in the control group (P<0.05). The levels of Bax, Caspase-3, Caspase-9 and PARP degradation

products in the siTIGAR transfection group were significantly higher than those in the control group (P<0.05). See Table 4.

Group	Relative expression of Bcl-2	Relative expression of Bax	Relative expression of Caspase-3	Relative expression of Caspase-9	Relative expression of PARP	Relative expression of PARP Degradation Products
Control group	0.94±0.39	0.98±0.34	0.96±20.62	1.02±0.29	1.03±0.39	0.97±0.20
siTIGAR transfection group	0.46±0.15	1.38±0.51	1.44±23.20	1.37±0.42	0.80±0.15	1.24±0.35

Table 4: Effect of TIGAR knockout on the expressions of apoptosis-related markers.

Effect of TIGAR knockout on epithelial/mesenchymal markers

The level of E-caderin in the siTIGAR transfection group was significantly higher than that in the control group (P<0.05). The level of vimentin in the siTIGAR transfection group was significantly lower than that in the control group (P<0.05). See Table 5.

Group	E-caderin	Vimentin
Control group	1.03±0.10	1.01±0.25
siTIGAR transfection group	1.27±0.19	0.54±0.13

Table 5: Effect of TIGAR knockout on the expressions of apoptosis-related markers.

Discussion

Today, NSCLC chemotherapy re-sistance has become an important factor restricting the overall survival benefits of patients. Among them, acquired re-sistance can be formed after DOX is used for a long term[10]. How to search for new drugs and therapies for patients with lung cancer chemotherapy resistance has become a hot issue in the medical com-munity. TIGAR is a transcriptional target of P53 gene that plays an important role in the regulation of programmed cell death[11]. Previous research results suggested that TIGAR can inhibit glycolysis and promote the PPP process[12]. The disorder of the levels of glycolysis and PPP may provide sufficient metabolic energy, nucleotide, amino acid and fatty acid, etc. for tumor cells to proliferate. TIGAR plays a role in glycolysis and PPP regulation by converting fructose 2, 6-bisphosphate into fructose-6-phosphate[13]. The effect of TIGAR on glucose metabolism is closely related to the regulation of the activity of glucose-6-phosphate dehydrogenase (G6PD)[14]. TIGAR has been shown to influence the transformation of glucose metabolic pathway from anaerobic oxidative phosphorylation to aerobic glycolysis, so it may be a key factor af-fecting the malignant metabolic abnormality of regulatory mechanism[15]. Enhanced TIGAR expression may relieve the autophagy injury of neurons under high glucose and regulate the activity of G6PD effectively[16]. Nevertheless, no definite conclusion has been reached as to whether TIGAR can influence chemotherapy resistance of tumor cells by reg-ulating metabolic pathway.

Previous studies showed that TIGAR can be transported to mitochondria to get involved in the regulation of mitochondrial membrane potential and maintain redox homeostasis[17]. Thus, it is speculated that TIGAR's ability to regulate the energy metabolism of tumor cells may influence drug resistance during cancer treatment. Some scholars contend that TIGAR is able to stimulate the transformation of epithelial cells of NSCLC into mesenchymal cells, lower the expressions of EMT transcription factor and E-caderin, and significantly increase the expression of vimentin[18]. What's more, the overexpression of TIGAR is likely to weaken the glycolytic ability of tumor cells and induce MCF7 cells' tamoxifen resistance[19]. In recent years, studies have revealed that TIGAR is related to the occurrence of chemotherapy resistance. TIGAR is overexpressed in bortezomib-resistant multiple myeloma cells and dasatinib-resistant chronic lymphocytic leukemia cells[20, 21]. The overexpression of TIGAR can result in enhanced mitochondrial activity, post-pone the apoptosis process of tumor cells and speed up the EMT phenotype switching of NSCLC cells[22].

Our study implied that TIGAR knockout can inhibit the EMT phenotype switching and apoptosis process of DOX-resistant lung cancer cell line-A549 cell line. Mean-while, TIGAR was highly expressed in DOX-resistant lung cancer cells, which was consistent with previous reports and further proved that the expression of TIGAR was probably related to the metastasis of tumor cells and can be used as a potential therapeutic target for acquired DOX resistance[23]. A large number of experiments and clinical studies verify that the overpro-duction and accumulation of ROS/RNS play a key role in each stage of the oc-currence and development of malignant tumor[24-26]. The rise of ROS level may induce DNA damage and initiate the pathway of programmed cell death. TIGAR has been proved to scavenge ROS in a quick and effective way. The fall of ROS level may aggravate DNA damage[27]. According to the results of this study, TIGAR knockout can effectively improve the intracellular level of ROS, and significantly lower the level of NADPH,

while the number of colonies decreases accordingly. Apoptosis belongs to programmed cell death. It is currently believed that its occurrence is regulated by multiple cascade signaling pathways, including the Bcl-2 family members. The overexpression of Bcl-2 is likely to inhibit apoptosis of a variety of solid tumor cells, and closely related to acquired drug resistance[28]. The down-regulation of Bax can accelerate the malignancy of cells and promote the cells' resistance to chemotherapy. The activation of Caspase-3 plays a vital role in the cell death pathway mediated by mitochondria and possibly results in activation of pro-apoptotic PARP and induce apoptosis[29]. As indicated by our findings, TIGAR knockout can lower the expression level of Bcl-2 and increased the expressions of Bax and Caspase-9 in A549/DOX cells. In addition, TIGAR knockout also significantly improve the levels of Caspase-3 and PARP protein, while is probably re-lated to the promotion of A549/DOX apoptosis after TIGAR knockout[30].

According to the results of this study, the down-regulation of the whole PARP expression implies that compared with A549/DOX cell line, the ability of TIGAR-deficient A549-DOX cell line to repair DNA damage is weakened. PARP-1 is a key transcription regulator in vivo and participates in the EMT pathway of tumor cells. The augment of PARP degradation products in TIGAR knockout A549/DOX cell line supports the change in the apoptosis of untreated A549-DOX cell line[31]. Foreign scholars contend that TIGAR is one of the important modifiers of the reaction of PARP inhibitor, which suggests that there is a correlation be-tween the functions of TIGAR and PARP[32].

EMT plays a critical role in the reg-ulation of embryonic development, repair of tissue damage, the invasion and me-tastasis of tumor. The decrease of the expression of E-cadherin, a kind of cal-cium-dependent adhesion protein in ep-ithelial cells, is one of the major features of EMT, and also an important precon-dition for the loss of stability of epithelial cell-cell adhesion[33]. In addition to the down-regulation of epithelial biomarkers, the up-regulation of mesenchymal bi-omarkers, for example, vimentin, is also strongly linked with the invasion and metastasis of lung cancer cells. Currently, the role of EMT in the progression and drug resistance of a variety of tumors, such as pancreatic cancer, bladder cancer and breast cancer, has been verified[34]. In out work, in TIGAR knockout A549/DOX cells, the expression of vimentin decreased, while the expression of E-cadherin grew, which demonstrated that the inhibition of TIGAR can effectively reverse the EMT process. Previous studies showed that chemotherapeutic drugs may stimulate the up-regulation of TIGAR, and TIGAR-mediated NADPH ensured the survival and proliferation of tumor cells by repairing post-chemotherapy injury[35, 36]. NADPH is a major ROS scavenger and PPP metabolite. There are reports that TIGAR can directly reduce the migration of tumor cells by inhibiting PPP-mediated TIGAR.

To sum up, TIGAR knockout can inhibit the EMT of DOX-resistant NSCLC and be used as a potential therapeutic target for patients with chemotherapy resistance.

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