Original Article
Dicer reverses the chemotherapy drug resistance in lung cancer cells and is negatively regulated by EZH2

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Abstract: Drug resistance is one of the major obstacles to limit the efficiency of chemotherapy in lung cancer patients. Dicer is a cytoplasmic Rnase III endonuclease and plays a key role in the biosynthesis of miRNAs and siRNAs to regulate the progression and invasion of cancer, however, the role of Dicer in drug resistance of lung cancer cells was still not clarified. In the present study, the Dicer levels in three lung cancer cell lines were detected by western blotting analysis. The results demonstrated that decreased levels of Dicer were observed in H1299 cells, H460 cells, and A549 cells, compared with WI38 cells. Moreover, we used cisplatin-resistant A549/DDP cells and the parental cisplatin-sensitive A549 cells as cell models. Western blotting analysis result showed the level of Dicer in A549/DDP cells was significantly decreased compared with that in A549 cells (**P<0.01). Knocking down the expression of Dicer promoted cell proliferation in A549 cells and Dicer was mainly located in the cytoplasm of lung cancer cells. We further to explore the molecular mechanism of Dicer in drug resistance by western blotting analysis. Dicer levels were negatively associated with P-glycoprotein (Pgp) and enhancer of zeste homolog 2 (EZH2). Importantly, the level of Dicer was gradually increased as the increasing concentration of DZNep, the inhibitor of EZH2. All the data demonstrated that lower levels of Dicer contributed to chemotherapeutic drug resistance in lung cancer cells and it was negatively regulated by EZH2 in the progression of lung cancers. Dicer might be a potential target in the course of drug-resistant chemotherapy of lung cancers.

Keywords: Lung cancer, dicer, EZH2, drug resistance

Introduction

Lung cancer, also known as primary bronchogenic carcinoma, is a malignant tumor that originated in the bronchial mucosal epithelium [1]. There are mainly two types of lung cancer including non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for about 85% of lung cancer and SCLC accounts for 15% of lung cancer [2]. Currently, NSCLC is the commonly seen lung cancer with higher morbidity and mortality rates, especially in developed countries. The clinical progress of NSCLC is slower than SCLC. Comprehensive treatment is usually used in the therapy of NSCLC, including surgery, radiotherapy, chemotherapy and molecular targeted therapy [3, 4]. Chemotherapy is one of the main methods to treat human cancers, however, the effect is gradually less efficient and the prognosis is poor due to the chemotherapeutic drug resistance [5, 6]. Thus, it is important to clarify the mechanism of occurrence, development, invasion and metastasis, especially chemotherapeutic drug resistance in lung cancers.

Dicer is a cytoplasmic endonuclease, which belongs to Rnase III family. It is a key enzyme in the biosynthesis of miRNAs and siRNAs [7, 8]. Normally, mature endogenous miRNAs get through the following ways: the genes encoding miRNAs are located in introns of host genes
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and share their regulatory elements [9]. Primary miRNAs (pri-miRNAs) are transcribed by RNA polymerase II as large RNA precursors [10]. Then, the pri-miRNAs are processed in the nucleus by multiple steps and the key components are including the RNase III enzyme Drosha4, and the double-stranded-RNA-binding protein, Pasha/DGCR85 [11, 12]. Next, pri-miRNAs are translocated into the cytoplasm by the karyopherin exportin 5 (Exp5) and Ran-GTP complex6, which is processed by the RNase III enzyme Dicer in cytoplasm to generate the mature miRNA with a double-stranded RNA approximately 22 nucleotides in length [13].

Till now, several paper has claimed that the expression level was involved in the tumorigenesis and dysregulation of Dicer plays an important role in the progression and invasion of various cancers, such as gastric cancer [14], prostate cancer [15, 16], breast cancer [17, 18], colorectal cancer [19, 20], ovarian cancer [21, 22], epithelial skin cancer [23], bladder cancer [24]. Moreover, the levels of Dicer worked as an indicator of prognosis for cancers [25, 26]. Zhao, H. has found that Dicer expression was decreased in cervical cancer tissues, and the lower expression levels of Dicer in cancer tissues were associated with advanced tumor stages (P = 0.03) and with metastasis (P = 0.01) [27]. Lombard, A. P. has also found that Dicer protein ablation leads to increased cell invasion, higher levels of matrix metalloproteinase-2, and decreased levels of key miRNAs shown to inhibit invasion in bladder cancer cells, suggesting that decreased Dicer levels may portend a more malignant phenotype [28].

However, it is not clearly clarified that the relationship between Dicer levels and chemotherapeutic drug resistance in lung cancer cells. In the present study, we explored the role of Dicer in cisplatin resistant lung cancer cells A549/DDP and further to investigate the molecular mechanism of Dicer in A549/DDP cancer cells. It would give new clues and be used as a theoretical reference for the drug resistance of lung cancer.

Materials and methods

Cell lines and agents

The human non-small cell lung cancer cell line H1299, human large cell lung cancer cell line H460, human lung adenocarcinoma cell line A549 was kept in our laboratory. The normal control cell line WI38 was obtained from Ruiqi Biological reagents Ltd. (Shanghai, China). The lung cancer cells were cultured in Dulbecco’s Modified Eagles Medium (DMEM) with 10% fetal bovine serum (FBS). The DMEM medium and FBS were purchased from hyclone corporation (Logan, Utah, USA). The chemotherapy drug Cisplatin was obtained from Sigma Aldrich (St. Louis, MO). The MTT agent, 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (Cat. No. M2128) was purchased from Sigma-Aldrich Corporation. Nuclear and cytoplasmic protein extraction kit (Cat. No. P0028) was obtained from Beyotime Institute of Biotechnology (Shanghai, China). Enhanced chemiluminescence (ECL) reagents were from Amersham Life Science Co., Ltd.

Cell transfection

The lung cancer cells (2×10^4 cells per well) were planted into 24-well plate. After 8 hours, the cells were transfected by Dicer shRNA and negative control shRNA. Human Dicer shRNA Plasmid (Cat. No. sc-40489-SH) and Control shRNA Plasmid-A (Cat. No. sc-108060) was obtained from Santa Cruz. Transfection of cells was performed by using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol.

MTT assay

Cell viability was determined by MTT assay. In one experiment, A549 cells and A549/DDP cells were plated into 96-well plate. The cells were cultured for 8 hours to adhere to the plate and treated with increasing concentrations of cisplatin for 24 h, 48 h and 72 h, respectively. In another experiment, lung cancer cell line A549 was plated into 48-well plate and transfected with Dicer shRNA and negative control shRNA for 48 hours and 72 hours, and cell viability was determined by MTT assay. Then, four hours before test, 10 μL of MTT solution (5 mg/mL) was added into the cultured medium of lung cancer cells and re-incubated at 37°C in a atmosphere of 5% CO₂. The absorbance of each well was tested and the value was read at 490 nm.

Antibodies

Anti-Dicer antibody (ab13502) was a rabbit polyclonal IgG and obtained from Abcam corpo-
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ration. Anti-β-actin antibody was purchased from TransGen Biotechnology (Beijing, China). P-gp P-glycoprotein antibody (ab36743) was obtained from Abcam. Anti-EZH2 Antibody (Catlog. No. #4905) was obtained from Cell Signaling Technology. Anti-histone H3K27me3 antibody (Cat. No. A15024) was a polyclonal rabbit IgG and purchased from Thermo Fisher Scientific Corporation. Anti-β-tubulin antibody (Cat. No. ab6046) and anti-Lamin B1 antibody (Cat. No. ab74393) was a polyclonal rabbit IgG and obtained from Abcam corporation.

Western blotting analysis

The cell lysates were prepared by RIPA buffer and the total proteins were separated with 10% SDS-PAGE. The total proteins were transferred to PVDF membrane at constant 400 mA for 2 hours and the membrane was blocked with 5% non-fat milk in PBST buffer. Then, the membrane was incubated with primary antibody overnight at 4°C. The membrane was washed for 3 times with PBS buffer, each time for 10 min. The membrane was incubated with HRP-conjugated secondary antibody for 30 min at room temperature. At last, chemiluminescent ECL reagent kit (Millipore) was used for development.

Statistical analysis

The MTT assay was set three replicates and repeated twice. The data was analyzed by SPSS20.0 software (SPSS Inc., Illinois, USA), which was shown as mean value ± S.D. P<0.05 was considered as statistically different.

Results

Decreased levels of Dicer are observed in lung cancer cell lines

Dicer, an RNase III-family nuclease, plays an important role to initiate RNA interference (RNAi). In order to detect the levels of Dicer in lung cancer cells, western blot was used to test the levels of Dicer in lung cancer cell lines, including H1299 (human non-small cell lung cancer cell line), H460 (human large cell lung cancer cells) and A549 (human lung adenocarcinoma cells), and normal control cell line WI38. As shown in Figure 1, the level of Dicer was significantly decreased in lung cancer cell lines than that in WI38 cells (**P<0.01). The results demonstrated that dicer might work as a tumor suppressor gene during the lung carcinogenesis.

Lower level of Dicer contributes to chemotherapeutic drug resistance in lung cancer cells

In order to identify whether Dicer was involved in the drug resistance in lung cancer cells, we used DDP-sensitive cell line A549 and its parental subline A549/DDP as cell models. Inhibition rate of DDP in A549 and A549/DDP
was determined by MTT assay, and IC50 value was calculated by Graphpad 5.0 software. As shown in Figure 2A, IC50 value of DDP in A549 cells was 97.4 μM, 33.1 μM and 11.30 μM in 24 h, 48 h and 72 h, respectively. Comparatively, IC50 value of DDP in A549/DDP cells was 490.9 μM, 129.0 μM and 71.27 μM in 24 h, 48 h and 72 h, which was 5.04 fold, 3.90 fold and 6.31 fold of that for A549 cells, respectively.

Then, we detected the expression level of Dicer in DDP-sensitive A549 cells and its parental DDP-resistant A549/DDP cells by western blotting analysis. As shown in Figure 2B, the level of Dicer in A549/DDP cells was significantly decreased compared with that in A549 cells (**P<0.01), suggesting that lower levels of Dicer may contribute to drug resistance in lung cancer cells.

Knock down the expression of Dicer promotes cell proliferation in A549 cells

In order to further confirm the role of Dicer in lung cancer cells, we used Dicer shRNA to knock down the endogenous Dicer in A549 cells. Western blotting results demonstrated that the levels of Dicer were obviously decreased after transfection for 48 hours in A549 cells (Figure 3A), and there was significant statistical difference between the cells transfected with Dicer shRNA and negative control shRNA (**P<0.01).

Moreover, cell viability was detected by MTT assay. A549 cells were transfected with Dicer shRNA and negative control shRNA and cultured for 48 hours (Figure 3C) and 72 hours (Figure 3D). The OD490 value was detected.
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and the results demonstrated that the cell viability was significantly increased in Dicer shRNA-transfected cells than that in negative control shRNA-transfected cells. The results demonstrated that knock down the expression of Dicer would significantly promote the cell proliferation in lung cancer cells.

Transfection with Dicer specific shRNA obviously decreases the levels of cytoplasmic Dicer

In order to determine the dicer location in lung cancer cells, the total proteins in cytoplasm and nucleus were extracted according to kit protocols and western blotting was used to determine the cytoplasmic and nuclear level of Dicer in lung cancer cells. As shown in Figure 4, Dicer was mainly located in cytoplasm of lung cancer A549 cells. Moreover, lung cancer A549 cells were transfected with Dicer shRNA for 48 hours. The level of Dicer in cytoplasm was obviously decreased, while the amount of Dicer in nucleus was not obviously changed.

The multi-drug resistance associated protein, P-glycoprotein was the first cloned resistance protein and usually highly expressed in drug resistant cancer cells. In the present study, we detected the expression levels of Pgp in cisplatin-sensitive A549 cells and cisplatin-resistant A549/DDP cells by western blotting analysis. As shown in Figure 5, the results demonstrated that A549/DDP cells had a lower expression level of Dicer and a higher level of Pgp, suggesting that A549/DDP cells could be sensitized to...
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Figure 4. Transfection with Dicer specific shRNA obviously decreases the levels of cytoplasmic Dicer. A549 cells were transfected with Dicer shRNA and negative control shRNA for 48 hours. The cytoplasmic and nuclear proteins were extracted according to the kit protocols. The expression levels of Dicer in cytoplasm and nucleus of A549 cells were detected by western blotting analysis.

Figure 5. The resistance protein P-glycoprotein (Pgp) expression level is negatively associated with Dicer level. A. A549 cells were plated into 48 well-plate and cultured for 24 hours. The levels of Dicer and Pgp were detected by western blotting analysis. B. The bands were captured and analyzed by Graphpad 5.0 software. The gray value of Dicer and Pgp was shown in histogram. **P<0.01, compared with that of A549 cells.

The chemotherapeutic drug DDP by up-regulating the levels of Dicer or down regulating the level of multidrug resistance protein Pgp.

Dicer was negatively associated with EZH2 and regulated by EZH2 in lung cancer cells

Polycomb protein histone methyltransferase enhancer of Zeste homologue 2 (EZH2) is reported to contribute to the progression of tumors and frequently overexpressed in human malignancies. We further explored the relationship between Dicer and EZH2 in human lung cancer cells. As shown in Figure 6A, the expression level of EZH2 in A549 was obviously lower than that in A549/DDP cells. However, the level of Dicer in A549 was higher than that in A549/DDP cells. The data demonstrated that the
expression of EZH2 contributed to drug resistance in lung cancer cells and which was negatively correlated with the level of Dicer.

Next, the lung cancer A549 cells were transfected with Dicer shRNA for 48 hours, and western blotting analysis was used to determine whether knock down the endogenous Dicer would affect the expression level of EZH2 in A549 cells. As shown in Figure 6B, the level of EZH2 in Dicer shRNA-transfected cells was not obviously changed compared with that in negative control shRNA-transfected A549 cells.

In order to further determine that whether EZH2 regulated the role of Dicer in lung cancer cells, we used 3-deazaneplanocin A (DZNep) to treat A549 cells, which was a histone methyltransferase inhibitor and selectively suppressed trimethylation of lysine 27 on histone H3 (H3K27me3). The lung cancer cells were treated with increasing concentrations (0 μM, 0.1 μM, 1.0 μM and 10 μM) of DZNep for 24 hours. As shown in Figure 6C, the expression levels of EZH2 was gradually decreased as the increasing concentration of DZNep, as well as the level of H3K27me3. However, the level of Dicer was gradually increased as the increasing concentration of DZNep. All the results demonstrated that the expression level of Dicer was negatively regulated by EZH2 in the upstream of Dicer.

Discussion

The dysregulation of several miRNAs is now observed and has been extensively studied in lung cancers, such as miRNA-296-3p [29], miRNA-26b [30], miRNA-1271 [31], miRNA-1469 [32], miRNA-200c [33], etc. DICER is the key enzyme that cleaves pre-microRNAs into 21-25 nucleotide duplex in the differentiation and development [34, 35]. It has been reported that reduced expression of Dicer associated with poor prognosis in lung cancer patients [36], however, the molecular mechanisms of Dicer in acquired chemotherapeutic drug resistance are still not clearly clarified. Till now, che-
motherapy remains one of the mostly used treatment for lung cancers, but the drug resistance still is the obstacle that make the chemotherapy do not achieve fully satisfactory outcome and prognosis. In the present study, we investigated the role of Dicer in lung cancer cells and further to explore the molecular mechanism of Dicer on chemotherapeutic drug resistance of lung cancer cells.

Firstly, we detected the levels of Dicer in three lung cancer cell lines, including human non-small cell lung cancer H1299 cells, human large cell lung cancer H460 cells, and human lung adenocarcinoma cells A549, compared with that in normal lung cells WI38, a lung-derived cell line from human embryonic fibroblast. The results demonstrated that the level of Dicer was significantly decreased in lung cancer cell lines than that in WI38 cells suggesting Dicer worked as a tumor suppressor gene during the lung carcinogenesis. This was consistent with the founding in prostate cancer that down-regulation of Dicer was associated with cell proliferation and apoptosis [16]. Next, we also used a pair of lung cancer cell lines, cisplatin-sensitive A549 cells and cisplatin-resistant A549/DDP cells, both of which were cultured from a parental subline as cell model. Western blotting analysis results demonstrated that Dicer in A549/DDP cells was significantly decreased compared with that in A549 cells (**P<0.01). Moreover, this was further confirmed by MTT assay that knocking down the endogenous level of Dicer promoted cell proliferation in A549 cells no matter in 48 hours or 72 hours. All the results obviously showed that lower levels of Dicer contributed to drug resistance in lung cancer cells.

We further to investigate the molecular mechanism of Dicer on the drug resistance of lung cancer cells. In lung cancer cell line A549 and A549/DDP cells, the expression levels of Dicer, Pgp and EZH2 was detected by western blotting analysis and the results obviously demonstrated that dicer levels were negatively associated with P-glycoprotein (Pgp) and enhancer of zeste homolog 2 (EZH2). More importantly, Dicer was regulated by the inhibitor of EZH2, DZNep, in a dose-dependent manner, which was consistent with the results from Cai, L. [37].

In conclusion, all the data revealed that lower levels of Dicer contributed to chemotherapeutic drug resistance in lung cancer cells and the level of Dicer was negatively regulated by EZH2. It was proved that Dicer could be used as an efficient target in the therapy of drug-resistant lung cancer.

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Disclosure of conflict of interest

None.

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References

[6] Xiao HL, Yang ZT, Han F and Wei HX. Association of glutathione S-transferase (GST) genetic polymorphisms with treatment outcome of cisplatin-based chemotherapy for advanced...
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[28] Lombard AP, Lim RM, Nakagawa RM, Vidalillo KD, Libertini SJ, Platero AJ and Mudryj M. Dicer ablation promotes a mesenchymal and inva-
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