ORIGINAL ARTICLE

Depleted aldehyde dehydrogenase 1A1 (ALDH1A1) reverses cisplatin resistance of human lung adenocarcinoma cell A549/DDP

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Abstract

Background: Cisplatin is the standard first-line chemotherapeutic agent for the treatment of non-small cell lung cancer (NSCLC). However, resistance to chemotherapy has been a major obstacle in the management of NSCLC. Aldehyde dehydrogenase 1A1 (ALDH1A1) overexpression has been observed in a variety of cancers, including lung cancer. The purpose of this study was to investigate the effect of ALDH1A1 expression on cisplatin resistance and explore the mechanism responsible.

Methods: Reverse transcriptase-PCR was applied to measure the messenger RNA expression of ALDH1A1, while Western blot assay was employed to evaluate the protein expression of ALDH1A1, B-cell lymphoma 2, Bcl-2-like protein 4, phospho-protein kinase B (p-AKT) and AKT. A short hairpin RNA was used to knockdown ALDH1A1 expression. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to determine the effect of ALDH1A1 decrease on cell viability. The cell apoptotic rate was tested using flow cytometry assay.

Results: ALDH1A1 is overexpressed in cisplatin resistant cell line A549/DDP, compared with A549. ALDH1A1 depletion significantly decreased A549/DDP proliferation, increased apoptosis, and reduced cisplatin resistance. In addition, the phosphoinositide 3-kinase (PI3K)/AKT pathway is activated in A549/DDP, and ALDH1A1 knockdown reduced the phosphorylation level of AKT. Moreover, the combination of ALDH1A1-short hairpin RNA and PI3K/AKT pathway inhibitor LY294002 markedly inhibited cell viability, enhanced apoptotic cell death, and increased cisplatin sensitivity.

Conclusion: These results suggest that ALDH1A1 depletion could reverse cisplatin resistance in human lung cancer cell line A549/DDP, and may act as a potential target for the treatment of lung cancers resistant to cisplatin.

Introduction

Lung cancer is the most common malignancy and the leading cause of cancer-related mortality worldwide, accounting for approximately 28% and 26% of all male and female cancer deaths, respectively.[1] Furthermore, in 2012, a total of 1.8 million new cases of lung cancer occurred, representing approximately 13% of the total cancers diagnosed.[2] In China, 546 259 lung cancer-related deaths occurred in 2013, approximately twice the number of deaths in 1990.[3] Non-small cell lung cancer (NSCLC) is the most common subtype, representing approximately 85% of all lung cancers. Despite the existence of various chemotherapeutic agents, the five-year survival rate for NSCLC remains poor at less than 15%.[4]

Cisplatin is the standard first-line chemotherapeutic agent for the treatment of human lung cancer, as well as for other cancers, and is combined with other drugs. However, the intrinsic and rapid development of acquired resistance to chemotherapy agents remains a major obstacle in the treatment of lung cancer.[5,6] Thus, one of the

most promising methods to markedly enhance chemotherapeutic efficacy in lung cancer is the reversal of cisplatin resistance. Therefore, further investigation on the mechanism responsible for cisplatin resistance is needed.

Aldehyde dehydrogenase 1A1 (ALDH1A1) is a detoxifying enzyme responsible for oxidizing intracellular aldehydes, which can inactivate integral chemotherapeutic agents.[7] ALDH1A1 overexpression is associated with poor overall and recurrence-free survival in NSCLC patients.[8] The aim of our study was to determine whether the direct targeting of ALDH1A1 by short hairpin RNA (shRNA) could enhance the chemosensitivity of lung cancer cells to cisplatin. Our results have shown that ALDH1A1 is potentially an important therapeutic target for human lung cancer cells.

Methods

Cell culture and reagents

A549, A549/DDP, PC9, and PC9/GR cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cell culture reagents were purchased from Corning (Life Technologies, Inc., Gaithersburg, MD, USA). In order to maintain cisplatin resistance, 2 μg/mL of cisplatin was added to the culture medium.

Cell transfection

A549/DDP cells were seeded in six-well plates and when the density reached 90%, cells were transfected with ALDH1A1 short hairpin RNA (shRNA) or a random sequence shRNA applied as scramble shRNA by lipofectamine 2000 (Invitrogen, San Diego, CA, USA), according to the manufacturer's protocol. ALDH1A1, shRNA, and scramble shRNA were established by Shanghai GenePharma Co., Ltd. (Shanghai, China).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

A total of 5×10^3 A549 or A549/DDP cells were plated in 96-well flat bottom plates and exposed to various concentrations of cisplatin. After 48 hours, 10 μg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in phosphate buffered saline (PBS) was added into each well for four hours. After removing the medium, 150 μL of dimethyl sulfoxide (DMSO) was added into each well to dissolve the formazan crystals. The optical density (OD) of each well was then measured using a microplate reader at 560 nm. The inhibition rate (%) after cells were treated and incubated with various concentrations of cisplatin was calculated as $[1 - (OD\ value\ in\ treatment\ group/OD\ value\ in\ control\ group)] \times 100\%$.

Reverse real-time transcriptase-PCR

Total RNA was extracted from cells using a TRIzol reagent (Invitrogen), according to manufacturer's instructions. Two micrograms of total RNA was used to synthesize complementary DNA (cDNA) using Moloney-Murine Leukemia Virus reverse transcriptase (Promega Corporation, Madison, WI, USA). Gene expression was detected by reverse transcription (RT)-qualitative PCR using FastStart Universal SYBR Green Master (Roche Diagnostics, Indianapolis, IN, USA) and gene specific primers according to standard procedures. The primers used were as follows: ALDH1A1, forward 5′-ACTCCCAAGCACGCTTAGTGCTC-3′, reverse 5′-TCGTCATGTCTTAGCCAGCT-3′; β-actin, forward 5′-CTGGCGCTAACTCGAGCACC-3′, reverse 5′-AAGTGTGGCGTGTAGGATG-3′.

Western blot assay

Total cellular proteins were extracted by lysis in radioimmunoprecipitation assay buffer and phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA), and subsequently centrifuged at 13 800g for 20 minutes. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, transferred onto a polyvinylidene fluoride membrane (Millipore Corporation, Billerica, MA, USA), blocked with 5% milk, and probed with the following primary antibodies at 1:1000 dilution in 5% nonfat milk at 4°C overnight with gentle shaking: ALDH1A1, phospho-protein kinase B (p-AKT), AKT (Cell Signaling Technology Inc., Beverly, MA, USA), B-cell lymphoma 2 (BCL; Wanlei Life Sciences, Shenyang, China), Bcl-2-like protein 4 (BAX), and β-actin (Bioworld Technology Co., Ltd., Nanjing, China). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Bioworld Technology Co., Ltd.) at 1:10 000 dilution for two hours at 4°C and visualized by an electrochemiluminescence kit (Pierce Biotechnology).

Flow cytometry assay

Cells were digested with 0.25% trypsin and fresh media were added to terminate digestion. Using an annexin V-APC/7-AAD Apoptosis Detection Kit (KeyGEN BioTECH, Nanjing, China), cells were stained according to the manufacturer's instructions after centrifuge at 352g for five minutes and twice washing with cold PBS. Finally, the cells were analyzed using FACS Calibur Flow Cytometry

(Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Statistical analysis

Data were presented as mean ± standard deviation (SD). Graphs were prepared using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Statistical significance between the experimental groups was evaluated by Student's *t*-test and $P < 0.05$ was considered significant.

Results

Aldehyde dehydrogenase 1A1 (ALDH1A1) was overexpressed in cisplatin resistant cell line A549/DDP

The cisplatin resistance of A549/DDP was first confirmed through MTT assay. Cell proliferation was higher in A549/

DDP compared with A549 when treated with different concentrations of cisplatin (Fig 1a). The inhibitory concentration (IC)50 values of A549 and A549/DDP were 12.98 ± 2.13 μM and 105.91 ± 3.70 μM, respectively (Fig 1b). The expression level of ALDH1A1 was measured by RT-qPCR and Western blot assay. A549/DDP and PC9/GR cells expressed higher expression of ALDH1A1 than their parent cells (A549 and PC9) in messenger (m) RNA or protein levels (Fig 1c,d). These results revealed that ALDH1A1 is associated with cisplatin resistance.

ALDH1A1 knockdown could inhibit proliferation and enhance apoptosis in A549/DDP cells

A shRNA was designed to decrease the expression of ALDH1A1. RT-qPCR and Western blotting were used to evaluate the knockdown effect of ALDH1A1-shRNA. ALDH1A1 expression markedly declined in both mRNA
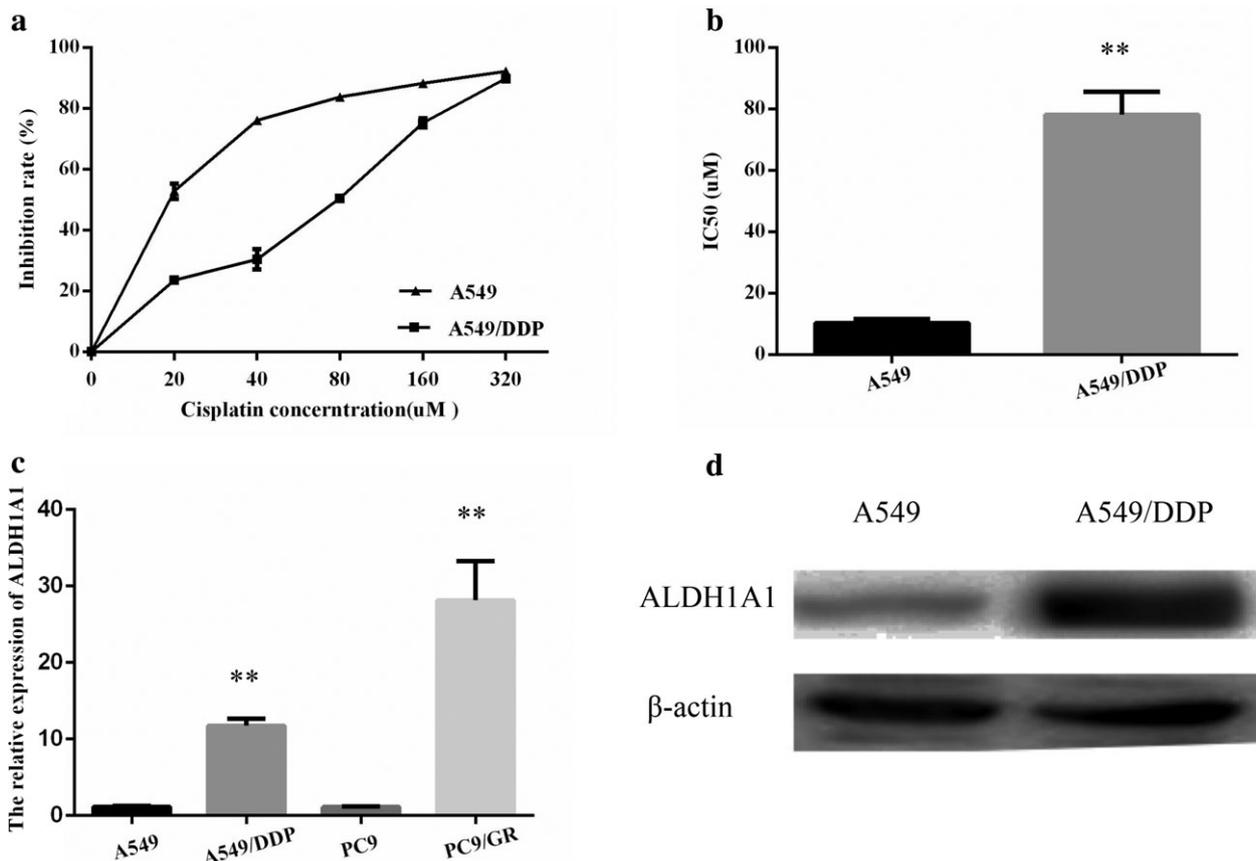
Figure 1 Inhibitory concentration (IC)50 and aldehyde dehydrogenase 1A1 (ALDH1A1) expression level of cisplatin resistant cells, A549/DDP, and its parent cells, A549. (**a**) A549 and A549/DDP were treated with various concentrations of cisplatin (0, 20, 40, 80, 160, 320 μM). (**b**) IC50 of A549 and A549/DDP to cisplatin. (**c**) Reverse transcriptase-qualitative PCR was used to explore ALDH1A1 messenger RNA expression levels in A549, A549/DDP, PC9 and PC9/GR cells; β-actin was used as an internal control. (**d**) Western blot to evaluate ALDH1A1 protein expression in A549 and A549/DDP cells; β-actin was used as an internal control. $P < 0.05$ was considered significant (*$P < 0.05$, **$P < 0.01$).

and protein levels (Fig 2a,d). In order to explore the effect of ALDH1A1 knockdown on A549/DDP cell resistance to cisplatin, MTT assays were performed. The data demonstrated that the proliferation of A549/DDP cells transfected with ALDH1A1-shRNA and sensitivity to cisplatin were significantly suppressed compared with the control group (Fig 2b,c). Apoptosis related gene BCL-2 expression was decreased, while BAX expression was increased in the shRNA group compared with the scramble shRNA group. In addition, cells with decreased ALDH1A1 expression
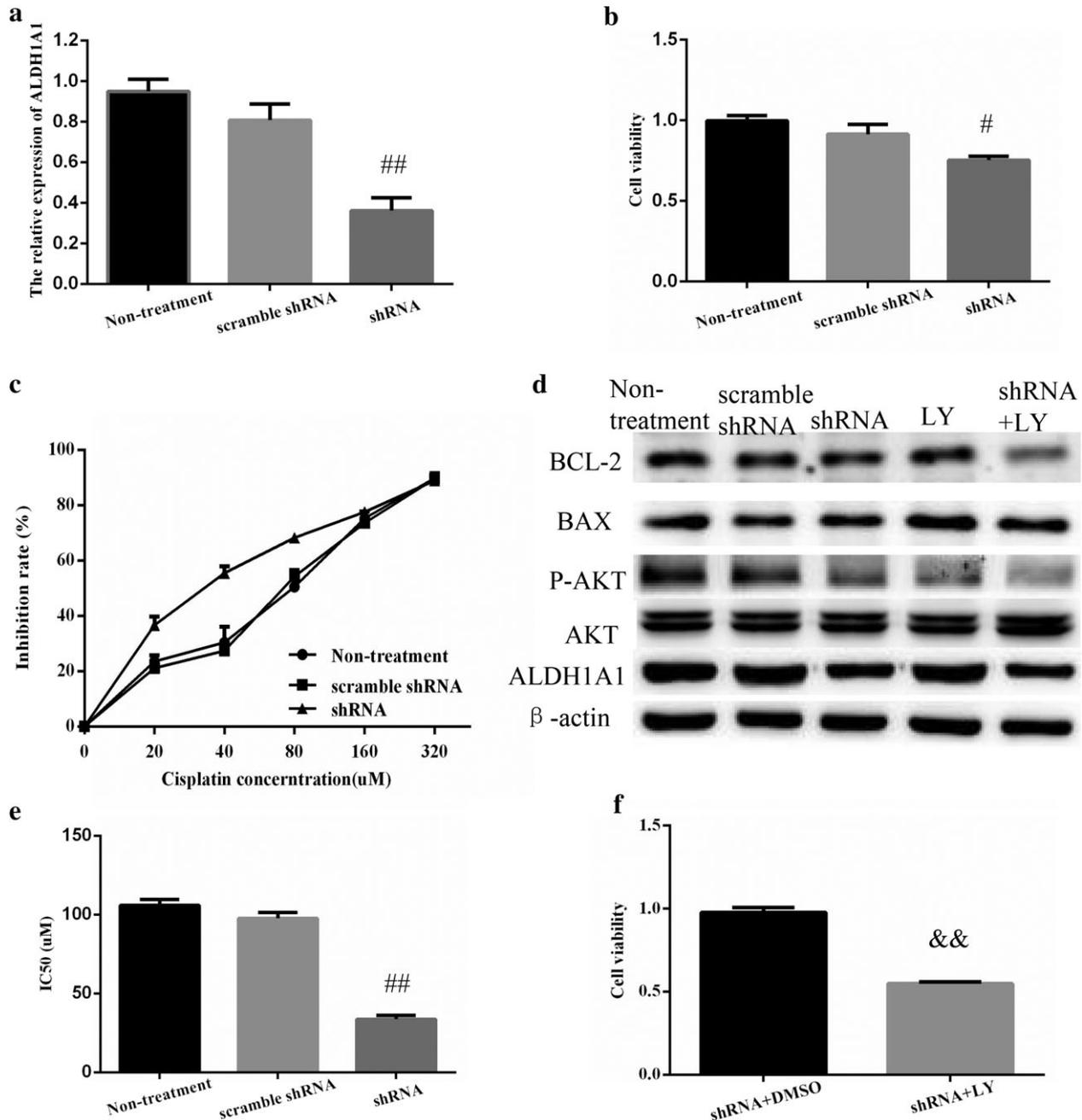
Figure 2 Aldehyde dehydrogenase 1A1 (ALDH1A1)-short hairpin (sh)RNA inhibits ALDH1A1 expression, cell proliferation, and cisplatin resistance in A549/DDP cells. (**a**) Cells were collected for reverse transcriptase-qualitative PCR to test ALDH1A1 expression. (**b**) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to measure cell proliferation ability. (**c**) MTT assay to evaluate the effect of ALDH1A1 decrease on cisplatin resistance. (**d**) Western blot assays to detect B-cell lymphoma 2 (BCL-2), Bcl-2-like protein 4 (BAX), phospho-protein kinase B (p-AKT), AKT and ALDH1A1 expression. (**e**) Inhibitory concentration (IC)50 values of cells transfected with scramble shRNA or shRNA. (**f**) Cell viability markedly declined when treated with LY294002. (#, compared with scramble shRNA group; &, compared with shRNA + dimethyl sulfoxide (DMSO) group; #, & $P < 0.05$; ##, && $P < 0.01$.)

were more sensitive to cisplatin, as IC50 values of cisplatin were lower in ALDH1A1-shRNA transfected cells. IC50 values of cisplatin decreased from 97.64 ± 3.64 μM to 33.65 ± 2.42 μM after ALDH1A1 decreased in A549/DDP cells (Fig 2e). Apoptosis in ALDH1A1-shRNA-tranfected cells was detected by flow cytometry. The apoptotic rate in the shRNA group was significantly higher compared with the scramble shRNA group (Fig 3c,d).

ALDH1A1 depletion could inhibit activation of the PI3K/AKT signal pathway, induce apoptosis, and decrease cell proliferation and cisplatin resistance

Western blot assay was used to investigate the impact of ALDH1A1 knockdown on phosphoinositide 3-kinase (PI3K)/AKT signal pathway activity in A549/DDP cells. As displayed in Figure 2d, p-AKT expression in A549/DDP cells transfected with ALDH1A1-shRNA was significantly suppressed, compared with the scramble shRNA group. In order to explore whether LY294002, a PI3K inhibitor, could improve the sensitivity of ALDH1A1-suppressed A549/DDP cells to cisplatin, the proliferation and IC50 value of cells treated with DMSO or 10 μM of LY294002 with downregulated ALDH1A1 expression was measured by MTT. As shown in Figure 2f, proliferation in the DMSO group was significantly higher than in the LY294002 group. The cell proliferation and IC50 value of the DMSO group was higher than in the LY294002 group, showing that cells treated with LY294002 were more sensitive to cisplatin than DMSO (Fig 3a,b). Apoptosis in the ALDH1A1-shRNA-tranfected cells treated with DMSO or LY294002 was detected. The apoptosis rate increased from $28.02 \pm 1.03\%$ to $38.14 \pm 4.19\%$ demonstrating that
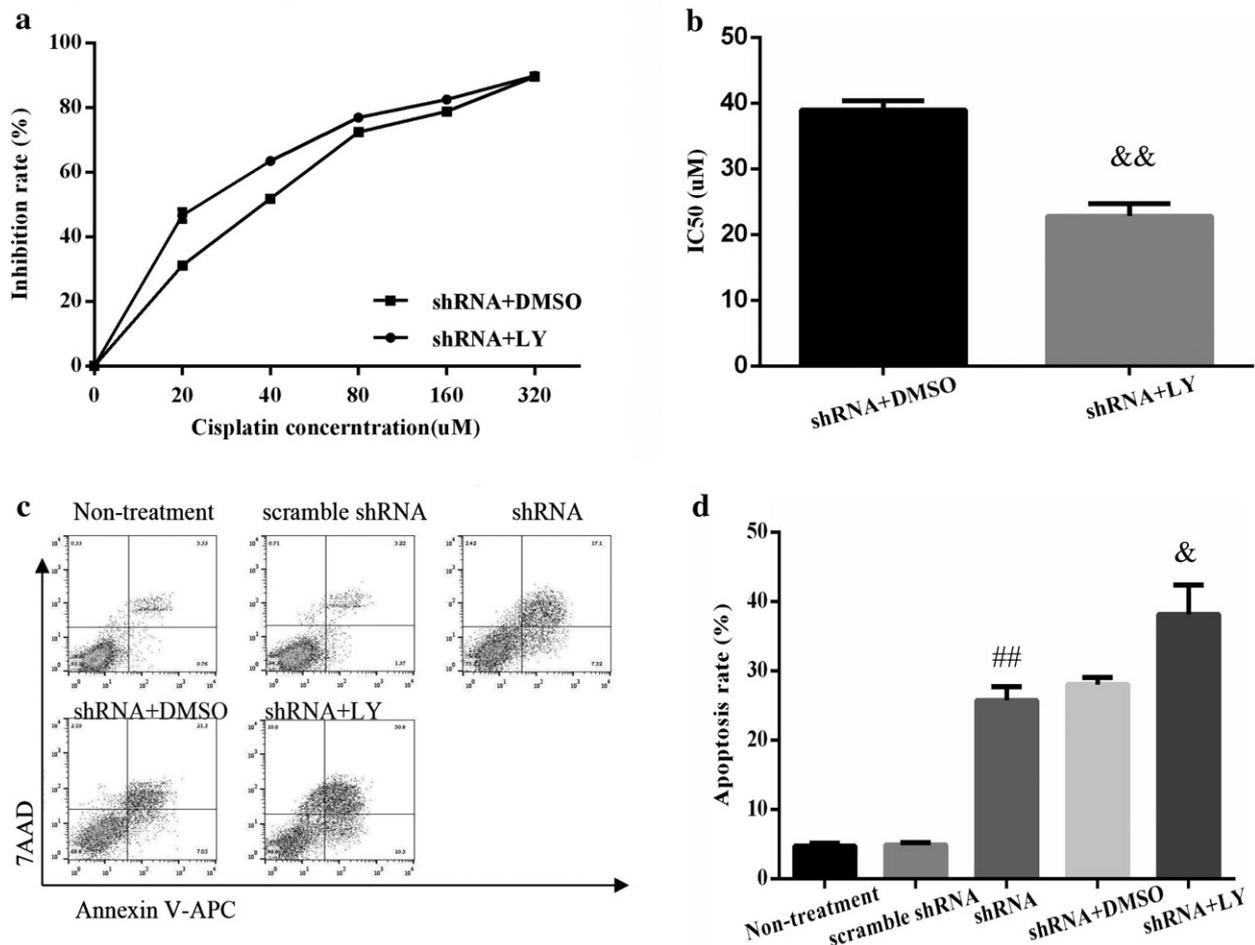
Figure 3 The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signal pathway is associated with cisplatin resistance and aldehyde dehydrogenase 1A1 (ALDH1A1) knockdown could inhibit AKT phosphorylation. (**a,b**) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to measure the effect of LY294002 on cisplatin sensitivity and inhibitory concentration (IC)50 of A549/DDP cells with decreased ALDH1A1 expression. (**c,d**) Flow cytometry analysis of non-treatment, scramble short hairpin (sh)RNA, shRNA, shRNA + dimethyl sulfoxide (DMSO), shRNA + LY group. (#, compared with scramble shRNA group; &, compared with shRNA + DMSO group; #, & $P < 0.05$; ##, && $P < 0.01$.)

LY294002 could induce apoptosis in A549/DDP cells transfected with ALDH1A1-shRNA (Fig 3c,d).

Discussion

Non-small cell lung cancer is one of the most fatal malignancies with the highest prevalence among all tumors worldwide. Cisplatin is one of the most important chemotherapeutic agents in the clinical management of NSCLC. The development of cisplatin resistance has been a major barrier in the treatment of NSCLC. Thus, investigation of the mechanisms responsible for cisplatin resistance is urgently needed.

Aldehyde dehydrogenase 1A1 is an intracellular enzyme that is widely expressed in adult organs, such as the liver, kidney, brain, and lungs. It oxidizes retinaldehyde to retinoic acid, and thereby regulates a variety of biological processes, such as cell proliferation, differentiation, and apoptosis.[9,10] Previous studies have shown that ALDH1A1 expression is associated with tumor progression and poor prognosis in several cancers, including lung, gastric, ovarian, and breast cancers.[8,11–13] A retrospective analysis of ALDH1A1 expression in 205 early stage NSCLC patients was conducted using immunohistochemistry. ALDH1A1 overexpression was found in 68.7% of primary tumors and predicted poor survival.[8] Another study, including data of 921 ALDH1A1+ breast cancer patients and 2353 controls from 15 studies analyzed the relationship between ALDH1A1 protein expression and clinicopathological and prognostic parameters.[14] The results demonstrated that higher ALDH1A1 expression is associated with tumor progression and poor prognosis. Tumor size, histological grade, and the possibility of lymph node metastasis (LNM) were higher in ALDH1A1+ than in ALDH1A1-cases.[14] Recently, several studies have demonstrated that ALDH1A1 also plays an important role in drug resistance.[15,16] Decreased ALDH1A1 expression by appropriate small interfering RNA could increase 4-hydroperoxycyclophosphamide (4-HC)-mediated toxicity in lung cancer cell line A549. Furthermore, all-trans retinoic acid downregulates ALDH1A1 expression and upregulates 4-HC cytotoxicity.[15]In addition, high ALDH1A1 expression is related to cyclophosphamide resistance in breast cancer patients.[16]

Cisplatin plays a vital role in NSCLC treatment; however, cisplatin resistance in NSCLC remains a challenge. Whether ALDH1A1 is associated with cisplatin resistance in NSCLC cell line A549 and the probable mechanism is not yet known. In this study, we explored the potential role of ALDH1A1 in the maintenance of cisplatin resistance. ALDH1A1 was overexpressed in A549/DDP cells compared with their parent A549 cells. We examined ALDH1A1 expression in gefitinib resistant PC9/GR cells and their parent PC9 cells, which showed that ALDH1A1 was overexpressed in PC9/GR cells, indicating that ALDH1A1 may also be related with gefitinib resistance. ALDH1A1 silencing dramatically reduced cisplatin resistance in A549/DDP cells by decreasing proliferation and increasing apoptosis. The apoptotic rate of A549/DDP cells with decreased ALDH1A1 expression was higher than in the control group. The regulation of cell apoptosis is complex and involves a number of genes. The BCL-2 family plays a vital role in both the inhibition and promotion of apoptosis. The ratio between BCL-2 and BAX is the predictor of apoptosis, rather than BCL-2 or BAX alone. In this study, the downregulation of ALDH1A1 markedly decreased BCL-2 expression and increased BAX expression, resulting in a reduced BCL-2/BAX ratio, thus enhancing cell apoptosis (Fig S1).

The PI3K/AKT pathway participates in several cellular functions, from differentiation to proliferation and apoptosis. Mounting studies have elucidated that the PI3K/AKT pathway is critical for carcinogenesis and drug resistance, including tyrosine kinase inhibitors, radiation, and cytotoxins.[17] In NSCLC cells, PI3K/AKT pathway inhibition could restore sensitivity to gefitinib, an epidermal growth factor receptor-tyrosine kinase inhibitor, via reduced epithelial-mesenchymal transition and decreased BAX/Ku70 interaction.[18,19] We found that the downregulation of ALDH1A1 led to diminished levels of p-AKT (Fig S1), revealing that the PI3K/AKT pathway also plays a vital role in cisplatin resistance.

In conclusion, our results indicate that ALDH1A1 is associated with cisplatin resistance in A549/DDP cells, and that activation of the PI3K/AKT signal pathway may be the responsible mechanism. Drugs that target ALDH1A1 could be considered for chemotherapeutic regimens to overcome cisplatin resistance in NSCLC.

Acknowledgments

Disclosure

No authors report any conflict of interest.

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Supporting Information

Additional Supporting Informationmay be found in the online version of this article at the publisher's website:

Figure S1 Knocking down aldehyde dehydrogenase 1A1 (ALDH1A1) induced a declined BCL-2/BAX ratio and downregulation of phosphorylated protein kinase B (AKT) expression. (**a**) The ratio of B-cell lymphoma 2 and Bcl-2-like protein 4 densitometry in Figure d. (**b**) Densitometry quantification of AKT phosphorylation in Figure d. $P < 0.05$ was considered significant (#, compared with scramble short hairpin [sh]RNA group; &, compared with shRNA + dimethyl sulfoxide [DMSO] group; #, & $P < 0.05$).