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PDE3A is hypermethylated in cisplatin resistant non-small cell lung cancer cells and is a modulator of chemotherapy response

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Abstract. – OBJECTIVE: In this study, we aimed to investigate the mechanism of PDE3A downregulation in chemoresistant non-small cell lung cancer (NSCLC) cells, its functional role in chemotherapy response and association with survival outcomes.

MATERIALS AND METHODS: The raw data of GDS5247 was downloaded from GEO datasets and reanalyzed. The expression of PDE3A in patients with NSCLC and its DNA methylation status were analyzed in NSCLC cohort in TCGA database. Cisplatin resistant A549/Cis cells and the parental A549 cells were used as the *in vitro* cell model. The association between PDE3A expression and overall survival (OS) and progression-free survival (PFS) in NSCLC patients with adenocarcinoma or squamous cell carcinoma, as well as the median OS and PFS, were assessed by Kaplan-Meier curves using Kaplan-Meier plotter.

RESULTS: PDE3A was significantly downregulated in chemoresistant NSCLC cells. Heat map of PDE3A expression and methylation in NSCLC patient cohort in TCGA database indicated a negative association between PDE3A expression and DNA methylation in lung adenocarcinoma. A549/Cis cells treated with 5-AZA-dC, a demethylation reagent, had significantly restored PDE3A expression. High PDE3A expression was associated with favorable OS (HR: 0.53, 95% CI: 0.41-0.68, $p<0.0001$) and PFS (HR: 0.54, 95% CI: 0.39-0.75, $p<0.001$) in patients with adenocarcinoma. However, in patients with squamous cell carcinoma, high PDE3A expression was associated with unfavorable OS (HR: 1.56, 95% CI: 1.08-2.24, $p=0.017$) and PFS (HR: 1.83, 95% CI: 1.02-3.29, $p=0.04$).

CONCLUSIONS: PDE3A is downregulated in chemoresistant NSCLC cells due to DNA hypermethylation. Enforced PDE3A expression can sensitize A549/Cis cells to cisplatin. High PDE3A expression is associated with better OS and PFS in patients with adenocarcinoma, but not in patients with squamous cell carcinoma.

Key Words:
PDE3A, Methylation, Cisplatin, NSCLC.

Introduction

Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-related deaths across the world[1]. For the patients with advanced NSCLC, cisplatin based chemotherapy is still the most effective therapeutic strategy[2]. However, cancer usually develops chemoresistance after repetitive administration of chemotherapeutic drugs[3]. Therefore, it is necessary to understand the molecular mechanisms underlying the resistance. Some recent studies found that epigenetic alterations are closely related to chemoresistance, such as DNA methylation[4,5]. The key enzymes involved in DNA methylation include *de novo* methyltransferases DNMT3A and DNMT3B and DNA methylation maintenance methyltransferase DNMT1[6], which are usually upregulated in multiple chemoresistant cancer cell lines[7,8]. Hypermethylation of DKK3, RUNX3 and SLFN11 directly confer increased chemoresistance to human NSCLC cells[8-10]. Cyclic nucleotide phosphodiesterases (PDEs) is a family of the enzyme that breaks phosphodiester bonds, including the phosphodiester bond in the second messenger cAMP[11]. In fact, the balance of intracellular cAMP level is mainly regulated by adenylate cyclases (cAMP-generating enzymes)

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and PDEs (cAMP-degrading enzymes)[12]. Therefore, PDEs are important modulators of cellular responses to diverse extracellular stimuli[13]. Previous studies reported that the members of phosphodiesterase 3 (PDE3), including PDE3A and PDE3B are involved in regulation of cancer cell invasion and cell motility[14,15]. The PDE3 inhibitor, such as cilostazol, can inhibit DNA synthesis and cell viability in some lung cancer cell lines[16]. PDE3A depletion leads to 6-(4-(diethylamino)-3-nitrophenyl)-5-methyl-4,5-dihydropyridazin-3(2H)-one (DNMDP) resistance in hundreds of cancer cell lines[17], suggesting that PDE3A may play an important role in cancer cell responses to chemotherapeutic regents. In this study, by reanalysis of one previous microarray, we found that PDE3A is downregulated in chemoresistant NSCLC cells. Also, we demonstrated that PDE3A downregulation is a result of DNA methylation, while PDE3A overexpression sensitized A549/Cis cells to cisplatin. The following data mining of available annotated microarray data showed that high PDE3A expression is associated with better overall survival (OS) and progression-free survival (PFS) in patients with adenocarcinoma, but not in patients with squamous cell carcinoma.

Materials and Methods

Bioinformatic Data Mining

One previous study[18] compared the transcriptional profiles between cisplatin-resistant H460 NSCLC cells and the parental H460 cells by using the Affymetrix Human Gene 1.0 ST Array. The raw data of this microarray was downloaded from GEO datasets (accession No. GDS5247) and reanalyzed. The expression of PDE3A in patients with NSCLC and the corresponding DNA methylation status were analyzed using the NSCLC cohort in TCGA database, using the UCSC Xena browser (http://xena.ucsc.edu/). The association between PDE3A expression and OS and PFS in NSCLC patients with adenocarcinoma or squamous cell carcinoma, as well as the median OS and PFS, were assessed by drawing Kaplan-Meier curves using Kaplan-Meier Plotter, which is an online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data of 2435 NSCLC patients[19].

Cell Culture and Transfection

The human NSCLC cell line A549 and the corresponding cisplatin resistant A549/Cis cells were obtained from China infrastructure of Cell Line Resources (Beijing, China). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 μg/mL penicillin, and 100 U/mL streptomycin.

Cell Transfection

The PDE3A human Lentifect™ purified lentiviral particles and the empty control vector were obtained from Genecopoeia (Rockville, MD, USA). A549/Cis cells were infected with the lentiviral particles or the negative controls in the presence of polybrene.

Western Blot Assay

In brief, the cell samples were lysed to extract total protein. Then the protein samples were denatured and loaded in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for separation. After that, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. Then, the membranes were incubated with primary antibodies against PDE3A (ab169534, Abcam, Cambridge, MA, USA) and β-actin (ab3280, Abcam). After the incubation, membranes were washed and further incubated with secondary antibodies coupled to HRP. Then, the protein bands were visualized by using the SuperSignal™ West Femto Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA) according to manufacturer's instruction.

QRT-PCR Analysis

Total RNAs in the cell samples were extracted using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and were reverse-transcribed to get cDNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Then, PDE3A was measured using qRT-PCR analysis with the following primers (F, 5'-TTGATGCCAGGAAAATGGAT-3' and R, 5'-CCTTCTCCTCGTCCTCTTCC-3') and the SYBR® Select Master Mix (Applied Biosystems, Foster City, CA, USA) in an ABI 7900HT Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA). GAPDH was detected as the endogenous control.

Cell Counting Kit-8 (CCK-8) Assay

A549 cells and A549/Cis cells with or without enforced PDE3A expression were plated onto 96-well cell culture plates at a density of 2 x 10^4 cells/well. Then, the cells were treated with varying concentrations of cisplatin for 72 h. After the treatment, cell viability was measured using

the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to manufacturer's instruction. Briefly, CCK-8 solution was added to each well and the plate was placed in a cell incubator for 4 h. Cell viability was reflected by the absorbance at 450 nm determined by a 96-well spectrophotometry. IC50 value was determined by creating dose-response curves.

Flow Cytometric Analysis of Active Caspase-3

A549/Cis cells with PDE3A overexpression alone or in combination with cisplatin treatment (50 μmol/L) for 48 h were subjected to flow cytometric analysis of active caspase-3 using the GaspGLOW™ Fluorescein Active Caspase-3 Staining Kit (Biovision, Mountain View, CA, USA) according to the manufacturer's protocol. Fluorescence was examined by a FACSCalibur (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

Data were presented as means ± SD. All analyses were performed with SPSS19 software package (SPSS Inc., Chicago, IL, USA). Data were analyzed for statistical significance by two-tailed Student's *t*-test or ANOVA with Student-Newman-Keuls test as a post hoc test. The difference between the Kaplan-Meier curves was assessed using the log-rank test. $p<0.05$ was considered statistically significant.

Results

PDE3A is Decreased, while DNMT3A is Increased in Cisplatin Resistant NSCLC Cells

Our reanalysis of the raw data of GDS5247 identified some of the most downregulated genes in cisplatin resistant H460 cells compared to the parental H460 cells (Figure 1A). PDE3A (Figure 1A, *red arrow* and Figure 1B) was among the downregulated genes, which has been reported as a target for anti-proliferative cancer therapy[17]. Interestingly, by analyzing the upregulated genes in cisplatin resistant H460 cells, we found that DNMT3A, a catalyzer of DNA methylation[6], was significantly upregulated (Figure 1C-D). In fact, global hypermethylation is an important mechanism of chemoresistance in NSCLC[20]. Therefore, we decided to further investigate whether PDE3A downregulation is related to its methylation status.

Demethylation Restores PDE3A Expression in A549/Cis Cells

Then, we explored the heat map of PDE3A expression and methylation in NSCLC patient cohort in TCGA database. The results indicated that increased DNA methylation might be associated with decreased PDE3A expression in lung adenocarcinoma (Figure 2A, *black frame*), but not in lung squamous cell carcinoma (Figure 2A). Then, we investigated PDE3A expression in cisplatin resistant lung adenocarcinoma cell line A549/Cis cells and in the parent A549 cells. The results showed that A549/Cis cells had significantly lower PDE3A expression at both mRNA and protein levels (Figure 2B). A549/Cis cells treated with 5-AZA-dC, a demethylation reagent, had significantly restored PDE3A expression (Figure 2C).

Enforced PDE3A Expression Sensitizes A549/Cis Cells to Cisplatin

Then, we investigated the functional role of PDE3A in cell response to cisplatin. A549/Cis cells were transfected with lentiviral PDE3A expression particles or the negative control (Figure 3 A). Drug sensitivity assay showed that PDE3A overexpression resulted in decreased cisplatin IC50 in A549/Cis cells (Figure 3 B). By performing flow cytometric assay, we found that PDE3A overexpression alone had no effect on activation of caspase-3 in A549/Cis cells (Figure 3 C-D), but it increased cisplatin-induced activation of caspase-3 (Figure 3 C-D).

High PDE3A expression is associated with better OS and PFS in adenocarcinoma, but not in squamous cell carcinoma

Data mining in Kaplan-Meier plotter showed that high PDE3A expression was associated with favorable OS (HR: 0.53, 95% CI: 0.41-0.68, $p<0.0001$) and PFS (HR: 0.54, 95% CI: 0.39-0.75, $p<0.001$) in patients with lung adenocarcinoma (Figure 4 A and C). The median survival of OS and PFS in high PDE3A and low PDE3A cohort of adenocarcinoma were 136.33 *vs.* 72 months and 45.3 *vs.* 19 months, respectively (Table I). However, in patients with squamous cell carcinoma, high PDE3A expression was associated with unfavorable OS (HR: 1.56, 95% CI: 1.08-2.24, $p=0.017$) and PFS (HR: 1.83, 95% CI: 1.02-3.29, $p=0.04$) (Figure 4 B and D). The median survival of OS and PFS in high PDE3A and low PDE3A cohort of squamous carcinoma were 49.97 *vs.* 78 months and 10 *vs.* 35 months, respectively (Table I).

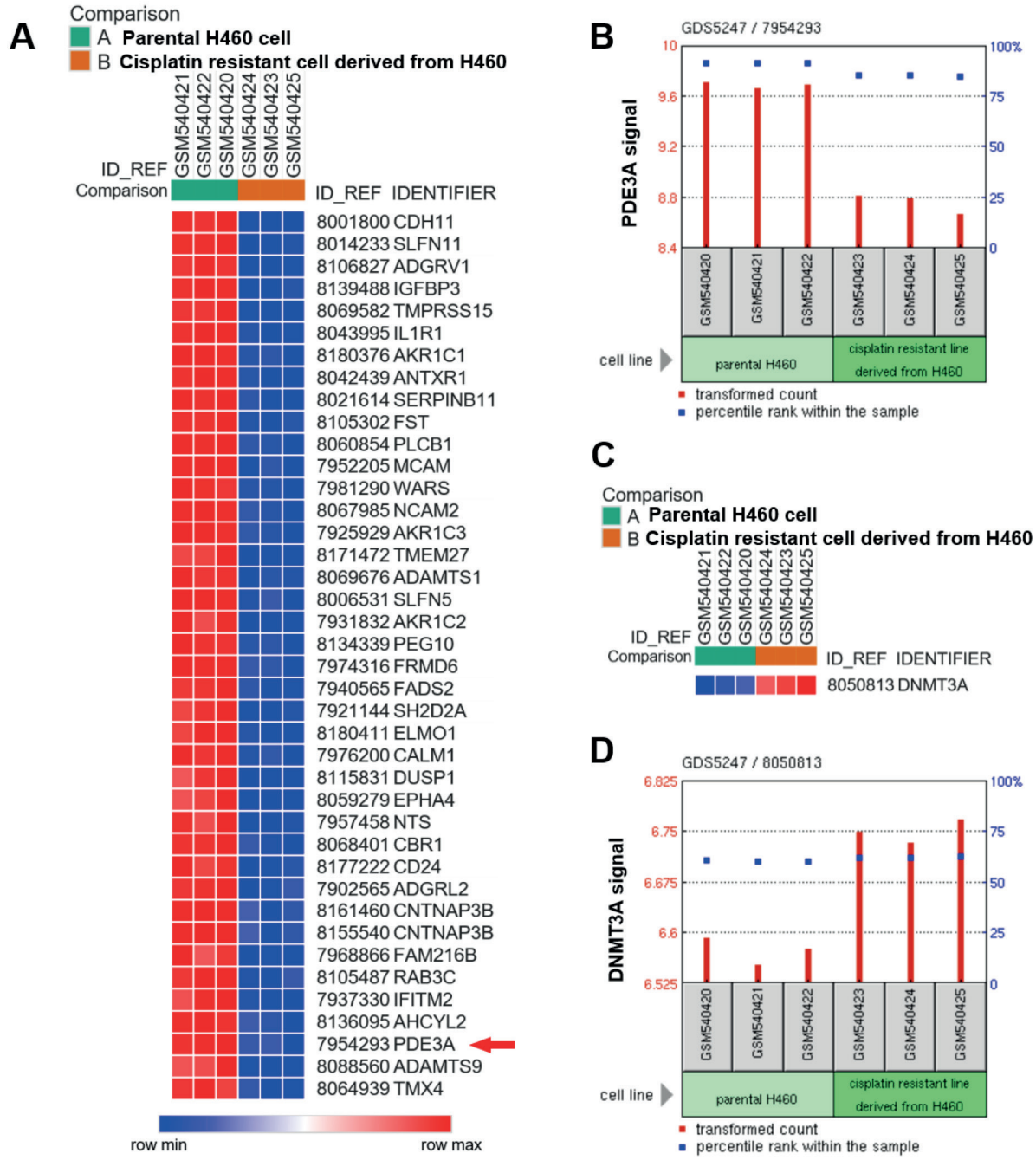

Figure 1. PDE3A is decreased, while DNMT3A is increased in cisplatin resistant NSCLC cells. ***A,*** Heat map of 40 dysregulated genes in cisplatin-resistant H460 NSCLC cells and the parental H460 cells. Red: up-regulation; Blue: down-regulation. Image was obtained by reanalysis of the raw microarray data of GDS5247. ***B,*** PDE3A microarray signal values. Data was analyzed by using the tool provided by GEO datasets. ***C,*** Heat map of DNMT3A in cisplatin-resistant H460 NSCLC cells and the parental H460 cells. D. DNMT3A microarray signal values.

Discussion

DNA methylation is one of the important mechanisms of chemoresistance in NSCLC. Hypermethylation mediated downregulation of RUNX3 can induce docetaxel resistance in human lung adenocarcinoma cells by activating AKT signaling[10]. SLFN11 is downregulated in cisplatin/carboplatin resistant ovarian cancer and NSCLC cells due to promoter CpG island hypermethyla-

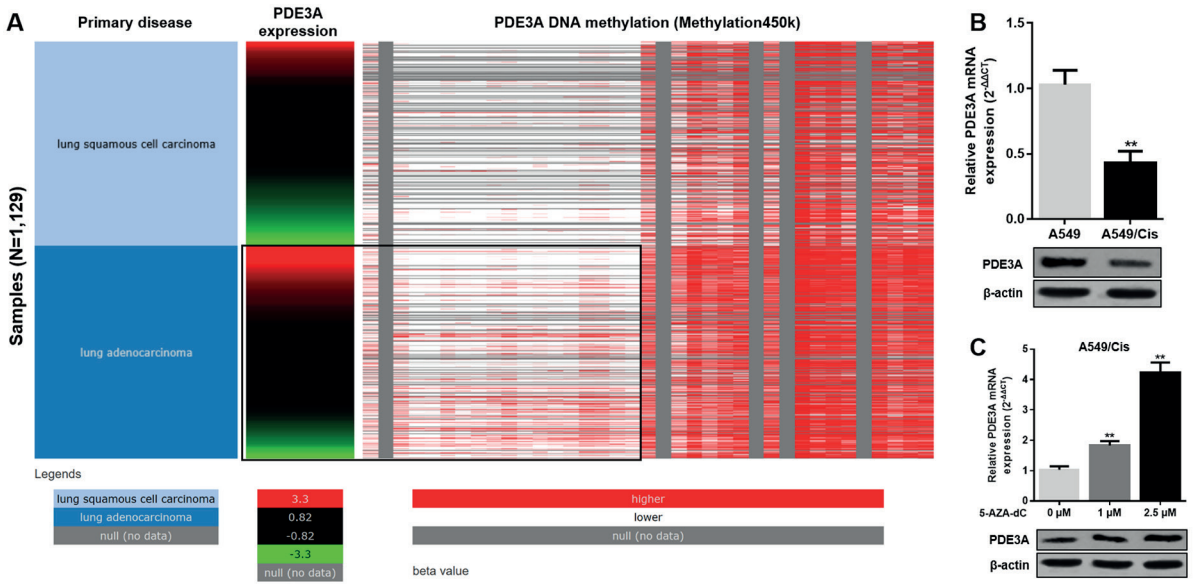

Figure 2. Demethylation restores PDE3A expression in A549/Cis cells. ***A,*** Expression and methylation heat map of PDE3A in NSCLC patient cohort in TCGA database (N=1,129). Data was obtained by using UCSC Xena. ***B,*** QRT-PCR (up) and Western blot (down) analysis of PDE3A expression in A549 and A549/Cis cells. ***C,*** QRT-PCR (up) and Western blot (down) analysis of PDE3A expression in A549/Cis cells 24 h after treatment with 1 or 2.5 μM 5-AZA-dC. **, $p<0.01$.

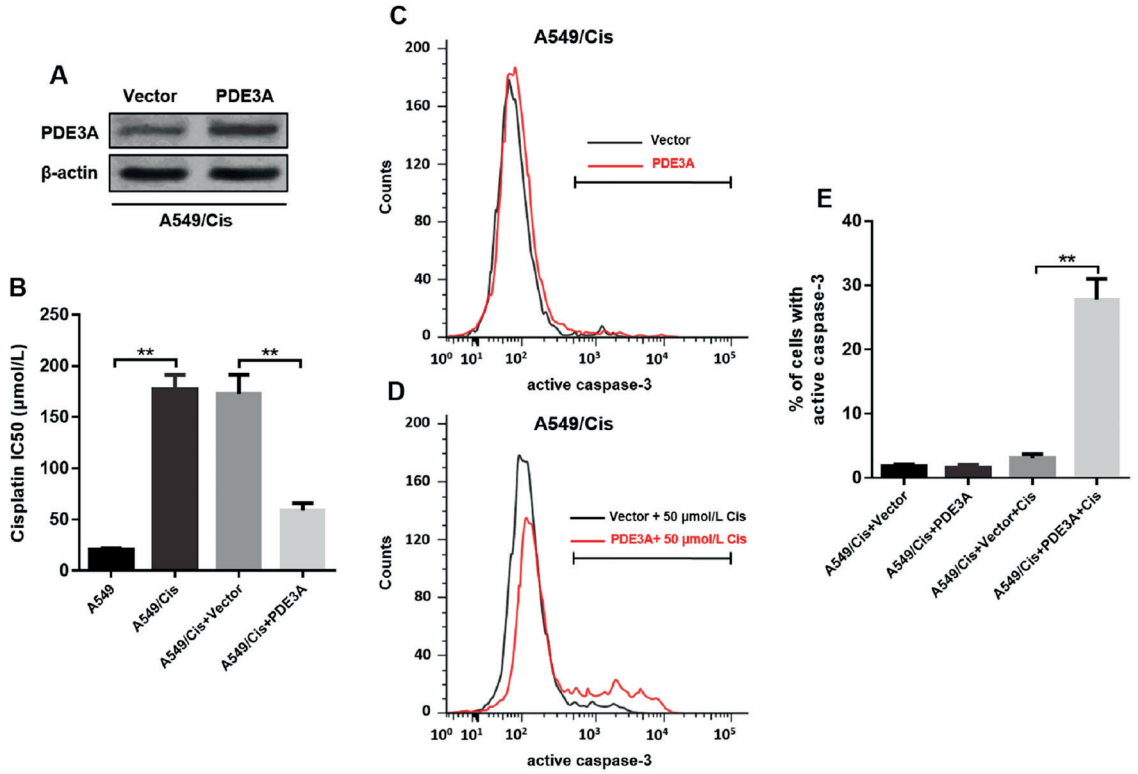

Figure 3. Enforced PDE3A expression sensitizes A549/Cis cells to cisplatin. ***A-D.*** Kaplan-Meier curves of the association between PDE3A expression and OS (A-B) and PFS ***(C-D)*** in patients with adenocarcinoma (A and C) or with squamous cell carcinoma (B and D). Data was obtained by using Kaplan-Meier Plotter. Analysis was performed by using the JetSet best probe set with auto-selected best cutoff.

tion, which also indicates a poor response to both cisplatin and carboplatin treatments[9]. Another recent study demonstrated that a panel of candidate genes, including GAS1, TIMP4, ICAM1 and WISP2, were downregulated by DNA methylation and induced cisplatin resistance in NSCLC[5]. Restoration of GAS1 in A549/Cis cells could increase sensitivity to cisplatin and induce proliferation inhibition, cell cycle arrest, apoptosis and *in vivo* growth retardation[5]. In this study, by reanalysis of the raw data of GDS5247, we found that PDE3A is significantly downregulated in cisplatin resistant H460 cells. The following bioinformatic analysis based on NSCLC patient data in TCGA database indicated that PDE3A downregulation might be associated with DNA hypermethylation in adenocarcinoma. In A549/Cis cells, PDE3A is downregulated compared to the parental A549 cells. Demethylation treatment significantly restored PDE3A expression in A549/Cis cells. Some PDE3/4 inhibitors, such as zardaverine, anagreli-de and quazinone, have previously been reported to have cell-cytotoxic characteristics in some cancer cell lines. For example, The PDE3 inhibitor quazinone, and the PDE 3/4 inhibitor zardaverine can effectively inhibit growth and induce cell apoptosis in Hela cells[21], while zardaverine shows potent and selective antitumor activity against hepatocellular carcinoma[15]. Another recent work found that the sensitivity to DNMDP across 766 cancer cell lines is correlated with the expression of PDE3A[17]. DNMDP exerts chemotoxic effect via promoting an interaction between PDE3A and SLFN12[17]. PDE3A and/or SLFN12 depletion significantly decreased DNMDP sensitivity of the cancer cells[17]. These findings suggest that PDE3A might be an important modulator of cell responses to chemotherapeutic reagents. Therefore, we further investigated the functional role of PDE3A in cisplatin responses of lung adenocarcinoma cells. Our results showed that PDE3A restoration had no effect on cell apoptosis in A549/Cis cells. However, it significantly enhanced cisplatin induced cell apoptosis. These results suggest that PDE3A can sensitize A549/Cis cells to cisplatin. Since PDE3A is involved in drug responses of NSCLC cancer cells, we further studied its association with survival outcomes in the patients. Data mining based on annotated microarray data suggests that high PDE3A expression is associated with favorable OS and PFS in patients with lung adenocarcinoma. However, in patients with squamous cell carcinoma, high PDE3A expression is associated with unfavorable OS and PFS. Therefore, we infer that the expression level of PDE3A might be a useful indicator of chemotherapy response and survival in patients with NSCLC.

Table I. Median OS and PFS in NSCLC patients with adenocarcinoma or squamous cell carcinoma.

	Adenocarcinoma		Squamous cancer	
PDE3A expression cohort	Low	High	Low	High
Median OS (months)	72	136.33	78	49.97
Median PFS (months)	19	45.3	35	10

Conclusions

PDE3A is downregulated in chemoresistant NSCLC cells due to DNA hypermethylation. Enforced PDE3A expression can sensitize A549/Cis cells to cisplatin. High PDE3A expression is associated with better OS and PFS in patients with adenocarcinoma, but not in patients with squamous cell carcinoma.

Conflict of interest

The authors declare no conflicts of interest.

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