Original Paper

PLAUR Confers Resistance to Gefitinib Through EGFR/P-AKT/Survivin Signaling Pathway

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Key Words

Exosome • PLAUR • EGFR • Survivin • Gefitinib-resistance

Abstract

Background/Aims: Tyrosine kinase inhibitor gefitinib significantly improves the survival of patients with non-small-cell lung cancer (NSCLC) by inhibiting epidermal growth factor receptor (EGFR) tyrosine kinase. However, patients eventually develop resistance to gefitinib through uncharacterized mechanisms. It is known that plasminogen activator urokinase receptor (PLAUR) plays an important role in cell proliferation, migration and apoptosis. However, the role of PLAUR, particularly exosomal PLAUR in gefitinib resistance in NSCLC has not been reported. The aim of this study is to determine the relationship between PLAUR and gefitinib resistance. **Methods:** In this study, a tethered cationic lipoplex nanoparticle (TCLN) biochip containing molecular beacons was used as probes to detect PLAUR mRNA in plasma exosomes from patients with gefitinib-sensitive and -resistant NSCLC. *In vitro*, Real-time PCR was used to examine the expression of PLAUR mRNA and Western blot was applied to examine the expression of related proteins. The gene knockdown was achieved by Lentivirus based RNA silence technique. The cell counting kit-8 assay and EdU incorporation were used to examine cell proliferation. The flow cytometry was applied to determine cell apoptosis and cell cycle, while the mitochondrial membrane potential was measured by JC-1 dye assay. Signaling pathway affected by PLAUR knockdown was identified by cDNA Microarray. The effect of PLAUR knockdown on tumorigenesis was analyzed *in vivo*. **Results:** We found that the exosomal PLAUR mRNA in the plasma of gefitinib-resistant NSCLC patients was significantly increased compared to that of gefitinib-sensitive NSCLC patients. The PLAUR mRNA and soluble PLAUR protein were also significantly increased in gefitinib-resistant human lung adenocarcinoma PC9R cells compared to gefitinib-sensitive PC9 cells. Silencing PLAUR in PC9R cells impaired mitochondrial membrane potential and increased cell apoptosis via EGFR/p-AKT/survivin

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signaling pathway. Furthermore, EGFR was upregulated in the gefitinib-resistant PC9R cells, and knockdown of EGFR significantly increased cell apoptosis. **Conclusions:** Taken together, our results demonstrated that PLAUR induces gefitinib-resistance through EGFR/p-AKT/survivin signaling pathway in gefitinib-resistant human lung adenocarcinoma cells. PLAUR could be a novel therapeutic target for gefitinib-resistant NSCLC patients.

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Introduction

Lung cancer is the leading cause of cancer death worldwide [1]. Approximately 80% of primary lung cancer is non-small-cell lung cancer (NSCLC) and most of the patients are diagnosed at advanced stages [2]. Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), such as gefitinib, show a significant efficacy in the treatment of NSCLC [3]. However, most patients eventually develop drug resistance to TKIs. Approximately 70% of the acquired resistance could be attributed to a T790M mutation or a c-MET gene amplification [4-6]. It is necessary to understand other molecular pathways in order to develop new biomarkers and pharmacological targets for gefitinib-resistant NSCLC.

Exosomes are small membrane-encapsulated fluid particles that contain proteins and RNA from their cells of origin and some of which are specific for certain tumors. Furthermore, exosomes are remarkably stable in blood, making them ideal cancer biomarkers [7]. For example, in ovarian cancer, the release of exosomes is positively correlated with cancer invasiveness [8]. However, because of their small size (<100 nm in diameter), exosomes are challenging to detect [7, 9]. The tethered cationic lipoplex nanoparticle (TCLN) assay is a recently developed technology that reliably detects mRNAs in exosomes [10, 11].

Plasminogen activator urokinase receptor (PLAUR) is a glycosyl-phosphatidylinositol (GPI)-anchored membrane protein [12]. PLAUR is associated with cell signaling because it forms a multi-protein complex with neighboring transmembrane receptors, such as integrins [13], FPR-like receptor-1/lipoxin A4 receptor (FPRL-1) [14], and EGFR [15]. Inhibition of PLAUR decreases tumor growth, invasion, angiogenesis and metastasis in various cancers, including NSCLC [16-18]. PLAUR is cleaved and becomes soluble, which is released into blood and other body fluids, binds to cell surface, and elicits various responses [19, 20]. Soluble PLAUR has been identified as a plasma biomarker of myeloma [21], prostate cancer [22, 23], ovarian cancer [24], colorectal cancer [25], small cell lung cancer [26], respiratory cancer [27], cervical cancer [28], and breast cancer [29].

EGFR overexpression is frequently observed in various cancers, including lung cancer, and is associated with decreased survival and increased risk of recurrence [30-32]. EGFR overexpression might activate NF- κ B and PI3K/Akt/mTOR pathways, and lead to drug resistance [33, 34]. EGFR has tyrosine kinase dependent activities, which are important for cell proliferation, and tyrosine kinase independent functions that are essential for cancer cells growth. Knockdown of EGFR leads to inactivation of phosphorylated AKT (p-AKT), phosphorylated extracellular signal-regulated kinase (p-ERK) and cell apoptosis [35-40]. Consistent with these findings, EGFR knockout animals are embryonic lethal [41, 42]. However, mice with kinase-inactive mutants are not [43]. Since the p-AKT/survivin pathway plays an important role in drug resistance in NSCLC, targeting EGFR itself, instead of its tyrosine kinase activity, could be a more efficient way to treat NSCLC, especially when the cancer cells are resistant to TKIs [44, 45].

In this study, we found that exosomal PLAUR mRNA in plasma from gefitinib-resistant lung adenocarcinoma was significantly increased compared to that from gefitinib-sensitive lung adenocarcinoma. The mRNA and the soluble PLAUR protein were also significantly increased in gefitinib-resistant human lung adenocarcinoma PC9R cells compared to gefitinib-sensitive PC9 cells. Silencing PLAUR in PC9R cells decreased the mitochondrial membrane potential and increased cell apoptosis via EGFR/p-AKT/survivin signaling pathway. Furthermore, EGFR was upregulated in the gefitinib-resistant PC9R cells, and downregulation of EGFR significantly induced cell apoptosis.

Materials and Methods

Patients and blood samples for detecting exosomal PLAUR mRNA

A total of 28 lung adenocarcinoma patients, 17 were sensitive to EGFR TKIs and 11 had acquired resistance to EGFR TKIs, were enrolled for study at the Department of Pulmonary Medicine, Zhongshan Hospital, Fudan University. The study was conducted with the approval of the ethics committee of our institution (Approval number B2016-154R). EDTA plasma samples were collected from these patients. The Invitrogen total exosome precipitation reagent (Thermo Fisher Scientific, MA, USA) was used to isolate exosomes from human plasma samples according to the manufacturer's instructions. The detection of exosomal PLAUR mRNA using tethered cationic lipoplex nanoparticles (TCLNs) was previously described [10, 11]. Briefly, to make TCLN biochips, a thin Au layer (15 nm) was deposited on a glass cover slip using a Denton e-beam evaporator (DV-502A, Denton Vacuum, NJ, USA). Mixed thiol self-assembled monolayers (SAMs), as an anchoring membrane, were formed on the Au layers. Cationic lipoplex nanoparticles, containing the PLAUR molecular beacons (MBs, custom synthesized by Sigma-Aldrich, MO, USA), were tethered onto the glass slide surface by a biotin-avidin linkage. All the MBs were labeled with Fluorescein amidite (FAM). Total Internal Reflection Fluorescence (TIRF) Microscopy (Nikon, Japan) was used to detect the expression of PLAUR mRNA in the exosomes. A custom-developed MATLAB program was applied to analyze the images by generating a binary mask to remove the background and measure the sum intensity of each image. In this study, PBS was used as control to define the background. Any fluorescence signals from the samples that were equal or lower than the signals observed in the PBS controls were defined as background in image analysis.

Cells and Cell culture

The human lung adenocarcinoma cell line PC9 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). To develop gefitinib-resistant PC9R cells, we continuously exposed gefitinib-sensitive PC9 cells to sequentially increasing concentrations of gefitinib [46]. The gefitinib-sensitive PC9 and -resistance PC9R cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin/streptomycin. The gefitinib resistance of PC9R cells was maintained by adding 1 μ M gefitinib (Selleckchem, TX, USA), which is an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, in the culture media. The cells were grown as monolayers in a humidified atmosphere containing 5% CO₂ at 37°C.

Soluble PLAUR quantification

The supernatants from the cultured cells were collected and centrifuged for 5 min to remove dead cells and cellular debris. An enzyme-linked immunosorbent assay (ELISA) for PLAUR was used to measure the soluble PLAUR in the cell supernatant according to the manufacturer's instructions (#DUP00, R&D Systems, MN, USA).

Lentiviral construction and infection

Short hairpin RNA (shRNA) vectors against the PLAUR genes shPLAUR-1 and shPLAUR-2, and against the EGFR genes shEGFR-1 and shEGFR-2 were obtained from The RNAi Consortium (TRC). Lentiviral plasmids, containing GV112-shPLAUR-1, -shPLAUR-2, -shEGFR-1, -shEGFR-2, and -negative, were obtained from GeneChem (Shanghai, China). Lentiviruses carrying overexpression human EGFR (GenBank accession number NM_005228) lentiviral vectors (GV358) were from GeneChem. The lentiviral particles were produced by transfecting HEK 293T cells with the lentiviral plasmids. For viral infection, PC9R cells were plated in 6-well plates, grown to 50-70% confluence, incubated with medium containing virus and 4 μ g/mL polybrene at an MOI (multiplicity of infection) of 10. The infected cells were then incubated with various concentrations of gefitinib after 24 hours of infection.

Cell proliferation and viability assay

The Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Japan) was used to assess cell proliferation. Briefly, cells were plated in 96-well plates at approximately 1000 cells per well with 200 μ L of culture medium. After 24 hours, 10 μ L of CCK-8 solution was applied to each well, and the plates were incubated for 1 hour at 37°C. Finally, the light absorbance values at 450 nm were determined using a microplate reader

(Multiskan, Thermo Fisher Scientific, MA, USA) with a reference wavelength of 650 nm. All experiments were conducted at least in triplicate.

EdU incorporation assay

Cells were incubated with 10 μ M EdU (5-ethynyl-2'-deoxyuridine, Thermo Fisher Scientific, MA, USA) for 4 hours and then fixed in 3.7% formaldehyde in PBS for 15 minutes at room temperature. EdU incorporation was detected according to manufacturer's recommendations. Confocal imaging was performed using a Nikon A1R confocal laser scanning microscope system (Nikon Corp., Tokyo, Japan). PC9R cells positive for EdU and Hoechst 33342 staining were counted using Image J (v. 1.42, Wayne Rasband, NIH) and were used to calculate the percentage of EdU-positive cells.

Detecting Apoptosis by Flow Cytometry

An Annexin V-FITC and propidium iodide (PI) double staining kit (Thermo Fisher Scientific, MA, USA) was used to analyze cellular apoptosis. The transfected PC9R cells were seeded in 6-well plates (5×10^5 cells/well) and were treated with 1 μ M gefitinib. Cells were then digested with trypsin (Gibco® Trypsin-EDTA, Thermo Fisher Scientific, MA, US), washed with PBS three times, suspended in 500 μ l of binding buffer, and were then incubated with 5 μ l of FITC-conjugated Annexin-V and 3 μ l of PI for 15 minutes at room temperature in the dark. The stained cells were detected using the BD FACS Aria II flow cytometer (BD biosciences, CA, USA).

Cell cycle analysis

The transfected PC9R cells were seeded in 6-well plates (5×10^5 cells/well) and were treated with 1 μ M gefitinib. The treated cells were then collected, washed with PBS, and fixed in 70% ethanol for 24 hours at 4°C. The fixed cells were stained with PI in the dark for 30 minutes at room temperature. Finally, cell cycle distribution was analyzed by flow cytometry (BD FACS Aria II flow cytometer, BD biosciences, CA, USA).

Mitochondrial membrane potential measurement

The MitoProbe™ JC-1 assay kit (Thermo Fisher Scientific, MA, USA) was used to detect changes in mitochondrial membrane potential. The assay was performed according to the manufacturer's instructions, and the results were obtained by the BD FACS Aria II flow cytometer. JC-1 forms J-aggregates emitting red fluorescence at 590 nm in healthy mitochondria, and J-monomers emit a green fluorescence at 490 nm in depolarized mitochondria. An increased ratio of J-monomers indicates mitochondria damage.

Quantitative RT-PCR

Total RNA was extracted using the TRIzol reagent (Thermo Fisher Scientific, MA, USA), and cDNA was synthesized using reverse transcriptase (TOYOBO, Japan). The RNA (1%) was reverse transcribed into complementary deoxyribonucleic acid, and 20 ng of complementary DNA was used as the template for RT-PCR. The amplification cycling parameters (40 cycles) were set as follows: 15 seconds at 95°C; 15 seconds at 60°C and 45 seconds at 72°C. The primer sequences included the following:

PLAUR sense 5'- GCTTGTGGAAGAAGGAGAA-3',

PLAUR anti-sense 5'- CCTCGGTAAGGCTGGTGAT-3'.

β -actin sense 5'- CTGGCACCCAGCACAATG -3',

β -actin anti-sense 5'- CCGATCCACACGGAGTACTTG -3'.

The gene expression was normalized to β -actin and were calculated by $2^{-\Delta\Delta CT}$. The RT-PCR assay was performed at least 3 separate times in triplicate.

Western blot assay

Total proteins from PC9, PC9R, and PC9R cells transfected with various lentiviruses (shNEG, shPLAUR-1, shPLAUR-2, shEGFR-1, shEGFR-2, shNEG-O, shEGFR-O) was extracted using a RIPA kit (Beyotime Biotechnology, China). They were separated on polyacrylamide gels and were transferred to PVDF membranes. The membranes were incubated with anti-PLAUR (Abcam, MA, USA), anti-EGFR (Cell Signaling Technology (CST), MA, USA), anti-phospho-Akt (Ser473, CST), anti-survivin (CST), anti-cleaved caspase-3 (Asp175, CST), anti-cleaved PARP (Asp214, CST), and anti-actin (CST) antibodies at 4°C overnight and were

then incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G at room temperature for 1 hour. Proteins were visualized using Pierce ECL Western blotting substrate and autoradiography. The blots were analyzed using Quantity One 4.6.

Intracellular signaling array

The cell extracts from PC9R cells transfected with shNEG and shPLAUR-1 were prepared and analyzed using the PathScan intracellular signaling array kit (Catalog no. 7323S; CST) and the PathScan stress and apoptosis signaling antibody array kit (Catalog no. 12856S; CST) according to the manufacturer's instruction. The PathScan Intracellular Signaling Array Kit simultaneously detects eighteen phosphorylated or cleaved intracellular signaling molecules. The PathScan Stress and Apoptosis Signaling Antibody Array simultaneously detects nineteen apoptosis-related signaling molecules, including cleaved caspases (caspase 3 and 9) and PARP.

cDNA array and pathway analysis

Total RNA was extracted from the negative control cells and the PC9R cells transfected with shPLAUR-1 using the RNeasy Plus Mini Kit (Qiagen, MD, USA). The extracted RNA was converted to double-stranded cDNA and was amplified using a OneArray plus RNA amplification kit (Phalanx Biotech Group, Taiwan). The Cy5-labeled RNA targets were hybridized to the Human Whole Genome OneArray (Phalanx Biotech Group, Taiwan). The intensity of signals was measured by the Agilent Microarray Scanner (G2505C, Agilent, USA) and was analyzed by the Rosetta Resolver System (Rosetta Biosoftware). Testing was performed at least in triplicate, and the statistical analyses were performed using the proprietary modeling techniques from the Rosetta Resolver.

Tumorigenicity evaluation

The animal experiments were approved by the Institutional Animal Care and Use Committee at Zhongshan Hospital from Fudan University, China. Four- to 6-week-old male BALB/c nude mice were obtained from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China). PC9R cells transfected with shNEG and shPLAUR-1 were subcutaneously injected into the right flanks of nude mice. The tumor volume was monitored and was calculated according to the following formula: Volume = length x width²/2. After one month, tumors were dissected out and weighed. Ki67 immunohistochemical staining was applied to identify the proliferating cells in the paraffin sections of xenograft tumors. The Ki67-positive cells were quantified in randomly selected fields from each tissue section using Image J.

Statistical analysis

Data are expressed as the means ± SD of at least three independent experiments. A one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test was applied to compare among different groups. Student T-test was applied to compare between 2 different groups. A p-value of <0.05 was considered statistically significant.

Results

Overexpression of exosomal PLAUR mRNA is correlated with gefitinib resistance

To evaluate the role of PLAUR in EGFR TKI resistance, we collected EDTA plasma samples from 17 individuals with lung adenocarcinoma who were sensitive to EGFR TKIs, and 11 individuals with lung adenocarcinoma who had acquired resistance to EGFR TKIs. The clinical characteristics of these 28 patients are listed in Table 1. There were no significant differences in the clinical characteristics between the EGFR TKI-sensitive and resistant patients. The expression of exosomal PLAUR mRNA in these patients was examined using a tethered cationic lipoplex nanoparticle based assay. It was found that, the levels of PLAUR mRNA in plasma exosomes from gefitinib-resistant lung adenocarcinoma were significantly higher than those of gefitinib-sensitive lung adenocarcinoma (p < 0.05) (Fig. 1), indicating that high level of PLAUR could be related to gefitinib resistance.

Table 1. The clinical characteristics of EGFR TKI-sensitive and -resistance lung adenocarcinoma patients

	Patient No.	EGFR TKI-sensitive	EGFR TKI-resistance	P
Total No.	28	17	11	
Age, median years (range)	62.68 (59.5-65.8)	61.9 (58.2-65.7)	63.8 (57.4-70.2)	0.396
Sex				0.315
	Female	16	11	
	Male	12	6	
Smoking				0.295
	Non-smokers	23	15	
	Smokers	5	2	
Tumor Stage				1
	IV	28	17	

PLAUR mRNA and secreted PLAUR protein are upregulated in gefitinib-resistant PC9R cells

Gefitinib-resistant PC9R cells were developed by continuous exposure of gefitinib-sensitive PC9 cells to sequentially increasing concentrations of gefitinib [46]. To validate gefitinib resistance in PC9R cells, the viability of PC9 and PC9R cells upon gefitinib treatment was measured by CCK-8 assay. It was found that the viability of the PC9 cells was dramatically reduced when the cells were incubated with 0.1 μ M gefitinib, whereas the viability of the PC9R cells was not significantly decreased by up to 5 μ M gefitinib treatment (Fig. 2A).

The expression of PLAUR mRNA in the gefitinib-sensitive PC9 and -resistant PC9R cells was investigated to understand the potential role of PLAUR in the development of gefitinib resistance. The RT-PCR result revealed that PLAUR mRNA was significantly increased in the gefitinib-resistant PC9R cells compared to that of the gefitinib-sensitive PC9 cells (Fig. 2B). Similarly, the ELISA results showed that soluble PLAUR protein in the supernatant of PC9R cells was significantly higher than that of the PC9 cells (Fig. 2C). However, the Western blot results showed that PLAUR protein levels in the PC9R cells were not significantly different from that of the PC9 cells (Fig. 2D). These results demonstrated that the mRNA and soluble form of PLAUR protein were significantly upregulated in gefitinib-resistant PC9R cells, indicating a role of PLAUR in inducing gefitinib resistance.

PLAUR mediates gefitinib resistance in PC9R cells

To further confirm the role of PLAUR in mediating gefitinib-resistant in PC9R cells, we downregulated PLAUR in PC9R cells by transfecting either PLAUR shRNA or negative shRNA into PC9R cells. The RT-PCR result revealed that both shPLAUR-1 and shPLAUR-2, two shRNA sequences targeting PLAUR gene, significantly reduced PLAUR mRNA expression in the transfected PC9R cells (Fig. 3A). The ELISA results revealed that shPLAUR-1 and shPLAUR-2 both significantly decreased soluble PLAUR protein levels in the supernatant of transfected PC9R cells (Fig. 3B).

Fig. 1. Exosomal PLAUR mRNA as a biomarker of gefitinib resistance. A,B) Representative Total Internal Reflection Fluorescence (TIRF) Microscopy images obtained from EGFR TKI-sensitive and -resistant patients. Scale bar: 10 μ m. C) Exosomal PLAUR mRNA in the plasma of individuals with lung adenocarcinoma sensitive (n=17) or resistant (n=11) to EGFR TKIs examined using the TCLN biochips.

To address the cellular functions of PLAUR signaling in gefitinib resistance, we transfected PC9R cells with PLAUR shRNA or negative shRNA for 24 hours and then incubated them with 1 μ M gefitinib for 96 hours. A morphological examination of the transfected PC9R cells demonstrated that shPLAUR-1 and shPLAUR-2 resulted in decreased cell proliferation (Fig. 3C). To verify this, CCK8 assay was performed and it was found that both shPLAUR-1 and shPLAUR-2 significantly inhibited the growth of the transfected PC9R cells compared to the negative control after treatment with 0.0, 0.1, 1.0, 2.5, 5.0, and 10.0 μ M gefitinib (Fig. 3D). In addition, cell shrinkage and pyknosis of nuclei of PC9R cells transfected with PLAUR shRNA were observed under a transmission electron microscope (Fig. 3E).

Furthermore, the effects of silencing PLAUR on the proliferation of transfected PC9R cells following gefitinib treatment was measured using EdU incorporation. The results showed that there were approximately 50% EdU positive cells in the negative control group and 30% in the shPLAUR-1 and shPLAUR-2 groups with 1 μ M gefitinib treatment (Fig. 4A and B), suggesting that silencing PLAUR significantly reduced the proliferation of transfected PC9R cells after gefitinib treatment.

To further confirm the effects of PLAUR downregulation on gefitinib resistance in PC9R cells, we performed an annexin V-FITC/PI double staining assay and a flow cytometry analysis. The apoptosis rate of PC9R cells transfected with shPLAUR-1 or shPLAUR-2 was significantly increased compared to that of the negative control when incubated with 1 μ M gefitinib (Fig. 5A and B). We also explored the effect of PLAUR downregulation on cell cycle and found that the number of cells in S phase of PC9R cells transfected with shPLAUR-1 or shPLAUR-2 was significantly decreased compared to the negative control (Fig. 5C and D).

Fig. 2. PLAUR mRNA and secreted PLAUR protein upregulated in gefitinib-resistant PC9R cells. A) CCK8 assay on the gefitinib dose response in gefitinib-resistant PC9R and -sensitive PC9 cells. B) RT-PCR on PLAUR mRNA in PC9R and PC9 cells. C) The ELISA results of soluble PLAUR in the supernatant of PC9R and PC9 cells. D) The Western blot of PLAUR protein of PC9 and PC9R cells. Data represented as the mean $\pm$ SD. N=3, * p <0.05 versus the PC9 cells.

Fig. 3. PLAUR mediates gefitinib resistance in PC9R cells. A) RT-PCR on the PLAUR mRNA expression. B) ELISA on the amount of soluble PLAUR protein in the cell supernatants. Data represented as the mean $\pm$ SD. N=3, * = p <0.05 versus the negative control. C) Bright-field images of the PC9R cells after transfection with PLAUR shRNAs or negative shRNA and treated with 1 μ M gefitinib. D) CCK-8 assay on the viability of PC9R cells treated with indicated concentrations of gefitinib. Data represented as the mean $\pm$ SD. N=4 * = p <0.05 versus the negative control. E) TEM images of PC9R cells treated with 1 μ M gefitinib.

Knockdown of PLAUR induces mitochondria dysfunction and caspase activation

To examine the effect of PLAUR on mitochondrial membrane function, we stained the PLAUR shRNA or negative shRNA stably transfected PC9R cells with JC-1, which emits a red fluorescence as J-aggregates in intact mitochondria and is green for JC-1 monomers in damaged mitochondria. We found that the JC-1 monomer ratio of PC9R cells transfected with shPLAUR-1 or shPLAUR-2 was significantly increased compared to that of the negative control when the cells were treated with 1 μ M gefitinib (Fig. 6A and B), indicating impaired mitochondria membrane in the transfected PC9R cells.

The PathScan intracellular signaling array kit and the PathScan stress and apoptosis signaling antibody array kit were then used to monitor the apoptosis-related protein expression. It was found that the levels of cleaved caspase-3 and PARP in PC9R cells transfected with shPLAUR-1 were significantly increased compared with the negative control cells (Fig. 7A and B). Western blot analysis further confirmed that cleaved caspase-3 and PARP significantly increased in PC9R cells transfected with shPLAUR-1 or shPLAUR-2 (Fig. 7 C-E). These results suggested that knockdown of PLAUR induce cell apoptosis by increasing expression of apoptosis related proteins and impairing mitochondrial membrane.

Silencing PLAUR inactivates the EGFR/p-AKT/survivin pathway

To further explore the molecular mechanism

Fig. 4. Silencing PLAUR decreased the proliferation of PC9R cells. A) EdU in PC9R cells treated with 1 μ M gefitinib. B) Quantification of EdU-positive cells (%). Data represented as the mean $\pm$ SD. N=3, * = p<0.05 versus the negative control.

Fig. 5. Flow cytometric analysis on cell apoptosis and cell cycle in PC9R cells. A) FITC-annexin V/PI stained and flow cytometry analysis on PC9R cells treated with 1 μ M gefitinib. B) Quantification of apoptotic cells (%). C) flow cytometry on cell cycle of PC9R cells treated with 1 μ M gefitinib. D) Quantification of cell numbers at different cell cycle. Data represented as the mean $\pm$ SD. N=3, * = p<0.05 versus the negative control.

underlying PLAUR-mediated gefitinib resistance in PC9R cells, a whole human cDNA array was used to identify related pathways. We found that silencing PLAUR significantly downregulated genes involved in proteoglycans molecules, such as EGFR, CAV2, and ESR1; focal adhesion and actin cytoskeleton molecules, such as ITGB4, ITGB5, and FYN; and the PI3K-Akt signaling pathways (Fig. 8A). To verify this, Western blot analysis was performed and showed that total EGFR, p-AKT and survivin were significantly decreased in PC9R cells transfected with shPLAUR-1 or shPLAUR-2 (Fig. 8B-E).

EGFR is overexpressed in PC9R cells and inhibits cell apoptosis

To investigate the potential role of EGFR in the development of PLAUR-mediated gefitinib resistance, we compared the expression levels of EGFR in gefitinib-sensitive PC9 and -resistant PC9R cells. Western blot analysis revealed that the total EGFR protein level was significantly increased in the gefitinib-resistant PC9R cells compared to that of gefitinib-sensitive PC9 cells (Fig. 9A). To explore the effects of EGFR in the gefitinib-resistant PC9R cells, we silenced EGFR expression in PC9R cells by transfecting EGFR shRNA or negative shRNA into PC9R cells. Western blot results demonstrated that both shEGFR-1 and shEGFR-2, two shRNA sequences targeting the EGFR gene, significantly reduced the expression of EGFR in transfected PC9R cells (Fig. 9B). The CCK8 assays revealed that both shEGFR-1 and shEGFR-2 significantly inhibited the growth of the transfected PC9R cells compared to the negative control after treatment with 0.0, 0.1, 1.0, 10.0, and 20.0 μ M gefitinib (Fig. 9D). The annexin V-FITC/PI double staining and flow cytometry analysis showed that the apoptosis rates of PC9R cells transfected with shEGFR-1 or shEGFR-2 significantly increased compared to that of the negative control when incubated with 1 μ M gefitinib (Fig. 9E and F). Furthermore, the JC-1 monomer ratio of PC9R cells transfected with shEGFR-1 or shEGFR-2 was significantly increased compared to that

Fig. 6. Downregulation of PLAUR decreases mitochondrial membrane potential. A) Histograms of flow cytometry of JC-1 staining. B) Quantification of JC-1 monomer ratio after gefitinib incubation. Data represented as the mean $\pm$ SD. N=3, * = $p < 0.05$ versus the negative control.

Fig. 7. PLAUR regulates cleaved caspase-3 and PARP. PC9R cells were incubated with 1 μ M gefitinib. Chemiluminescent images of the PathScan intracellular signaling array A) and the PathScan stress and apoptosis signaling antibody array B) Red box: cleaved PARP; Green box: cleaved caspase-3. C) Western blot of cleaved caspase-3 and PARP. D) and E) Quantification of cleaved caspase-3 and PARP normalized to β -actin. Data represented as the mean $\pm$ SD. N=3, * = $p < 0.05$ versus the negative control.

of the negative control when incubated with 1 μ M gefitinib (Fig. 9G and H), indicating that suppressing EGFR expression decreases mitochondrial membrane potential and induces cell apoptosis.

PLAUR downregulation - induced cell apoptosis is attenuated by overexpression of EGFR

To further explore the regulation of PLAUR by EGFR, PC9R cells were first transfected with lentiviruses carrying overexpressing human EGFR lentiviral vector shEGFR-O, and then transfected with shPLAUR-1. Western blot analysis revealed that the total EGFR protein level was significantly increased in EGFR overexpressed PC9R cells (Fig. 10A and B). The CCK8 assays revealed that the cell proliferation of the PC9R cells transfected with shPLAUR-1 and shEGFR-O significantly increased compared to that transfected with shPLAUR-1 only (Fig. 10C), suggesting that PLAUR downregulation-induced apoptosis can be attenuated by overexpression of EGFR.

Knockdown of PLAUR suppresses tumor growth in nude mice

To determine whether knockdown of PLAUR suppresses lung tumor growth *in vivo*, mouse tumor xenograft models were established by using PC9R cells transfected with shPLAUR-1 and negative control vectors. After one week of cell injection, the mice produced visible tumors. It was noted that tumor volume of the PC9R cells transfected with shPLAUR-1 was significantly smaller than that of the tumor from cells transfected with the negative control (Fig. 11A-B). Furthermore, the tumor mass of PC9R cells transfected with shPLAUR-1 was significantly less than that of tumor from the cells transfected with the negative control after one month (Fig. 11C). To examine tumor cell proliferation, the tumor sections were immunohistochemically stained with Ki67. It was found that the percentage of Ki67-positive cells significantly decreased in tumor tissues from animals injected with PC9R cells transfected with shPLAUR-1 compared to that of the negative control (Fig. 11D and E).

Fig. 8. PLAUR regulates EGFR/p-AKT/survivin pathway. A) PC9R cells analyzed by whole human cDNA array. The expression changes of selected genes were presented. B) Western blot on total EGFR, p-AKT, AKT, and survivin. Quantification of Western blot C) EGFR, D) p-AKT and E) survivin were normalized to β -actin. Data represented as the mean $\pm$ SD. N=3, * = $p < 0.05$ versus the negative control.

Discussion

Exosomes are one of the most promising biomarkers for monitoring drug resistance in NSCLC. They are very stable in blood and contain molecular constituents of the tumor cells they come from, such as EGFR mutations [47, 48], K-ras, claudins and translocation ALK-EML4 [49]. However, it is very challenging to examine the levels of these molecular constituents in exosomes from plasma, which impedes examination of exosome from being widely used in clinics. In this study, we applied the newly developed TCLN assay, which contains molecular beacons to directly capture exosomes and detected mRNA within them [10, 11]. We found that exosomal PLAUR mRNA in the plasma from gefitinib-resistant lung adenocarcinoma was significantly increased compared to that of gefitinib-sensitive lung adenocarcinoma, indicating that exosomal PLAUR mRNA could be used as a biomarker for gefitinib resistance in NSCLC.

Elevated levels of soluble PLAUR protein are reported in lung cancer [27, 50] and other cancers [51]. PLAUR gene amplification is observed in breast cancer [52] and colorectal cancer [53]. However, PLAUR gene amplification in lung cancer has not been investigated. For the first time, we evaluated the expression of PLAUR mRNA in exosomes from NSCLC patients.

Fig. 9. EGFR upregulated in PC9R cells and EGFR knockdown induced cell apoptosis. A) Western blot of EGFR protein in PC9R and PC9 cells. B) Western blot of EGFR in PC9R cells transfected with shEGFR-1, shEGFR-2 and negative vector. C) CCK-8 on the viability of PC9R cells incubated with indicated concentrations of gefitinib. N=3. D) flow cytometry on PC9R cells treated with 1 μM gefitinib and stained with FITC-annexin V/PI. E) Quantification of apoptotic cells (%) of PC9R cells. F) Histograms of flow cytometry analysis of JC-1 staining. G) Quantification of JC-1 monomer ratio in PC9R cells incubated with 1 μM gefitinib. Data represented as the mean ± SD. N=3, * = p<0.05 versus the negative control.

Fig. 10. Overexpression of EGFR attenuates PLAUR-mediated gefitinib resistance. A Western blot of EGFR protein in PC9R cells with human EGFR overexpression shRNA vectors (shEGFR-O) and with control vector. B) EGFR expression in Western blot quantified and normalized to β-actin. C) CCK-8 on the viability of PC9R cells transfected with indicated shRNAs. Data presented as the mean ± SD. N=3, * = p<0.05 versus the negative control.

PLAUR protein exists in various states in cells [54]. Our RT-PCR results showed that PLAUR mRNA was significantly increased in the gefitinib-resistant PC9R cells compared to the gefitinib-sensitive PC9 cells. The ELISA results showed that soluble PLAUR in the supernatant of the PC9R cells was significantly higher than that of the PC9 cells. However, the Western blot results showed that the PLAUR protein level in the PC9R cells were not significantly different from that of the PC9 cells. One possible explanation is that the overexpressed PLAUR protein exists as a soluble form and secretes out of cells, leaving the PLAUR protein level inside the cells unchanged. These results demonstrated that the expression of mRNA and soluble form of PLAUR were significantly increased in the gefitinib-resistant PC9R cells, indicating a role for PLAUR in gefitinib resistance.

The molecular mechanism underlying PLAUR-mediated gefitinib resistance in NSCLC was investigated by establishing a gefitinib-resistant human lung adenocarcinoma PC9R cell line and examining the expression of PLAUR in these gefitinib-sensitive and -resistant human lung adenocarcinoma cells. Consistent with the clinical findings, we demonstrated that the exosomal PLAUR mRNA and the soluble PLAUR were significantly increased in gefitinib-resistant PC9R cells compared to gefitinib-sensitive PC9 cells. Silencing PLAUR in PC9R cells decreased the mitochondrial membrane potential and increased cleaved caspase-3 and PARP, suggesting that knockdown of PLAUR induced cell apoptosis via a mitochondrial-dependent pathway. To further explore the molecular mechanism, a whole human cDNA array was used to identify the related pathways. We found that downregulation of PLAUR significantly decreased expression of various genes involved in proteoglycan, focal adhesion, actin cytoskeleton, and PI3K-Akt signaling pathways.

Fig. 11. Knockdown PLAUR suppresses tumor growth in nude mice. A) Tumor tissues from the animals one month after injections of PC9R cells transfected with shPLAUR-1 or negative vector. B) The change of tumor volume. C) The statistical analysis of tumor weight. D) Ki67 immunohistochemistry staining of the xenograft tumors. E) Quantification of Ki67-positive cells. Data presented as the mean ± SD. N=5, * = p<0.05 versus the negative control.

EGFR has tyrosine kinase dependent and independent functions, and its tyrosine kinase independent activities are essential for cell survival [36, 38-40]. Knockdown of EGFR in cancer cells leads to inactivation of p-AKT and p-ERK, that are important for drug resistance and induces cell apoptosis [35-40, 44, 45], while deletion of EGFR in mice is embryonic lethal [41, 42]. The interaction between PLAUR and EGFR was previously studied. Glioblastoma cells expressing EGFR variant III release soluble PLAUR, which promotes cell migration and invasion [55], while PLAUR induces EGFR transactivation in MCF-7 cells [56] and T-HEp3 cells [15]. Although previous studies have demonstrated that PLAUR inhibits EGFR tyrosine kinase activity, those reports did not show the expression of EGFR regulated by PLAUR. For the first time, we found that knockdown of PLAUR significantly reduced EGFR protein expression in gefitinib-resistant NSCLC cells, suggesting that PLAUR may regulate cell apoptosis through the tyrosine kinase independent activities of EGFR.

Resistance to EGFR TKIs in various tumors is correlated with enhanced expression of EGFR [57], suggesting the involvement of EGFR in the resistance mechanism. In this study, we found that EGFR expression increased in gefitinib-resistant PC9R cells, indicating that EGFR contributed to the acquired EGFR TKIs resistance in the EGFR-mutant, gefitinib-sensitive cells. Thus, the resistance could be reversed by silencing EGFR in gefitinib-resistant cancer cells.

Survivin is a member of the inhibitor of apoptosis protein (IAP) family [58], and directly interacts with caspase-3 to regulate apoptosis [59]. Survivin expression is increased in many cancers and embryonic tissues [60] and is mediated by EGFR via the phosphatidylinositol 3-kinase/AKT signaling pathway in breast cancer cells [61]. In this study, we found that downregulation of PLAUR significantly decreased the expression of survivin, indicating that PLAUR can inhibit cell apoptosis through the EGFR/p-AKT/survivin pathway.

Conclusion

The results of this study suggest that PLAUR confers resistance to gefitinib through EGFR/p-AKT/survivin signaling pathway. Exosomal PLAUR mRNA could be used as a biomarker to monitor gefitinib resistance in NSCLC, and silencing PLAUR may reverse the acquired resistance.

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Authors have no financial, professional, or personal conflicts to disclose

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