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ORIGINAL RESEARCH

S100A6 Induces the Resistance to Gefitinib in Human Lung Adenocarcinoma PC9 Cell Lines with EGFR 19 Exon Mutations

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Introduction: S100 calcium binding protein A6 (S100A6) has been confirmed to be involved in the occurrence and development of various malignant tumors, including lung adenocarcinoma (LADC). The impact of S100A6 on the drug resistance of cancer cell lines is still uncovered. PC9 cells with EGFR 19 exon mutation is commonly used in lung cancer cell experiments.

Methods: In this work, PC9 cells over-expressing S100A6 (PC9/S100A6) was successfully constructed, and the PC9 cells were set as control group simultaneously. MTT assay was used to detect and compare the growth rates of the cells in two groups at different concentrations and time points of gefitinib, cisplatin, pemetrexed, bevacizumab.

Results: Data showed that the inhibitory rates of gefitinib (0.01μmol/L, 0.1μmol/L, 1μmol/L) on PC9/S100A6 cells significantly decreased at 24h, 48h, 72h ($P<0.05$). Additionally, the inhibitory effects on PC9/S100A6 cells were significantly lower for 10 μmol/L gefitinib (at 48h and 72h), 100 μmol/L gefitinib (at 72h), 2.5 μg/mL cisplatin (at 48h and 72h), and 10 μg/mL cisplatin (at 72h). However, there is no obvious difference in the inhibitory rate of cisplatin (2.5μg/mL 24h; 10μg/mL 24h, 48h; 5μg/mL, 20μg/mL, 40μg/mL 24h, 48h, and 72h), pemetrexed, bevacizumab on two groups.

Conclusion: Our results demonstrated that S100A6 can apparently promote the resistance to gefitinib of LADC PC9 cell lines with EGFR 19 exon mutations.

Keywords: S100A6, EGFR 19 exon mutation, PC9, gefitinib, drug resistance

Introduction

Worldwide, lung cancer is the principal cause of cancer-related death, leading an estimated 1.8 million deaths in 2022.[1] It is classified into non-small cell lung cancer (NSCLC) and small cell lung cancer pathologically, the former is divided into adenocarcinoma, squamous carcinoma, and large cell carcinoma.[2] Lung cancer has become the most deadly malignant tumor due to two main reasons. The first one is that lung cancer patients lack specific symptoms in the early stages, and the vast majority of patients are already in the disease progression stage when seeking medical treatment, losing the opportunity for surgical treatment.[3] On the other hand, adenocarcinoma is the largest subtype of lung cancer, characterized by high invasiveness, susceptibility to hematogenous and lymphatic metastasis, and poor sensitivity to traditional, commonly used treatments, such as radiotherapy, chemotherapy, angiogenesis inhibitors.[4] In the treatment of lung adenocarcinoma, several therapeutic agents are commonly used due to their clinical relevance and distinct mechanisms of action. Cisplatin, a platinum-based chemotherapeutic agent, is a cornerstone of traditional chemotherapy for lung cancer, known for its ability to form DNA adducts and induce apoptosis. Pemetrexed, a multitargeted antifolate, inhibits enzymes involved in purine and pyrimidine synthesis. Bevacizumab, a monoclonal antibody targeting vascular endothelial growth factor (VEGF), is used to inhibit angiogenesis and has shown benefits in combination with chemotherapy for advanced NSCLC.[5] In the past decade or so, the prognosis of some lung adenocarcinoma patients has been significantly improved due to the widespread use of molecular targeted drugs targeting EGFR mutations, ALK gene rearrangements, and ROS1 gene rearrangements.[6] Among them, EGFR mutations are commonly detected, have higher rates amongst

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Asians (38.8–64.0%), including deletions in exon 19 (E19 dels) and a point mutation in E21.[7] Tyrosine kinase inhibitors (TKIs) that block EGFR-derived signal transduction show excellent efficacy to patients with EGFR mutations. However, patients have to face the dilemma of targeted drug resistance after a period of EGFR-TKIs use.[8] The resistance mechanism of targeted drugs, such as EGFR-TKIs, has also become a hot topic in recent years.

S100A6 (Calcylin) is a small molecule, acidic, and stable protein that can regulate cell cycle and differentiation, and promote various biological behaviors of tumor cells.[9] In lung cancer, S100A6 was confirmed to selectively over-express in lung adenocarcinoma.[10,11] In this study, we explored the role of S100A6 in the resistance of lung adenocarcinoma cells to several drugs, including gefitinib, cisplatin, pemetrexed, and bevacizumab. Given the high incidence of EGFR mutations in lung adenocarcinoma and the clinical significance of gefitinib in treating these mutations, we specifically investigated the resistance to gefitinib in PC9 cells with EGFR 19 exon mutations.

Materials and Methods

Cell Culture

The lung adenocarcinoma cell line PC9, with EGFR 19 exon mutation, and normal lung epithelial cell (BEAS-2B) were purchased from the Procell Life Science & Technology Co., Ltd (Wuhan, China). The cells were preserved under 5% CO_2 at 37°C, expanded for one to two weeks in growth medium containing high glucose Dulbecco's modified Eagle medium (DMEM; Gibco, USA) combined with 100 U/mL streptomycin/penicillin (Gibco, USA) and 15% heated-inactivated foetal bovine serum (FBS, Sinopharm Chemical Reagent Co., Ltd, Shanghai, China).

RNA Extraction and qPCR

Trizol reagent (Ambion, 15596–026) was used to lyse the cells, then the mixture was centrifuged and the supernatant were collected. Before purity detection and RNA concentration using Nanodrop 2000 (Thermo Fisher Scientific, USA), RNA separation was completed by chloroform, 2-propanol, and 75% ethanol. Next, reverse transcription was conducted by Revert Aid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA), all steps were applied according to the kit instruction. The primers' information in PCR assay was as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer (5'-TCAAGAAGGTGGTGAAGCAGG-3'), GAPDH reverse primer (5'-TCAAAGGTGGAGGAGTGGGT −3'), S100A6 forward primer (5'-GACAAGCACACCCTGAGCAA-3'), S100A6 reverse primer (5'- TTCCGGTCCAAGTCTTCCAT −3').

Western Blotting

S100A6 protein levels in PC9 and BEAS-2B cell lines were detected by Western blotting assay. Firstly, cells were collected and dissolved by Total Protein Extraction Kit. The cell lysate was then centrifugated at 14,000 rpm for 10 min, added loading sample buffer, followed by heating at 95°C for 5 min, separating with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and finally transferring to nitrocellulose membrane. We then blocked the nitrocellulose filters using 5% non-fat milk and incubated them with primary antibody (ab181975; Abcam, Shanghai, China; 1:10000 dilution) at 4°C overnight. Simultaneously, the mouse GAPDH antibody was set as an endogenous control using. The secondary antibody against S100A6 (ab150077; Abcam, Shanghai, China; 1:600 dilution) were next added with horse radish peroxidase-labelled polymer. The membrane was re-rinsed and images were collected by enhanced chemiluminescence.

Cell Transfection

The above mentioned qPCR and Western blot assay confirmed PC9 cells have lower S100A6 expression compared BEAS-2B cells. Thus, we generated S100A6 over-expression lentivirus vector plasmid by connecting target gene with vector plasmid, and the information on origin of S100A6 human gene origin was referred to the website: "https://www.ncbi.nlm.nih.gov/gene/6277". The vector plasmid pLVX-mCMV-ZsGreen-IRES-Puro and target gene were digested by EcoR I and BamH before the connecting. Then, pLVX-mCMV-ZsGreen-IRES-Puro-Homo-S100A6, pspax2, and pmd2. G were co-transfected into 293T cells at a ratio of 2:1:1. After 72 h, we observed the fluorescence expression by

microscope, the supernatant were collected, filtrated with PVDF filter (Millipore), then we obtained thirty-fold concentrated stock after the ultracentrifugation (50,000× g, 4°C, 150min), resuspended and stored them at −80°C.

PC9 cells at logarithmic phase were collected and trypsinised to a density of 3×10^5/mL, plasmid with over-expressed S100A6 was added when their confluence reached 80–90%. The transfection efficiency was then detected by quantitative PCR and Western blotting.

MTT Assay

We applied MTT colorimetric assay to evaluate the effect of drugs on the cell proliferation rate. PC9 cells and PC9 cells with over-expressing S100A6 (PC9/S100A6) in logarithmic growth phase were inoculated into a 96 well plate at a density\3 of 5×10^3 cells per well overnight in incubator (37°C, 5% CO_2), sterile PBS (200μL) was added to pores around the cells at the same time. Cell grouping was carried out according to different drug concentrations and time points, with specific information as follows: cisplatin (2.5μg/mL, 5μg/mL, 10μg/mL, 20μg/mL, 40μg/mL; 24h, 48h, and 72h), pemetrexed (0.04nmol/L, 0.2nmol/L, 1nmol/L, 5nmol/L, 25nmol/L; 24h, 48h, and 72h), bevacizumab (0.0125μg/mL, 0.025μg/mL, 0.05μg/mL, 0.1μg/mL, 0.2μg/mL; 24h, 48h, and 72h), gefitinib (0μmol/L, 0.1μmol/L, 1μmol/L, 10μmol/L, 100μmol/L; 24h, 48h, and 72h). After the required time for cell culture, we added 100ul MTT to every well, incubated them at 37°C for 50 minutes before extracting the culture medium, adding DSMO (150μL), and measuring the absorbance values of each well by ELISA at 570nm.

Statistical Analysis

We applied Microsoft Excel 2019, SPSS 22.0 and GraphPad Prism 7.0 to collect, analyze data, make chart and calculate half maximal inhibitory concentration (IC50). *T* test was used to compare differences among two groups for the data with normal distribution, while rank sum test was applied for those without confirmation of normal distribution to analyze the differences. All the results of the OD value and cell inhibiting rates had three replications, displaying as mean±SD.

Results

Inhibition Rate of Cisplatin on PC9 and PC9/S100A6 Cells

In the drug inhibition test, among the four drugs, cisplatin showed the best inhibitory effect on both PC9 cells and PC9/S100A6 cells. Both low (2.5μg/mL) and high (40μg/mL) concentrations of cisplatin showed high inhibition rates on both groups in the early (24 hours) and late (72 hours) stages. At 24h, 48h, 72h, the IC50 of cisplatin was 9.135μg/mL (PC9), 9.293μg/mL (PC9/S100A6), 3.488μg/mL (PC9), 4.397μg/mL (PC9/S100A6), 1.903μg/mL (PC9), 2.719μg/mL (PC9/S100A6) respectively, as showed in Figure 1 and Table 1. Besides, compared with PC9 cells, the inhibitions of cisplatin on PC9/S100A6 cells were reduced at the concentrations of 2.5μg/mL (48h, 72h) and 10μg/mL (72h). However, there was no statistical difference in the inhibitory rate of cisplatin between the two groups at other concentrations/time points. Detailed information could be found in Table 2.

Inhibition Rate of Bevacizumab on PC9 and PC9/S100A6 Cells

Bevacizumab has a poorer inhibitory effect than other medicines on the two groups, with all of inhibition rates lower than 50% at various concentrations (0.0125μg/mL, 0.025μg/mL, 0.05μg/mL, 0.1μg/mL, 0.2μg/mL) and time periods (24h, 48h, 72h), no significant differences were observed between the two groups, as listed in Table 3. At 24h, 48h, 72h, the IC50 of bevacizumab was not determinable within the tested concentration range, as showed in Table 1.

Inhibition Rate of Pemetrexed on PC9 and PC9/S100A6 Cells

As showed in Table 4, overall speaking, the inhibitory effect of pemetrexed on PC9 cells and PC9/S100A6 cells was slightly better than that of bevacizumab, but extremely lower than that of gefitinib and cisplatin. The inhibition rate of the highest concentration of pemetrexed on both groups of cells was still less than 15%, which was less than one-sixth of the inhibition rate of high concentration cisplatin. Similarly, pemetrexed did not show a statistically significant difference in cell inhibition rates between two groups, and IC50 could not be calculated at any tested concentration, as Table 1 showed.

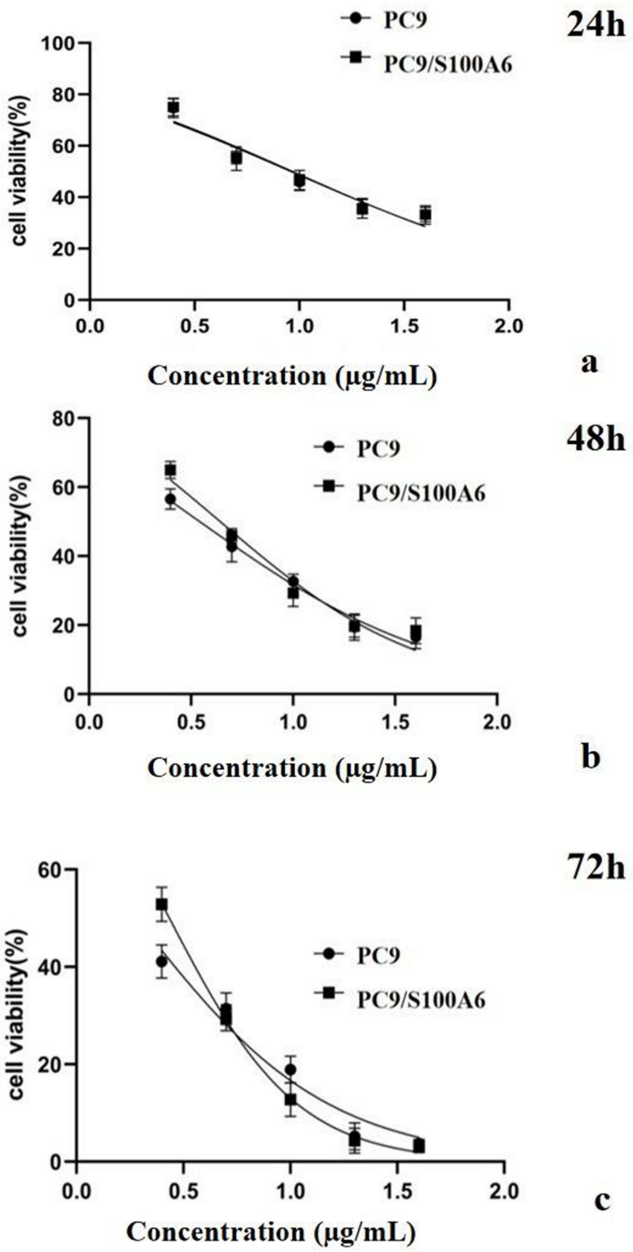

Figure 1 Inhibition rates of various concentrations of cisplatin on PC9 and PC9/S100A6 cells at 24h (**a**), 48h (**b**), and 72h (**c**).

Inhibition Rate of Gefitinib on PC9 and PC9/S100A6 Cells

Gefitinib showed a relatively high inhibitory rate on PC9 cells and PC9/S100A6 cells at 24 hours already. The IC50 of gefitinib was 0.3724μmol/L (PC9), 1.126μmol/L (PC9/S100A6), 0.1403μmol/L (PC9), 0.4998μmol/L (PC9/S100A6) at 48h, 72h, respectively, as listed in Figure 2 and Table 1. Compared with PC9 cells, gefitinib showed an obvious decrease in the inhibition rate of PC9/S100A6 cells, with $P<0.05$. Specifically, the significant decrease in inhibition of gefitinib for PC9/S100A6 cells (compared to PC9 cells) was observed for each of these three concentrations (0.01, 0.1, and 1μmol/L) at all three specified time points (24h, 48h, and 72h). In addition, the inhibitory rates of 10 μmol/L (48h, 72h) and 100μmol/L (72h) gefitinib on PC9/S100A6 cells apparent lower than that in PC9 cells, as showed in Table 5 ($P<0.05$).

Table 1 Comparison of IC50 Values of Four Drugs

	24h		48h		72h	
	PC9	**PC9/S100A6**	**PC9**	**PC9/S100A6**	**PC9**	**PC9/S100A6**
Gefitinib (μmol/L)	–		0.3724	1.126	0.1403	0.4998
Cisplatin (μg/mL)	9.135	9.293	3.488	4.397	1.903	2.719
Pemetrexed (nmol/L) Bevacizumab (μg/mL)	–					

Note: - represents not determinable within the tested concentration range.

Table 2 Inhibition Rates of Cisplatin on PC9 and PC9/S100A6 Cells at Different Concentrations and Time Points

Cisplatin (Concentrations, Time Points)	**OD Value (PC9)**	**OD Value (PC9/S100A6)**	***P* value**	**Inhibition Rates (%) (PC9)**	**Inhibition Rates (%) (PC9/S100A6)**	***P* value**
0μg/mL, 24h	0.942±0.02	0.940±0.02	0.97	−0.07±2.48	−0.03±2.57	0.99
0μg/mL, 48h	1.030±0.02	1.066±0.02	0.30	0.06±2.30	−1.19±0.72	0.63
0μg/mL, 72h	1.298±0.10	1.455±0.02	0.21	0.02±7.99	0.00±1.13	1.00
2.5μg/mL, 24h	0.683±0.02	0.701±0.02	0.46	27.45±2.29	24.82±2.12	0.45
2.5μg/mL, 48h	0.583±0.02	0.692±0.15	0.01*	43.48±1.65	35.05±1.36	0.02*
2.5μg/mL, 72h	0.534±0.02	0.770±0.02	0.00*	58.86±1.52	47.10±1.40	0.00*
5μg/mL, 24h	0.482±0.03	0.522±0.01	0.26	48.74±2.81	44.50±1.48	0.25
5μg/mL, 48h	0.440±0.03	0.490±0.01	0.15	57.29±2.47	54.03±1.09	0.29
5μg/mL, 72h	0.408±0.02	0.431±0.02	0.40	68.59±1.45	70.40±1.06	0.37
10μg/mL, 24h	0.389±0.02	0.438±0.02	0.16	58.66±1.81	53.40±2.44	0.16
10μg/mL, 48h	0.337±0.01	0.313±0.02	0.39	67.31±1.19	70.67±1.19	0.24
10μg/mL, 72h	0.245±0.02	0.186±0.02	0.08	81.10±1.24	87.22±1.38	0.03*
20μg/mL, 24h	0.290±0.02	0.336±0.02	0.17	69.14±1.78	64.25±2.33	0.17
20μg/mL, 48h	0.200±0.02	0.210±0.02	0.74	80.60±2.12	80.30±1.73	0.92
20μg/mL, 72h	0.068±0.02	0.063±0.01	0.85	94.79±1.22	95.65±1.01	0.62
40μg/mL, 24h	0.258±0.02	0.313±0.02	0.11	72.55±2.21	66.67±1.72	0.10
40μg/mL, 48h	0.171±0.02	0.196±0.02	0.43	83.45±1.89	81.58±2.04	0.54
40μg/mL, 72h	0.048±0.00	0.047±0.01	0.92	96.27±0.38	96.75±0.54	0.52

Note: * P<0.05.

Table 3 Inhibition Rates of Bevacizumab on PC9 and PC9/S100A6 Cells at Different Concentrations and Time Points

Bevacizumab (Concentrations, Time Points)	**OD Value (PC9)**	**OD Value (PC9/S100A6)**	***P* value**	**Inhibition Rates (%) (PC9)**	**Inhibition Rates (%) (PC9/S100A6)**	***P* value**
0μg/mL, 24h	0.934±0.02	0.935±0.02	0.96	0.03±2.29	−0.07±2.67	0.99
0μg/mL, 48h	1.012±0.02	1.078±0.02	0.04*	0.06±1.86	0.06±1.12	1.00
0μg/mL, 72h	1.382±0.02	1.415±0.02	0.21	0.00±1.22	0.02±1.62	0.99
0.0125μg/mL, 24h	0.946±0.02	0.946±0.02	0.99	−1.283±2.00	−1.103±1.87	0.95
0.0125μg/mL, 48h	1.019±0.02	1.087±0.02	0.07	−0.623±2.04	0.740±1.61	0.97
0.0125μg/mL, 72h	1.369±0.02	1.397±0.01	0.27	0.917±1.26	1.250±0.93	0.84
0.025μg/mL, 24h	0.921±0.03	0.920±0.02	0.99	1.313±2.34	1.713±2.25	0.94
0.025μg/mL, 48h	1.023±0.01	1.098±0.02	0.02*	−0.987±1.20	−1.793±1.56	0.70
0.025μg/mL, 72h	1.413±0.02	1.441±0.02	0.29	−2.267±1.17	−1.813±1.11	0.79
0.05μg/mL, 24h	0.929±0.02	0.930±0.02	0.98	0.500±1.81	0.643±2.53	0.97
0.05μg/mL, 48h	1.005±0.01	1.085±0.02	0.03*	0.757±1.43	−0.523±1.69	0.59
0.05μg/mL, 72h	1.390±0.02	1.427±0.02	0.21	−0.603±1.25	−0.850±1.22	0.89
0.1μg/mL, 24h	0.950±0.02	0.955±0.02	0.89	−1.677±2.14	−1.993±2.77	0.93

(*Continued*)

Table 3 (Continued).

Bevacizumab (Concentrations, Time Points)	OD Value (PC9)	OD Value (PC9/S100A6)	*P* value	Inhibition Rates (%) (PC9)	Inhibition Rates (%) (PC9/S100A6)	*P* value
0.1μg/mL, 48h	0.992±0.01	1.062±0.02	0.06	2.073±1.44	1.607±2.19	0.87
0.1μg/mL, 72h	1.370±0.02	1.397±0.02	0.34	0.847±1.16	1.297±1.31	0.81
0.2μg/mL, 24h	0.918±0.03	0.919±0.03	0.99	−0.747±3.60	1.853±2.85	0.60
0.2μg/mL, 48h	1.023±0.02	1.087±0.02	0.08	−1.020±1.95	−0.740±1.70	0.92
0.2μg/mL, 72h	1.365±0.02	1.387±0.02	0.46	1.230±1.47	2.003±1.21	0.71

Note: *P<0.05.

Table 4 Inhibition Rates of Pemetrexed on PC9 and PC9/S100A6 Cells at Different Concentrations and Time Points

Pemetrexed (Concentrations, Time Points)	OD Value (PC9)	OD Value (PC9/S100A6)	*P* value	Inhibition Rates (%) (PC9)	Inhibition Rates (%) (PC9/S100A6)	*P* value
0nmol/L, 24h	0.942±0.02	0.940±0.02	0.97	−0.070±248	−0.03±2.57	0.99
0nmol/L, 48h	1.030±0.02	1.066±0.02	0.30	0.06±2.30	0.00±1.67	0.98
0nmol/L, 72h	1.298±0.10	1.455±0.02	0.21	0.02±7.99	0.00±1.13	1.00
0.04nmol/L, 24h	0.914±0.02	0.911±0.02	0.92	2.83±2.58	3.12±2.624	0.94
0.04nmol/L, 48h	1.029±0.03	1.056±0.01	0.40	0.19±2.53	0.91±1.20	0.81
0.04nmol/L, 72h	1.276±0.02	1.430±0.01	0.00*	1.72±1.36	1.69±0.73	0.99
0.2nmol/L, 24h	0.891±0.03	0.899±0.02	0.85	5.28±3.13	4.40±2.37	0.83
0.2nmol/L, 48h	1.010±0.02	1.052±0.02	0.20	2.04±2.19	1.31±1.42	0.80
0.2nmol/L, 72h	1.303±0.01	1.431±0.02	0.00*	−0.41±1.16	0.96±1.22	0.46
1nmol/L, 24h	0.855±0.02	0.861±0.02	0.85	9.14±2.07	8.44±2.17	0.83
1nmol/L, 48h	0.987±0.02	1.020±0.02	0.28	4.30±1.99	4.32±1.63	1.00
1nmol/L, 72h	1.288±0.02	1.423±0.01	0.00*	0.80±1.20	2.22±0.71	0.37
5nmol/L, 24h	0.840±0.02	0.836±0.02	0.90	10.77±2.15	11.10±2.47	0.92
5nmol/L, 48h	0.983±0.01	1.005±0.02	0.41	4.66±1.32	5.75±1.79	0.65
5nmol/L, 72h	1.261±0.02	1.410±0.02	0.00*	2.88±1.25	3.09±1.42	0.92
25nmol/L, 24h	0.818±0.03	0.822±0.01	0.91	13.04±2.66	12.59±1.55	0.89
25nmol/L, 48h	0.946±0.02	0.982±0.02	0.25	8.28±1.49	7.88±2.08	0.89
25nmol/L, 72h	1.244±0.03	1.399±0.02	0.01*	4.16±2.08	3.82±1.27	0.90

Note: *P<0.05.

Discussion

In this study, we constructed PC9 cell line with over-expressed S100A6 to investigate the role of S100A6 in resistance of lung adenocarcinoma cells to common therapeutic drugs. Data showed that S100A6 promotes resistance of PC9 cells with EGFR 19 exon mutation to first-generation EGFR-TKI gefitinib (most concentrations/time points) and cisplatin (individual concentrations/time points), while it has no significant impact on the effects of other medicines. To the best of our knowledge, our study firstly explored the function of S100A6 in resistance analysis on lung adenocarcinoma to vascular endothelial growth factor inhibitors, chemotherapeutics, and EGFR-TKI.

Alterations to the kinase domain of EGFR caused by somatic activating mutations commonly occur in NSCLC.[12] EGFR mutations are observed up to 50% of East-Asian patients with NSCLC, and for those female patients who never smoked, the incidence is more higher.[13] Exon 19 deletions and exon 21 L858R point mutations represent the majority of EGFR mutations.[14] For NSCLC patients with EGFR mutations (adenocarcinoma mainly), small-molecule TKIs show better therapeutic efficacy by binding the adenosine triphosphate pocket of EGFR and inhibiting its downstream signal transduction.[15] Targeted drugs that are currently used include three generations, such as gefitinib and erlotinib (first-generation), afatinib and dacomitinib (second-generation), osimertinib (third-generation).[16] Related clinical trial showed 12-month rates of progression-free survival (PFS) is 24.9% in the gefitinib treating patients (adenocarcinoma 95.4%) and 6.7% in the patients received carboplatin-paclitaxel (adenocarcinoma 97.2%).[17] In the subgroup of patients who were

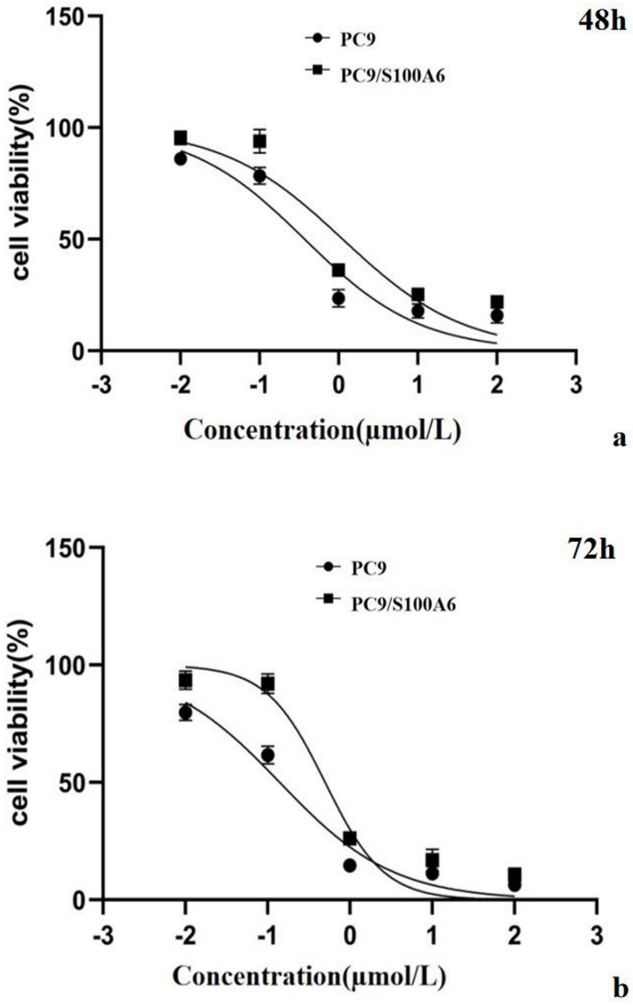

Figure 2 Inhibition rate of various concentrations of gefitinib on PC9 and PC9/S100A6 cells at 48h (**a**) and 72h (**b**).

positive for EGFR mutation, the PFS was significantly long among those received gefitinib. Despite the in-depth understanding of pathogenesis in lung adenocarcinoma and major therapeutic advance, the majority of these patients ultimately develop EGFR TKIs resistance after a period of time.[18] Several mechanisms are confirmed to be involved in

Table 5 Inhibition Rates of Gefitinib on PC9 and PC9/S100A6 Cells at Different Concentrations and Time Points

Gefitinib (Concentrations, Time Points)	OD Value (PC9)	OD Value (PC9/S100A6)	*P* value	Inhibition Rates (%) (PC9)	Inhibition Rates (%) (PC9/S100A6)	*P* value
0μmol/L, 24h	0.941±0.02	0.940±0.02	0.97	0.07±2.56	−0.07±2.48	0.97
0μmol/L, 48h	1.041±0.02	1.074±0.02	0.32	1.31±2.34	−0.06±1.84	0.67
0μmol/L, 72h	1.502±0.01	1.564±0.02	0.08	−0.02±0.98	0.02±1.47	0.98
0.01μmol/L, 24h	0.842±0.02	0.925±0.02	0.04*	10.52±1.63	1.70±2.41	0.04*
0.01μmol/L, 48h	0.818±0.02	1.019±0.02	0.00*	21.39±2.17	5.03±2.22	0.01*
0.01μmol/L, 72h	0.927±0.02	1.433±0.01	0.00*	38.26±1.45	8.40±0.81	0.00*
0.1μmol/L, 24h	0.842±0.02	0.925±0.02	0.04*	10.52±1.63	1.70±2.41	0.04*
0.1μmol/L, 48h	0.818±0.02	1.019±0.02	0.00*	21.39±2.17	5.03±2.22	0.01*
0.1μmol/L, 72h	0.927±0.02	1.433±0.01	0.00*	38.26±1.45	8.40±0.81	0.00*
1μmol/L, 24h	0.728±0.02	0.805±0.01	0.03*	22.67±2.03	14.45±1.52	0.03*

(*Continued*)

Table 5 (Continued).

Gefitinib (Concentrations, Time Points)	OD Value (PC9)	OD Value (PC9/S100A6)	*P* value	Inhibition Rates (%) (PC9)	Inhibition Rates (%) (PC9/S100A6)	*P* value
1μmol/L, 48h	0.259±0.02	0.380±0.02	0.01*	75.12±1.67	64.55±2.14	0.02*
1μmol/L, 72h	0.228±0.02	0.395±0.01	0.00*	84.79±1.31	74.72±0.70	0.00*
10μmol/L, 24h	0.669±0.02	0.750±0.02	0.07	28.94±2.33	20.30±2.59	0.07
10μmol/L, 48h	0.181±0.02	0.281±0.01	0.01*	82.64±1.70	73.78±1.30	0.01*
10μmol/L, 72h	0.162±0.02	0.238±0.00	0.02*	89.22±1.33	84.80±0.31	0.03*
100μmol/L, 24h	0.616±0.02	0.685±0.02	0.05	34.57±1.93	27.84±2.20	0.08
100μmol/L, 48h	0.164±0.02	0.234±0.01	0.04*	84.13±1.93	78.05±1.11	0.05
100μmol/L, 72h	0.083±0.02	0.157±0.01	0.02*	93.61±1.21	89.23±0.86	0.04*

Note: *P<0.05.

the resistant mutations: EGFR target-dependent mechanics of resistance, including T790M mutations, C797X mutations, G796R, G796S, etc, EGFR target-independent mechanisms of resistance, such as MET amplification, HER2 amplification, oncogenic fusion or chromosomal rearrangements.[19,20] Additionally, other rare mechanisms, like histologic and phenotypic transformations, are also reported in EGFR-TKIs resistance.[21] For gefitinib, data indicate that T790M mutation was found in 50–60% of patients receiving gefitinib, erlotinib or afatinib, of 18 gefitinib/erlotinib-resistant patients with lung cancer, 4 (22%) were detected having MET amplification.[22] Some ligands, such as HGF, could bind MET, thus inducing various biological effects, including morphogenic, mitogenic, antiapoptotic activities. The activation of PI3K/Akt driving TKI resistance, invasive tumor behaviors, could be also restored by the complex.[15]

The S100 protein family comprises a total of 25 human members and has a close association with carcinogenesis of many malignant tumors due to the rearrangement proneness of the chromosomal location.[23] S100A6 (Calcylin) is a small molecule, acidic, and stable protein with a non spherical secondary structure, consisting of two structural domains, two calcium binding regions, and no transmembrane region.[9] S100A6 has an important role in regulating cell cycle and differentiation, as well as mediating intracellular calcium signaling release. In the S100 protein family, S100A6 is the first member confirmed to be related to the differentiation of tumor cells.[23] In NSCLC, Luigi De Petris et al tested the expression of S100A6 in surgical specimens of 103 patients with stage I lung cancer. The results showed that in the surgically excised specimens, the expression of S100A6 was significantly higher in lung adenocarcinoma than in lung squamous cell carcinoma (25/51 vs 1/52).[10] In A549 lung adenocarcinoma cell line, S100A6 has been shown to promote tumor cell invasion and migration by acetylating P53.[24] In other kinds of cancers, thyroid cancer, cervical cancer, for instance, S100A6 could promote their development by activating PI3K/AKT/mTOR, which is also a vital downstream signalling pathway of EGFR.[25,26] However, overall, there is still a lack of research about the exact mechanisms on the correlation between S100A6 and EGFR TKIs resistance in lung adenocarcinoma. We hypothesize that S100A6 intersects with EGFR signaling pathways in lung adenocarcinoma via multiple mechanisms. S100A6 could enhance PI3K/AKT/mTOR pathway activation, compensating for EGFR inhibition by TKIs and promoting drug resistance. This may involve direct interactions with pathway components or modulation of influencing signaling molecules. Additionally, S100A6 might alter EGFR expression/activity or downstream effector function, impacting cancer cell sensitivity to TKIs. For example, it could affect EGFR or downstream target phosphorylation, influencing TKI efficacy. S100A6 may also interact with other resistance mechanisms like MET or HER2 amplification, further enhancing resistance to EGFR-TKIs. Future research should focus on clarifying the molecular interactions between S100A6 and the EGFR pathway and exploring S100A6's role in modulating other resistance mechanisms in lung adenocarcinoma. As for cisplatin resistance, the mechanism seems multi-factorial. Many factors, including tolerance or repair of cisplatin-DNA adducts, induction of anti-apoptotic signals, activated efflux from cell cytoplasm, epigenetic regulation conducted by miRNA, growth regulatory pathways deregulation, immune system suppression, are known to be associated with cisplatin resistance.[27] As there were only individual points of concentration/time showing S100A6 could enhance the cisplatin resistance of PC9 cells, further exploration is needed to confirm their relationship and underlying mechanisms.

The limitations of our overexpression model should be considered when interpreting these results. While overexpression studies are valuable for identifying potential roles of specific proteins, they do not necessarily reflect the endogenous regulation and interactions of these proteins in a physiological context. Further studies using additional models, such as gene knockdown or knockout approaches, would be necessary to confirm the causative role of S100A6 in gefitinib resistance. Additionally, mechanistic validation through studies that directly demonstrate how S100A6 modulates the EGFR signaling pathway or other cellular response pathways would provide stronger evidence for a causative relationship.

In conclusion, our results demonstrate a significant correlation between S100A6 overexpression and reduced sensitivity to gefitinib in lung adenocarcinoma PC9 cell lines with EGFR 19 exon mutations. While the data strongly suggest that S100A6 may contribute to gefitinib resistance, further mechanistic studies are required to establish a direct causative relationship. Future research should focus on elucidating the specific molecular mechanisms by which S100A6 modulates the cellular response to gefitinib, including its potential interactions with the EGFR signaling pathway and other resistance mechanisms.

Data Sharing Statement

The data that support the findings of this study are available on request from the corresponding author.

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Disclosure

The authors declare that they have no competing interests.

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