

Inhibition of Glutamine Transporter SLC1A5 Reverses Resistance to Erlotinib in Human Non-small Cell Lung Cancer

Zhuo Lu^{1#}, Tao Wang^{2#}, Zhen Chen³

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¹Department of Thoracic Surgery, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, P.R. China²Institute of Geriatrics, Jiangxi Provincial People's Hospital, the First Affiliated Hospital of Nanchang Medical College, Nanchang, Jiangxi, P.R. China³Zhejiang Key Laboratory of Organ Development and Regeneration, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, P.R. China⁴Department of Pathology, Jiangxi Maternal and Child Health Hospital, Nanchang, Jiangxi, P.R. China⁵Jiujiang Central Blood Station, Jiujiang, Jiangxi, P.R. China⁶School of Basic Medical Sciences, Nanchang University, Nanchang, Jiangxi, P.R. China

#These authors have contributed equally to this work.

Abstract

Purpose: The acquired resistance to erlotinib, an EGFR tyrosine kinase inhibitor (EGFR-TKI), is a great challenge in the targeted therapy of non-small cell lung cancer (NSCLC). Targeting glutamine import could re-sensitize the resistant cancer cells to erlotinib. Our results indicated that the glutamine transporter solute carrier 1 family member 5 (SLC1A5) could be a potential target for the treatment of NSCLC, and the application of GPNA, an SLC1A5 inhibitor, may reverse the acquired resistance to erlotinib.

Materials and Methods: The cell proliferation was measured by crystal violet staining assay. The expression levels of proteins were checked by western blot. The flow cytometry was used to analyze the cell cycle. CO-IP was used to detect the ubiquitination of indicated proteins.

Results: The expression of SLC1A5 in erlotinib-resistant cell lines (PC9ER, HCC827ER) was significantly higher than those in the erlotinib-sensitive cell lines (PC9, HCC827). The proliferation of erlotinib-resistant cells was more dependent on glutamine. Inhibition of SLC1A5 significantly suppressed the growth of NSCLC cells, especially for the erlotinib-resistant cells. Moreover, inhibition of SLC1A5 aggravated the inhibitory efficacy of erlotinib on cell proliferation in erlotinib-resistant cells and induced cell cycle arrest in G0/G1 phase. Mechanistically, SLC1A5 inhibition facilitated PDK1 degradation through the ubiquitin-proteasome pathway, and decreased the expression of p-AKT, Cyclin B1 and CDC42.

Discussion: Glutamine and its transporters play important roles in the development of various numbers of cancers. In this study, we showed that the proliferation of erlotinib resistance NSCLC cells exhibited greater dependence on glutamine. Our study highlighted the role of SLC1A5 in promoting the proliferation of NSCLC cells, especially in erlotinib-resistant NSCLC cells. The effect of SLC1A5 on cell growth in NSCLC was related with its metabolism regulation function and its impact on PDK1-AKT signaling pathway. Our results indicated that SLC1A5 would be a potential target for the treatment of EGFR-TKIs resistant NSCLC.

Keywords: SLC1A5; Erlotinib resistance; Glutamine; Cell cycle; Non-small cell lung cancer

Introduction

Lung cancer is the highest mortality and morbidity of cancer in the world. Non-small cell lung cancer (NSCLC) is the major type of lung cancer, accounting for more than 85% of all lung cancers [1, 2]. Clinically, the great majority of patients with NSCLC have been in advanced stage when diagnosed and have a dismal 5-year survival rate of approximately 15% [3, 4]. Significant advances have been achieved in the management of NSCLC over the last few years. Newer chemotherapy agents, targeted therapy and, more recently, immunotherapy, prolong survival and improve quality of life in patients with good performance status [5-7]. Among them, targeted therapy is pushing the boundary to significantly improve patient outcomes and quality of life [8, 9].

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), including erlotinib and gefitinib, can be used as a classical treatment for advanced NSCLCs harbouring certain mutations in the EGFR gene. EGFR-TKIs have a significant effect in improving the overall survival and progression-free survival of patients with advanced NSCLC [10-12]. However, after a period of clinical treatment, many patients with NSCLC would ultimately become resistance to EGFR-TKIs, which leads to poor prognosis. Some molecular mechanisms of acquired resistance have been elucidated such as T790M secondary

mutation, MET amplification, and so on [13-15]. However, many of the remaining mechanisms of resistance are still unknown. Therefore, more work needs to be done to reveal additional mechanisms and provide new therapeutic targets to reverse drug resistance.

Solute-linked carrier family 1A, member 5 (SLC1A5), also known as ASCT2, encodes a Na⁺-dependent neutral amino acid transporter [16]. SLC1A5 is a glutamine transporter that plays an important role in glutamine uptake, which is involved in energy supply, macromolecular synthesis, redox homeostasis, and nucleotide biosynthesis [17-19].

***Corresponding authors:** You-Kun Jie, Department of Pathology, Jiangxi Maternal and Child Health Hospital, Nanchang, Jiangxi, P.R. China, Tel: +86-791-86310716; E-mail: jyk1043@163.com

Cai-Feng Xie, School of Basic Medical Sciences, Nanchang University, Nanchang, Jiangxi, P.R. China, Tel: +86-791-83827160; E-mail: xiecaifeng@ncu.edu.cn

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More recently, SLC1A5 got more attention since its expression was elevated in various malignancies, including lung cancer, triple-negative basal-like breast cancer, and renal cell carcinoma [20-22]. In NSCLC, SLC1A5 was demonstrated as a major transporter of glutamine and its expression is closely associated with tumor aggressiveness and prognosis [20, 23-25]. Targeting SLC1A5 may affect glutamine uptake, cell growth, ATP generations, autophagy, and cell death in lung cancer cells, which are likely mediated in part by mTOR-AKT signaling [20, 24]. L- γ -Glutamyl-p-Nitroanilide (GPNA) was proposed as an SLC1A5 inhibitor [26] and has been subsequently widely used to this purpose [24, 27, 28], although it is known that it also inhibits other sodium-dependent amino acid transporters [29]. Currently, the role of SLC1A5 in the EGFR-TKIs resistant-NSCLC remains unclear.

PDK1 (3-phosphoinositide-dependent protein kinase 1) is one of downstream effectors of PI3K (phosphoinositide 3-kinase), which belongs to the family of AGC kinases [30]. Which phosphorylates AKT-Thr308 to activate the AKT kinase [31, 32] PDK1 participates in PI3K/AKT pathway in many cellular processes, containing cell cycle, cell survival, oncogenesis, and Epithelial-Mesenchymal Transition (EMT) [33]. Because of the auto-phosphorylation and activation property, there are few studies investigating the direct regulation of PDK1. It has been reported that PDK1 could be modified by mono-ubiquitination, although the function of PDK1 mono-ubiquitination is still unknown [34].

In several cancers, the expression of PDK1 is regulated via ubiquitination and degradation, then suppresses AKT kinase activity and oncogenic functions [35, 36]. Exploring the regulation of PDK1 protein will provide insights for understanding PI3K- AKT roles in biological process.

In this study, we will investigate the regulatory role of SLC1A5 in erlotinib-resistant NSCLC cells and try to clarify the exact molecular mechanism underlying that effect.

Materials and Methods

Cell culture

The non-small cell lung cancer (NSCLC) cells from ATCC were cultured in RPMI- 1640 (Invitrogen, C11875500BT) supplemented with 10% FBS (Excel, FCS) at 37°C in the presence of 5% CO₂. The erlotinib-resistant cell lines (PC9ER and HCC827ER) were obtained and cultured as previously described [37].

Reagents

Erlotinib hydrochloride was obtained from Bio vision Inc. L-Glutamic acid γ -(p-nitroanilide) (GPNA, SLC1A5 inhibitor) and SLC1A5 siRNA were obtained from Santa Cruz Biotechnology. MG132 was purchased from Bio vision (1791-5). Cycloheximide was bought from Sigma (CHX; C7698). Primary antibodies against β -actin (66009-1-Ig), SLC1A5 (20350-1-AP), PDK1 (17086-1-AP), CDK1 (19532-1-AP), CDC42 (10155-1-AP), Cyclin B1 (55004-1-AP) and UB (201-2-AP) were purchased from Proteintech. Primary antibody against Phos-AKT1 (Ser473) (9271S) was purchased from Cell signaling technology. Crystal violet and other analytical grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

siRNA transfection experiment

For knockdown experiments, the cells were seeded the day before SLC1A5 siRNA transfection to reach 50% confluence at the time of transfection. Trilencer-27 universal scrambled negative control siRNA duplex (SR30004; Origene Technologies) was used as a negative

control. The knockdown efficiency was determined by western blot using appropriate antibodies.

Cell growth assay

To carry out cell growth assays under glutamine (Gln) free condition, cells were seeded in 12-well plates at a density of 10⁵ cells per well in 2 ml of complete culture medium. The medium was changed to Gln free medium supplemented with 10% FBS on the following day. Medium was changed every 2 days. After 6 days, the cell number was counted. For cell growth assays with erlotinib or GPNA treatment, cells were seeded in 12-well plates at a density of 10⁵ cells per well in 2 ml medium with 10% FBS. On the following day, the medium was changed to RPMI 1640 + 10% FBS + DMSO or RPMI 1640 + 10% FBS + erlotinib, or RPMI 1640 + 10% FBS + GPNA. Medium containing erlotinib or GPNA was changed every 2 days. After 6 days, the cell number was counted. For cell growth assays following knockdown of SLC1A5, cells were transiently transfected with SLC1A5 siRNAs and seeded in 24-well plates at 10⁵ cells per well in 0.5 ml medium containing 10% FBS. The medium was changed every 2 days. Cells were fixed in 4% formaldehyde at the indicated times and stained with 0.1% crystal violet. Dye was extracted with 10% acetic acid and the relative proliferation rate was assessed from the increase in absorbance at 595 nm.

Western blot analysis

PC9, PC9ER, HCC827 and HCC827ER cells were cultured in 100-mm culture dishes at a density of 3 \times 10⁵ cells/dish. After treatment, cells were lysed in RIPA buffer (Beyotime, P0013), containing protease inhibitor cocktail (Sigma, P2714) and phenylmethylsulfonyl fluoride (DINGGUO, WB0180). Then, the cell lysates were centrifuged at 10,000 g for 20 min at 4°C. The supernatants were precleared with protein G agarose beads (Roche, 11243233001) for 1 h at 4°C. Then, the supernatants were combined with the indicated antibodies supplemented with protein G agarose beads to incubate overnight at 4°C. On the second day, the immunocomplexes were washed with lysis buffer. The supernatants were suspended with loading buffer and boiled for 10 min.

For western blotting, the proteins were subjected to 10% or 12% SDS-PAGE and transferred to PVDF membranes (Millipore, IPVH00010). The membranes were blocked with 5% skim milk (BD, 232,100) for 1 h at room temperature and incubated with the indicated antibodies overnight at 4°C. The membranes were washed 3 times at room temperature with 1 \times TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20) for 10 min every time, followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse (Thermo Fisher Scientific, 31,430) or 166 anti-rabbit (Thermo Fisher Scientific, 31,460) secondary antibodies. Protein bands were visualized after incubation using the Pro-Light chemiluminescence detection kit (TIANGEN, PA112-01) and a digital gel image analysis system (TANON 5500), and the band intensities were quantified with ImageJ software.

Cell cycle analysis

The cells were seeded in 6-well plates with each treatment. Then, the cells were harvested and washed with 1 \times PBS, and 70% alcohol in PBS was added to the cells on ice for 2 h; the cells were washed with 1 \times PBS and re-suspended with 400 μ l guava cell cycle reagent (Millipore, 4700-0160) (PI:585/29). After incubation at 37°C for 15 min, the cells were analyzed using a BD FACS Jazz™ Cell Sorter (Becton Dickinson).

Statistical analysis

Each value corresponded to the mean \pm Standard Error of Mean (S.E.M) of three independent experiments in triplicates. The statistical analyses were performed using GraphPad Prism 5.0 software. Statistical significance was tested by one-way ANOVA, with P-value of less than 0.05 considered statistically significant.

Results

The expression of SLC1A5 was elevated in erlotinib-resistant NSCLC cells

In this study, to examine the involvement of SLC1A5 in erlotinib resistance, we performed western blot to detect its expressions in erlotinib-resistant NSCLC cells (PC9ER and HCC827ER) and the parental erlotinib-sensitive NSCLC cells (PC9 and HCC827). Results showed that SLC1A5 expression was significantly higher in erlotinib-resistant cells than in the parental cells (Figure 1A and 1B). When cells were incubated with glutamine free medium or treated with GPNA, an inhibitor of SLC1A5, the cell growth was inhibited from Day 6 in PC9 and HCC827 cells (Figure 1C and 1E), while the growth of PC9ER and HCC827ER cells was inhibited since Day 4 (Figure 1D and 1F). Thus, the growth of erlotinib-resistant cells appears more dependent on SLC1A5 and glutamine metabolism than that of erlotinib-sensitive cells.

SLC1A5 inhibition reversed the resistance of HCC827ER to erlotinib

To evaluate the role of SLC1A5 in erlotinib resistance, cells were treated with GPNA or erlotinib. The combination treatment of GPNA and erlotinib showed greater efficiency in cell growth inhibition than the treatment with GPNA or erlotinib alone in PC9ER cells (Figure 2B), and there were no significant differences between the treatment with erlotinib alone and the combination treatment with GPNA and

erlotinib in PC9 cells (Figure 2A). Similar results were also observed in HCC827 and HCC827ER cells (Figure 2C and 2D). Moreover, the protein levels of SLC1A5 were examined and the results showed that both GPNA and erlotinib decreased the expression of SLC1A5 (Figure 2E and 2F).

To further demonstrate the role of SLC1A5 in erlotinib resistance in NSCLC cells, the expression of SLC1A5 was knocked down using SLC1A5 siRNA, and knockdown efficiency of SLC1A5 siRNA was determined by western blot. Consistent with the results of cells treated with GPNA, knockdown of SLC1A5 by siRNA significantly inhibited the growth of PC9ER and HCC827ER cells from Day 2 to Day 6 (Figure 2G and 2H), while the growth of PC9 and HCC827 was slightly inhibited by SLC1A5 siRNA (Figure 2I and 2J). These results indicate that SLC1A5 inhibition is a potential way to reverse the acquired resistance to erlotinib.

SLC1A5 inhibition resulted in cell cycle arrest in erlotinib-resistant cells

To further examine the role of SLC1A5 in cell proliferation, we detected the effects of SLC1A5 inhibition on cell cycle progression. Knockdown of SLC1A5 by siRNA in PC9ER cells induced an accumulation of cells at G0/G1 phase and a reduction of cells at S phases (Figure 3A). GPNA treatment also arrested the cell cycle at G0/G1 phase (Figure 3B). Same results were also observed in HCC829ER cells (Figure 3C and 3D). In this context, it is demonstrated that the inhibition of cell proliferation is associated with SLC1A5 mediated cell cycle arrest.

SLC1A5 promoted erlotinib resistance through PDK1-AKT signaling pathway in NSCLC cells

To further investigate the downstream signaling pathways of SLC1A5, the expression of regulators in PDK1-AKT signaling pathway were examined. Intriguingly, SLC1A5 knockdown by

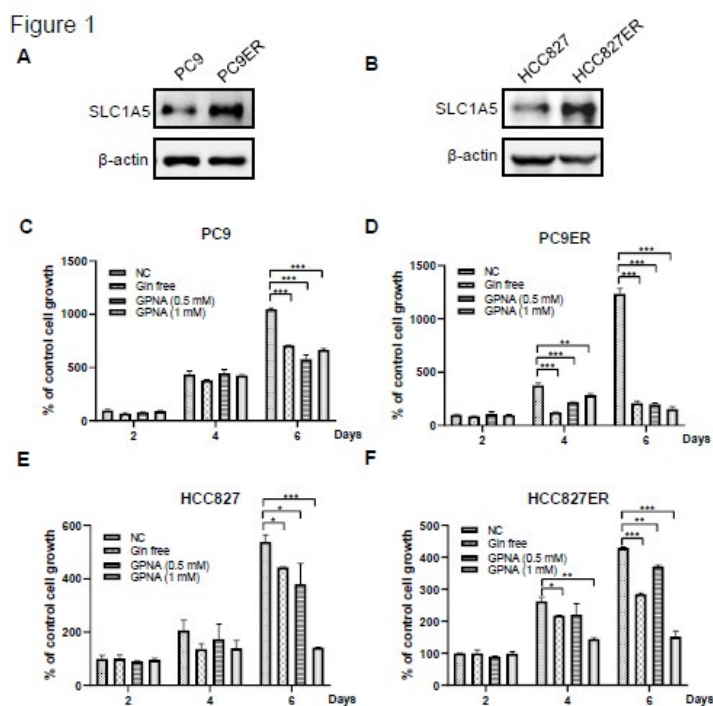


Figure 1: The expression of SLC1A5 was elevated in erlotinib resistant NSCLC cells. (A, B) The protein level of SLC1A5 was detected by western blot in PC9, PC9ER, HCC827 and HCC827ER cells. (C-E) Cell growth was determined in PC9, PC9ER, HCC827 and HCC827ER cells treated with glutamine free medium or SLC1A5 inhibitor GPNA (0.5 and 1mM). Values represent mean \pm S.E.M. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. negative control group.

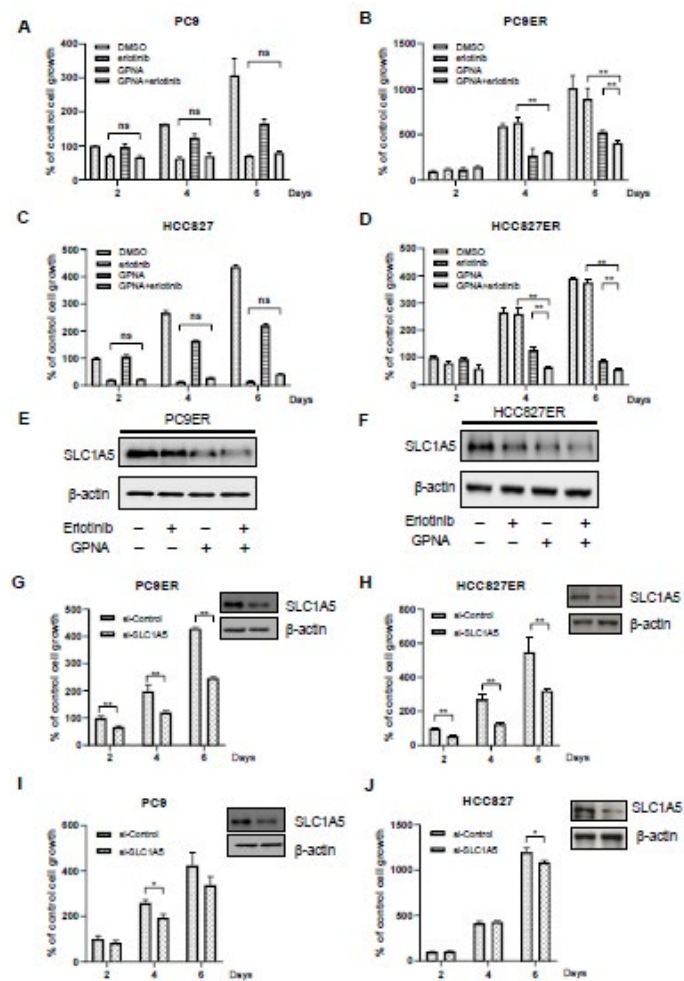


Figure 2: SLC1A5 inhibitor GPNA and siRNA reversed the resistance of HCC827ER to erlotinib. (A-D) The PC9, PC9ER, HCC827 and HCC827ER cells were treated with GPNA (1mM), erlotinib (1 μ M) or combination of them for 2, 4 and 6 days, cells growth was determined by crystal violet staining and the absorbance was measured at 595 nm. (E, F) The protein levels of SLC1A5 and β -actin were detected by western blot after treatment with GPNA (1 mM), erlotinib (1 μ M) or combination of them for 48h in PC9ER and HCC827ER cells. (G-J) SLC1A5 siRNA was transfected into PC9, HCC827, PC9ER and HCC827ER cells. The cell growth was detected after cells were cultured for 2, 4 and 6 days. The siRNA knockdown efficiency was determined by western blot. Values represent mean \pm S.E.M. *P < 0.05 and **P < 0.01 vs. negative control group.

siRNA significantly decreased the protein levels of PDK1 and p-AKT. Furthermore, CDC42 and Cyclin B1 were also down-regulated following SLC1A5 knockdown (Figure 4A). As expected, treatment with GPNA alone or the combination of GPNA and erlotinib markedly suppressed the expression of PDK1, p- AKT, CDC42 and Cyclin B1 in PC9ER cells (Figure 4B). These results suggest that SLC1A5 inhibition may decrease the activation of PDK1-AKT signaling pathway, resulting in the down regulation of CDC42 and Cyclin B and cell cycle arrest. Furthermore, for the first time, our study bridges cell cycle proteins with SLC1A5 and erlotinib resistance, and this work might shed new light on cancer treatment by targeting cancer metabolism.

SLC1A5 inhibition facilitates PDK1 degradation via the ubiquitin-proteasome pathway

GPNA treatment has been shown to increase protein ubiquitination and proteasomal degradation [38]. Thus, we speculate that SLC1A5 and glutamine metabolism might be involved in PDK1 degradation.

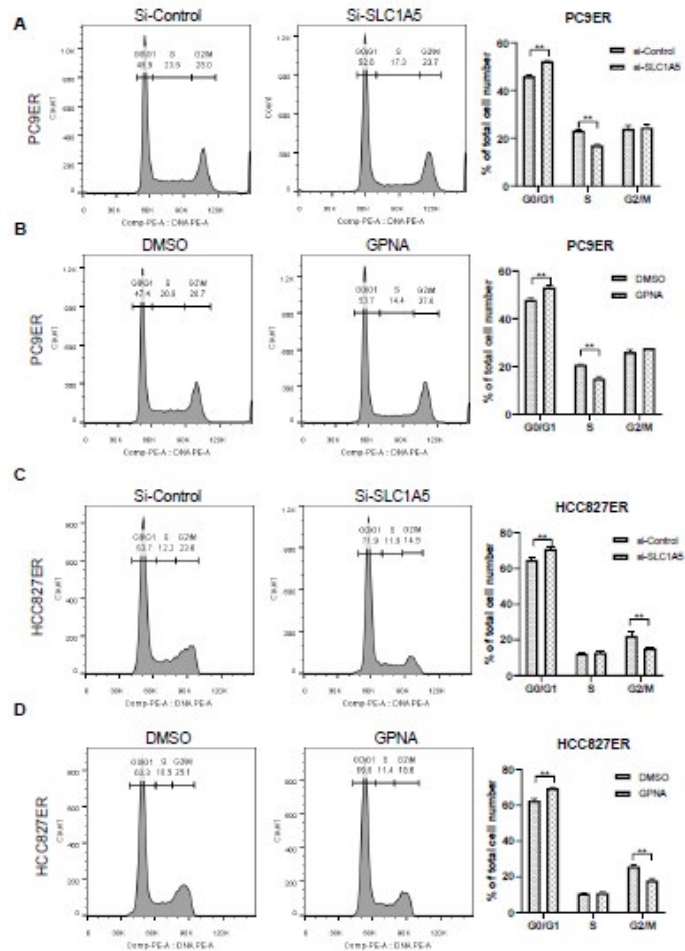


Figure 3: SLC1A5 inhibition resulted in cell cycle arrest at G0/G1 phase. (A) PC9ER cells were transfected with either si-Control or si-SLC1A5. After 48 h, cell cycle was analyzed by flow cytometry. (B) PC9ER cells were treated with either DMSO or GPNA. After 48 h, cell cycle was analyzed by flow cytometry. (C) HCC827ER cells were transfected with either si-Control or si-SLC1A5. After 48 h, cell cycle was analyzed by flow cytometry. (D) HCC827ER cells were treated with either DMSO or GPNA. After 48 h, cell cycle was analyzed by flow cytometry. The inserts show the quantification of the cell cycle analysis. Values represent mean \pm S.E.M. *P < 0.05 and **P < 0.01 vs. negative control group.

Our results showed that the ubiquitination of PDK1 was promoted by MG132 treatment (Figure 5A), suggesting that PDK1 protein was degraded in a proteasomal degradation pathway. Furthermore, GPNA treatment also promoted the ubiquitination of PDK1 (Figure 5B). To determine whether SLC1A5 regulates the stability of PDK1, we examined the protein levels of PDK1 in the presence of CHX under GPNA-treated condition. As shown in Figure 5C, treatment of GPNA accelerated the degradation rate of PDK1. These results suggest that inhibition of SLC1A5 promote the degradation of PDK1 through the ubiquitin-proteasome pathway, which might contribute to the impact of SLC1A5 on erlotinib response.

Discussion

Glutamine and its transporters play important roles in the development of various numbers of cancers [29, 30]. In this study, we showed that the proliferation of erlotinib resistance NSCLC cells exhibited greater dependence on glutamine. Thus, concentrating on factors that potentially affect the glutamine metabolism could be a promising therapeutic strategy for NSCLC. GPNA, a major inhibitor

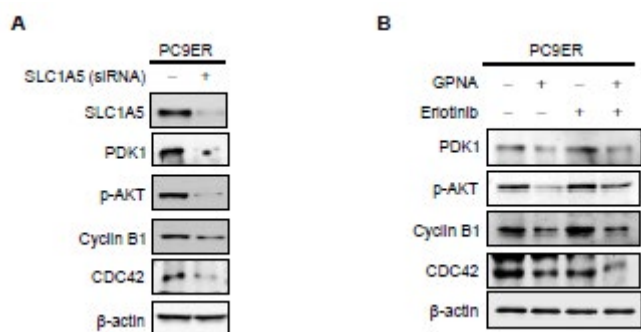


Figure 4: SLC1A5 affected PDK1-AKT signaling and its downstream signaling regulators. (A) The protein levels of PDK1, p-AKT, CDC42 and Cyclin B1 were detected by western blot at 48h after siSLC1A5 in PC9ER cells. The bands were quantified using Image software (ImageJ) and normalized to the levels of β -actin. (B) The protein levels of SLC1A5, PDK1, p-AKT, CDC42 and Cyclin B1 were detected by western blot after treatment with GPNA (1mM), erlotinib (1 μ M) or combination of them for 48h in PC9ER cells.

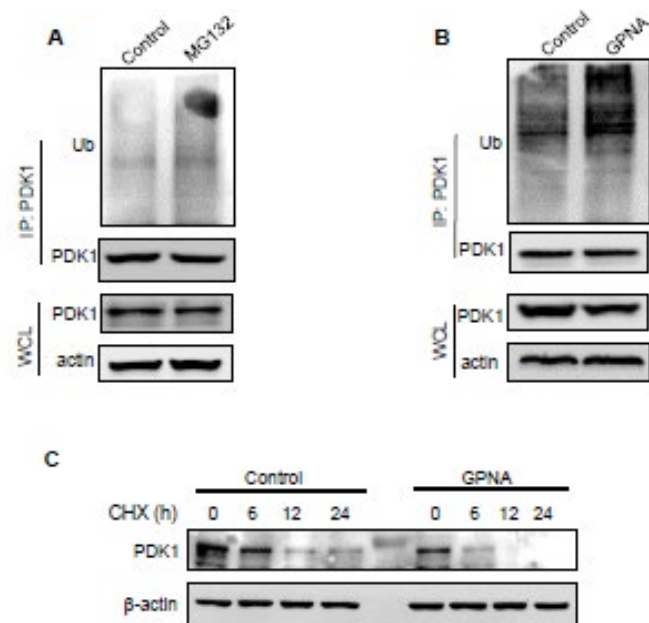


Figure 5: Inhibition of SLC1A5 increased PDK1 degradation via the ubiquitin-proteasome pathway. (A) PDK1 plasmids were transfected into PC9ER cells, followed by treated with MG132 for 24 h. The levels of PDK1 ubiquitination were detected by immunoprecipitation and western blot with anti-Ub and anti-PDK antibodies. (B) PC9ER cells were treated with GPNA for 24h. The levels of PDK1 ubiquitination were detected by immunoprecipitation and western blot with anti-Ub and anti-PDK antibodies. (C) PC9ER cells were treated with 25 μ g/ml CHX for different periods of time combined with or without GPNA, and then immunoblotted with antibodies against PDK1 and β -actin. PDK1 β a CHX (h) 0 6 12 24 B PDK1 IP: IP: PDK1WCLPDK1.

of SLC1A5 along with other members of SNAT family, has been widely used to block glutamine intake [24, 27, 28]. In different erlotinib resistance NSCLC cell lines, we showed that inhibition of SLC1A5 by GPNA significantly enhanced the inhibitory efficacy of erlotinib. Furthermore, GPNA or SLC1A5 siRNA induced cell cycle arrest at G0/G1 phase. Taken together, our results suggest that SLC1A5 may be a potential target to ameliorate the erlotinib resistance in NSCLC.

The involvement of SLC1A5 in erlotinib response led us to study the mechanism by which SLC1A5 affects the erlotinib efficacy. A previous study reported that SLC1A5 inhibition induced apoptosis [39]. In esophageal cancer cells, SLC1A5 down regulation induced

cell cycle arrest in G0/G1 phase, though the regulated mechanism still unknown [40]. In this study, the results of flow cytometry showed that inhibition of SLC1A5 by siRNA or GPNA in erlotinib resistant NSCLC cells arrested the cell cycle at G0/G1 phase. Cyclin B1 expression accumulates at the G2-M cell cycle transition, accompanied with its translocation to the nucleus. We also detected the expression of cell-cycle proteins and found that the protein levels of Cyclin B1 and CDC42 were reduced following SLC1A5 inhibition.

PI3K/AKT signaling pathway plays multiple roles in the regulation of cell cycle [41]. Accordingly, we consider that the inhibition of SLC1A5 might attribute to the inhibition of PI3K/AKT signaling pathway. Most strikingly, our work revealed that SLC1A5 induced the degradation of PDK1 and down-regulated the expression of p-AKT, which might contribute to the decreased expression of cell-cycle proteins and cell cycle arrest. These findings enriched our understandings of SLC1A5 and provided more experimental evidence for the idea of developing SLC1A5 as anti-tumor target.

Conclusions

Our study provides insights into understanding the role of SLC1A5 in promoting the proliferation of NSCLC cells, especially in erlotinib-resistant NSCLC cells. The effect of SLC1A5 on cell growth in NSCLC was related with its metabolism regulation function and its impact on PDK1-AKT signaling pathway. Our results indicated that SLC1A5 would be a potential target for the treatment of EGFR-TKIs resistant NSCLC.

Declarations

Conflicts of Interest

The authors declare that there are no conflicts of interests.

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Data availability statement

The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

Author Contributions

Zhuo Lu, Tao Wang: Project administration, Writing-original draft, Methodology, Validation, Software, Data curation. Zhen Chen: Project administration, Methodology. Shi-Yin Chen: Statistical consultation, Formal analysis. You-Kun Jie, Zhu-Yun Fu: Visualization, Conceptualization, Resources, Writing-review & editing, Supervision.

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