Metabolic reprogramming induced by inhibition of SLC2A1 suppresses tumor progression in lung adenocarcinoma

Yanfen Wang¹, Shanshan Shi²*, Yongling Ding¹, Zheng Wang¹, Shuang Liu¹, Jiajia Yang¹, Tongpeng Xu³

¹Department of Pathology, The Affiliated Hospital of Yangzhou University, Yangzhou University, Yangzhou, Jiangsu, P. R. China; ²Department of Pathology, Jinling Hospital, Clinical Medical School of Southern Medical University, Nanjing, Jiangsu, P. R. China; ³Department of Oncology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, P. R. China. *Equal contributors.

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Abstract: Lung adenocarcinoma (LAC) is one of the common reasons of cancer-related death with few biomarkers for diagnosis and prognosis. Solute carrier family 2 member 1 protein, SLC2A1, has been associated with tumor progression, metastasis, and poor prognosis in many human solid tumors. However, little is reported about its biological functions in lung adenocarcinoma. Here we observed a strong up-regulation of SLC2A1 in patients with LAC and found that SLC2A1 was significantly correlated with prognosis. Knockdown of SLC2A1 in LAC cells inhibits cellular proliferation and plate clone formation in vitro as well as suppression of glucose utilization. Meanwhile, silencing of SLC2A1 also suppresses tumor metastasis in vitro. Mechanistically, GSEA showed that genes in cell cycle pathway were prominently enriched in the higher SLC2A1 group. By a large-scale proteomic analysis, we revealed that cell cycle protein level was significantly increased in SLC2A1-high group. Collectively, our findings indicate that elevated SLC2A1 is a critical modulator in lung adenocarcinoma progression by altering glucose metabolism and the cell cycle pathway, and also suggest SLC2A1 as a promising target for lung adenocarcinoma therapy.

Keywords: SLC2A1, tumor growth, invasion, glycometabolism, cell cycle, lung adenocarcinoma

Introduction

Lung cancer is one of the most malignant tumors and is the leading cause of cancer-related mortality worldwide [1]. Among lung cancers, lung adenocarcinoma (LAC) is the most common histopathological subtype, and despite recent advances in the elucidation of molecular mechanisms and surgical and chemotherapeutic interventions, the prognosis of LAC has not been improved satisfactory [2, 3]. Therefore, the molecular mechanisms of LAC need to be revealed in further detail to identify efficient molecular markers, including predictive markers for LAC, and to establish favorable treatment strategies for LAC. It has been reported that several regulators of the glycolysis pathway are detected in several premalignant lesions and tumors, which suggests that these proteins may participate in early carcinogenesis and progression of cancer [4]. Warburg effect not only promotes tumor cell proliferation by providing cellular building blocks, but also favors tumor cell metastasis by acidified microenvironment through increased production of lactate [5]. Therefore, glycolysis pathway might be a potential targetable pathway for cancer therapy.

Solute carrier family 2 facilitated glucose transporter member 1 (SLC2A1), also known as glucose transporter 1 (GLUT1), is a crucial protein in the cellular energy metabolism pathway. In normal tissues, GLUT1 is limited to be expressed on erythrocytes and endothelial cells in the blood-brain barriers [6]. Recently, SLC2A1 has been demonstrated to be a pivotal rate-limiting element in the transport of glucose in malignancy cells and over-expressed in several different types of human carcinomas, including
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Liver, pancreatic, endometrial, and breast cancers, as well as lung cancer [7-10]. These studies suggest that SLC2A1 could be one of the driver genes in tumors. However, the expression pattern and cellular functions of SLC2A1 in LAC remain largely unexplored.

In this study, we observed that SLC2A1 was over-expressed in LAC tumors by dataset analysis and found the significant correlation between SLC2A1 expression and prognosis. We further explored the functional significance of SLC2A1 in LAC tumorigenesis and demonstrated that SLC2A1 promoted LAC cells growth and enhanced LAC cells migration in vitro via enhancement of glucose utilization.

Material and methods

Cell culture and reagent

Human LAC cell lines NCI-H1793, NCI-H1975, NCI-H23 and HCC827 were all purchased from American Type Culture Collection (ATCC, Manassas, VA). All of these cells were cultured in indicated medium according to ATCC protocols, and supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotics (100 μg/ml streptomycin and 100 units/ml penicillin) at 37°C in a humidified incubator under 5% CO₂ condition.

Quantitative real-time PCR

Total RNA was extracted from H1793 and HCC827 cells using Trizol reagent (Takara, Japan), and reversely transcribed through PrimeScript RT-PCR kit (Takara, Japan) according to the manufacturer’s instructions. Quantitative real-time PCR was performed with SYBR Premix Ex Taq (Takara, Japan) on a 7500 Real-time PCR system (Applied Biosystems, Inc. USA). Primer sequences used in this study were as follows: SLC2A1, forward: 5'-AAGGTGATCGAGGAGTTCTACA-3'; reverse: 5'-ATGCCCAACAAGA AAGATG-3'; β-actin, forward 5'-ACTCGTCGAGATCAGATCCATTGATCGTTTTCGTTG-3'; reverse 5'-GAAACTACCTTGCTGAGATGAGGTCTTTTG-3'. The stable SLC2A1 knockdown cells were selected in the presence of 2 μg/ml puromycin. The knockdown efficacy was tested by western blotting and real time PCR.

Cell viability assay

For cell viability assay, cells were seeded into a 96-well plate at 3 × 10⁵ cells per well with 100 μl culture medium and cultured at 37°C. The cell viability was quantified by Cell Counting Kit-8 (CCK-8, Dojindo, Japan). Briefly, by addition of 10% (v/v) CCK-8 to the culture medium and incubation for 1.5 h, cell viability was monitored by measuring absorbance at 450 nm using a Power Wave XS microplate reader (BIO-TEK). The experiment was performed in quintuplicate and repeated twice.
Cell invasion assay

The invasive ability of LAC cells was measured by transwell model (Corning, NY, USA) according to the manufacturer's instructions. 700 μl RPMI 1640 or DMEM medium containing 5% (v/v) FBS was added to the lower chambers. 2× 10^4 Cells in 100 μl 1640 or DMEM medium were seeded into the upper compartment coated with 100 μl matrigel (BD Bioscience, USA). After the cells were incubated for 48 h, the non-invading cells remained on the upper surface were scraped off. The invaded cells were fixed with 4% paraformaldehyde and stained with...
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Measurement of pyruvate, lactate and glucose

Cells were cultured in 24 well plates for 24 h. Pyruvate and lactate production was measured by the enzymatic method using a commercially available fluorescence-based assay kit (Abcam, US). The glucose in the conditioned media was quantified using the Glucose Assay kit (Sigma, Shanghai, China). Quantities of glucose consumed were normalized to the DNA content of each well and triplicate samples were analyzed.

3H-2-deoxyglucose transport assay

Cells were incubated in glucose-free Dulbecco’s modified eagle’s medium (DMEM) and then pulsed with 2 µCi of 3H-2-deoxyglucose (~60 pmol) (PerkinElmer, Nanjing, China) for 10 min. The monolayers were quantitated by liquid scintillation counting. The data is presented as counts per minute (CPM) per µg DNA.

Statistical analysis

Data were presented as the means ± SD. The SPSS software program (version 19.0; IBM Corporation) was used for statistical analysis. Graphical representations were performed with GraphPad Prism 5 (San Diego, CA) software. Survival curves were evaluated using the Kaplan-Meier method and differences between survival curves were tested by the log-rank test. The student’s t-test was used for comparison between groups. P < 0.05 was considered statistically significant.

Results

SLC2A1 is over-expressed in LAC tissues and predicts poor prognosis

To illustrate the expression pattern of SLC2A1 in LAC, we searched the mRNA expression level of SLC2A1 in four GEO datasets. The results showed that SLC2A1 expression was significantly up-regulated in LAC tissues comparing with paired normal pancreatic tissues using GSE7670 (Figure 1A, n = 27, P = 1.12E-10) and GSE32863 (Figure 1B, n = 54, P = 2.39E-23). Expression of SLC2A1 was also remarkably higher in the LAC tissues than the unpaired normal lung tissues as revealed by GSE10072 (Figure 1C, P = 2.15E-16) and GSE31210 (Figure 1D, P = 5.86E-21).

To evaluate the prognostic significance of SLC2A1 in LAC patients, the correlation between SLC2A1 expression and corresponding clinical follow-up information were analyzed using Kaplan-Meier method. We determined the prognostic value of SLC2A1 at mRNA level using two GEO datasets and TCGA RNAseq dataset. As shown in Figure 1E and 1F, in GEO datasets, patients with higher SLC2A1 level had significantly shorter survival time than those with a lower SLC2A1 level. Similarly, in TCGA dataset, patients with higher SLC2A1 level had marked shorter overall survival time and disease free survival time (Figure 1G and

Table 1. Relationship between SLC2A1 expression and clinicopathological features of LAC patients

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*, P < 0.05, **, P < 0.01.
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Taken together, these data above suggest that up-regulated SLC2A1 predicts poor prognosis and might contribute to tumor progression in LAC patients.

Relationship between SLC2A1 expression and clinical parameters in patients with LAC

To determine the clinical significance of SLC2A1 expression in LAC, the Chi-square test was used to assess the relationships between SLC2A1 mRNA expression and corresponding patients’ clinicopathologic parameters including age, gender, TNM stage, tumor size, lymph node metastasis and distant metastasis in TCGA data. The results showed that SLC2A1 mRNA expression in LAC tissues was significantly correlated with age ($P = 0.015$), gender ($P = 0.006$), TNM stage ($P = 0.018$), tumor size ($P = 0.005$), lymph node metastasis ($P = 0.011$), while no significant associations were observed between SLC2A1 expression and distant metastasis (Table 1).

Correlations between SLC2A1 mRNA expression and prognosis of LAC patients

We further evaluated the correlation between SLC2A1 expression and overall survival rate or disease-free survival rate of LAC patients in early or advanced TNM stage and in the presence or absence of lymphatic metastasis. Kaplan-Meier analyses showed that overall survival time was shorter in LAC patients with higher SLC2A1 expression regardless the state of lymphatic metastasis ($P = 0.019$). While

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**Figure 2.** SLC2A1 expression is correlated with disease-free survival rate dependent on TNM stage and lymph node metastasis. A: Comparison of overall survival in patients with or without lymph node metastasis was conducted based on SLC2A1 expression. B: Comparison of disease-free survival in patients with or without lymph node metastasis was conducted based on SLC2A1 expression. C: Comparisons of overall survival between lower SLC2A1 expression group and higher SLC2A1 expression group in early TNM stage (I-II) cohort and in advanced TNM stage (III-IV) cohort. D: Comparisons of disease-free survival between lower SLC2A1 expression group and higher SLC2A1 expression group in early TNM stage (I-II) cohort and in advanced TNM stage (III-IV) cohort. $P$ value was calculated by log-rank test.
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**Figure 3.** Knockdown of SLC2A1 inhibited LAC cells viability in vitro. Interfere efficacy in H1793 and HCC827 cells was detected by Western blotting (A) and quantitative real-time PCR (B and C). Cell counting kit-8 (CCK-8) assay showed silencing of SLC2A1 inhibited cell growth in H1793 (D) and HCC827 cells (E). Knockdown of SLC2A1 inhibited the colony formation ability of H1793 (F) and HCC827 (G) cells, Scale bar: 5 mm. shcon versus shRNA-1 or shRNA-2, **P < 0.01.
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the correlation between SLC2A1 expression and disease-free survival rate of LAC patients was dependent on lymphatic metastasis (Figure 2B) and TNM stage (Figure 2D). In the absence of lymphatic metastasis and early TNM stage, disease-free survival time was also shorter in LAC patients with higher SLC2A1 expression. In advanced TNM stage and the presence of lymphatic metastasis, disease-free survival rate of LAC patients had no significant associations with SLC2A1 level.

Suppression of SLC2A1 inhibits LAC cells growth in vitro

We established stable cell lines expressing short hairpin RNA targeting SLC2A1 in H1793 and HCC827 cells. Knockdown efficiency of SLC2A1 in the two cell lines was verified by western blot and real time PCR. As shown in Figure 3A-C, Stable expression of two short hairpin RNA (shRNA-1, shRNA-2) resulted in > 75% decrease in SLC2A1 expression. Cell counting kit-8 (CCK-8) assay showed that knockdown of SLC2A1 expression induced significant decreases in H1793 cellular viability (Figure 3D). Similarly, the HCC827 cells suppressed SLC2A1 expression grew slower than the control (Figure 3E). To further validate the role of SLC2A1 in cancer cell survival, clonogenic assay was performed. As shown in Figure 3F and 3G, suppression of SLC2A1 significantly reduced the colony formation of H1793 and HCC827 cells.

Silencing of SLC2A1 inhibits LAC cells invasion in vitro

By Transwell model, we investigated the effect of SLC2A1 on LAC cells invasion. We observed that fewer numbers of invaded cells in shRNA group compared with the control group in both H1793 (P < 0.01, Figure 4A) and HCC827 (P < 0.01, Figure 4B) cells.

Knockdown of SLC2A1 depresses LAC cells glucose utilization in vitro

Next, we sought to determine whether the LAC cells with decreased SLC2A1 protein had altered glucose utilization. As shown in Figure 5A, SLC2A1-shRNA HCC827 cells consumed fewer glucose than the control (P < 0.01). Similarly, HCC827 cells silencing of SLC2A1 took fewer 3H-2-deoxyglucose (3H-2-DOG) compared to the control cells (Figure 5B, P < 0.01). Meanwhile, we found a decrease in fructose-6-phosphate (Figure 5C, P < 0.001) and
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pyruvate production (Figure 5D, \( P < 0.001 \)) in SLC2A1-silencing HCC827 cells compared with vehicle cells. Furthermore, we found significantly decreased glyceraldehydes-3-phosphate production (G3P) in SLC2A1-silencing HCC827 cells compared with vehicle cells (Figure 5E, \( P < 0.001 \)). Similarly, there's a decrease in lactate production in SLC2A1-silencing HCC827 cells compared with vehicle cells (Figure 5F, \( P < 0.001 \)). These data demonstrate that knockdown of SLC2A1 expression leads to a derangement of glucose transport, glucose consumption and lactate secretion, indicating the critical roles of SLC2A1 in glycolysis and glucose metabolism. Overall, these data support that LAC cells with increased SLC2A1 protein had enhanced glucose utilization.

SLC2A1 correlates increased cell cycle protein level in lung adenocarcinoma

To illustrate the mechanism by which SLC2A1 promotes tumor progression in lung adenocarcinoma, Gene Set Enrichment Analysis (GSEA) was performed in the expression profiles of LAC tissues in TCGA. Based on SLC2A1 expression level in TCGA dataset, top fifty tissues were defined higher group and posterior fifty tissues were defined lower group. The difference of SLC2A1 expression between two groups was significant (Figure 6A). GSEA showed that genes in cell cycle pathway were prominently enriched in the higher SLC2A1 group, indicating that SLC2A1 is a potential promoter of cell cycle (Figure 6B). To test this,
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we analyzed the levels of cancer-related total and phosphorylated proteins using the reverse-phase protein arrays (PRPAs) located in the TCGA dataset. The result showed that cyclin B1, cyclin D1, cyclin E1, CHK2_pT68, CHK1_pS345, MET-pY1235, EGFR and EGFR_pY1173 was significantly higher in SLC2A1-high group (Figure 6C). In addition, knockdown of SLC2A1 expression induced significant decreases in Cyclin B1 and Cyclin D1 protein expression in H1793 and HCC827 cells (Figure 6D). Taken together, these results indicate that the oncogenic functions of SLC2A1 in lung adenocarcinoma may associate with cell cycle.

Discussion

SLC2A1 gene encodes GLUT1 protein and is one of the isoforms of the protein family of facilitative glucose transporters, which is highly concentrated in tissue endothelium and epithelium [11]. Considering glucose uptake is the first rate-limiting step in aerobic glycolysis, SLC2A1 is often found to be over-expressed in various cancer types, including oral, liver, lung, breast, and endometrial cancer, due to the higher demand of glucose [7, 9, 10, 12]. In lung cancer, SLC2A1 expression is upregulated at both the RNA and protein levels in premalig-
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Inhibitory lesions of lung squamous cell carcinoma, when compared with normal lung epithelial basal cells from the same patients [13]. In lung adenocarcinoma, two previous studies showed that SLC2A1 could be a prognostic marker [14, 15]. However, its underlying biological functions in LAC cells remains unknown.

In our study, by systematic analysis of several datasets from GEO and TCGA, we also observed that SLC2A1 level was commonly up-regulated in clinical specimens compared with the normal lung tissues. To address the prognostic value of SLC2A1 in LAC, we performed Kaplan-Meier survival analyses. Consistent with previous studies, our results indicated that SLC2A1 expression significantly correlated with patients' overall survival and disease free survival. Elevated expression of SLC2A1 was inversely associated with clinical outcomes of LAC patients.

Up-regulated SLC2A1 expression contributes to carcinogenesis and tumor progression. In this study, we analyzed the oncogenic activity of SLC2A1 in LAC cell lines. We established stable cell lines silencing SLC2A1 in H1793 and HCC827 cells to further explore the function of SLC2A1. Firstly, we observed that knockdown of SLC2A1 significantly inhibited LAC cell viability and colony formation. Secondly, silencing of SLC2A1 markedly suppressed LAC cells invasion. Finally, SLC2A1-silencing HCC827 cells took fewer 3H-2-DG, consumed fewer glucose and produced fewer fructose-6-phosphate, glyceroldehydes-3-phosphate, pyruvate and lactate compared with control. These results consistent with that in gastric cancer. Consistent with this observation, it was reported that overexpression of SLC2A1 promotes tumor cell proliferation and metastasis in gastric cancer by promoting glucose utilization [16]. And in disease progression from benign tissue to prostate cancer, glyceroldehydes-3-phosphate production was also increased [17]. Given the roles of SLC2A1 in enhancing glucose utilization, thus supporting energy requirements, providing enormous biosynthetic needs and promoting the secretion of lactate and further contributing to the acidification of tumor microenvironment, which ultimately favors tumor growth and progression, it is reasonable to expect the oncogenic functions in lung adenocarcinoma.

In conclusion, we find that elevated SLC2A1 expression is significantly correlated with LAC patients' prognosis. Meanwhile, knockdown of SLC2A1 inhibits the proliferation, colony formation, invasion and glucose utilization of LAC cells. These results suggest that SLC2A1 might acts as a candidate target for developing treatment of LAC.

Disclosure of conflict of interest

None.

Address correspondence to: Tongpeng Xu, Department of Oncology, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, Jiangsu, P. R. China. E-mail: tpengxu@sina.com

References

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