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ORIGINAL RESEARCH

Lactate Transporter SLC16A3 (MCT4) as an Onco-Immunological Biomarker Associating Tumor Microenvironment and Immune Responses in Lung Cancer

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Purpose: Lactate, a marker of tumor metabolic reprogramming, maintains the acidic microenvironment and also affects the metabolism and function of immune cells. SLC16A3 is responsible for the extracellular transport of lactate, which is a key component of glycolysis. However, the role of SLC16A3 in immune infiltration and immunosuppression of lung cancer is largely unknown. Our study explored the therapeutic and prognostic value of SLC16A3 in predicting immune infiltration and immune checkpoint efficacy of lung cancer.

Methods: SLC16A3 expression was evaluated with TCGA database. Kaplan–Meier analysis was performed for survival rates. GO and KEEG enrichment was conducted to determine predictive signaling pathways. We utilized TIMER and CIBERSORT to analyze the correlation between SLC16A3 and immunocyte infiltration as well as immune checkpoint. Interleukin and HIF-1a expression was measured with ELISA kit and flow cytometry separately.

Results: In comparison with normal tissues, SLC16A3 expression was significantly upregulated in both lung adenocarcinoma (LUAD) and squamous carcinoma (LUSC), which was closely related to poor prognosis. GO analysis indicated that SLC16A3 involved in different signal pathways in LUAD and LUSC and linked to HIF-1 signaling in LUAD. High SLC16A3 was correlated with immunosuppressive cells (Treg, Th2 and iDC), immune checkpoint (PD1, PD-L1, PVR, Tim-3, ITGAM) and immunosuppressive factors (foxp3, TGF-β) in LUAD not LUSC. Furthermore, SLC16A3 was identified to tightly interact with IL-8 which may induce microenvironment immune tolerance. Based on the clinical prediction, we performed experiments with LUAD A549 cells and showed reduced IL-8 and HIF-1a when treated with SLC16A3 knockdown. HIF-1a stimulation by dimethyloxalylglycine (DMOG) could restore IL-8 secretion in SLC16A3 downregulated cells.

Conclusion: Taken together, our results suggest that SLC16A3 contributes to a worse prognosis in lung cancer and may play an important role in immune microenvironment and evasion through HIF-1a-IL8 axis, which could be a novel therapeutic target for immunotherapy in lung cancer.

Keywords: SLC16A3 (MCT4), IL-8, lung cancer, immune evasion, bioinformatics analysis

Introduction

Recently, the role of lactate in facilitating biological behavior of tumors has attracted more and more attention. Lactate is not only a high-energy metabolic substrate, but also an important regulator in complicated tumor activities.[1] Accompanied by H^+efflux, lactate released into the microenvironment by tumor cells as a metabolite contributes to the formation of acidic barriers,[2] which suppresses proliferation and partially induces cell death in cytotoxic T lymphocyte (CTL).[3–5] Moreover, as a signal molecule, lactate could inhibit dendritic cell (DC) differentiation and reduce the release of inflammatory factors such

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as IL-6 and TNF via activation of GPR81.[6,7] The latest research has reported that lactate promoted the M2 polarization of tumor-associated macrophage (TAM) by modifying histone (as histone lactylation),[8] indicating a new approach for lactate in immune regulation. M2 polarization of TAM weakens the effective immune response and further promotes immunosuppression by preparing T cell cytokine production and enhancing PD-1 expression.[9,10] Therefore, lactate is a vital mediator connecting tumor basic metabolism, microenvironment and immune response.[11,12]

Lactate transport is one of the acidosis-enhancing processes that are mediated via monocarboxylate transporters (MCTs).[13] SLC16A3 (MCT4) is an H^+-coupled symporter highly expressed in metastatic tumors and at inflammatory sites undergoing hypoxia or the Warburg effect.[14] At these sites, extracellular lactate contributes to malignancy and immune response evasion. Deficiency of SLC16A3 resulted in intracellular accumulation of lactate and induced a reactive oxygen species (ROS)-dependent cellular apoptosis.[15] High SLC16A3 expression significantly correlated with aberrant cell proliferation, invasion and distant metastasis, indicating early recurrence and poor prognosis in colorectal cancer, hepatocellular carcinoma, gastric cancer, prostate cancer, bladder cancer, etc.[16–20] Increasing evidence indicates that selective inhibition of the lactate transporter SLC16A3 will bring promising clinical benefits.

Given that SLC16A3 has been shown to enhance proliferation and invasiveness in lung cancer,[21–25] little is known whether SLC16A3 is involved in immune responses to assist immune evasion. In this study, we analyzed relevant databases to predict differential expression, clinical prognosis, gene enrichment and the relationship with immune infiltration of SLC16A3 in both lung adenocarcinoma (LUAD) and squamous carcinoma (LUSC). Additionally, the correlation of SLC16A3 and immune checkpoints as well as tumor-derived interleukins were also evaluated and preliminarily examined with LUAD cell line.

Materials and Methods

RNA-Sequencing Data and Bioinformatics Analysis

The single-cell RNA sequencing (RNA-Seq) data of SLC16A3 was from the Human Cell Landscape (http://bis.zju.edu.cn/HCL/) to display the common distribution of SLC16A3 in various tissues. The differential expression of SLC16A3 of pan/lung cancers and adjacent normal tissues was obtained from the data of TCGA (https://cancergenome.nih.gov/) and analyzed with UCSC Xena database (https://xena.ucsc.edu/). Meanwhile, the SLC16A3 protein level was measured with Human Protein Atlas (http://www.proteinatlas.org/).

Survival information of lung cancer patients were obtained from TCGA and analyzed with Kaplan–Meier analysis. We then analyzed the prediction pathway of SLC16A3 and its associated proteins via Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) on metascape (https://metascape.org).

Comprehensive Correlation Analysis in Tumor-Infiltrating Immune Cells

Tumor immune estimation resource (TIMER, https://cistrome.shinyapps.io/timer) served for analysis of tumor-infiltrating immune cells, and the correlations between the infiltrating level of different subsets of immune cells and SLC16A3 were computably detected. The immune cells contained CD4+ T cells, CD8+T cells, B cells, macrophages, neutrophils, and dendritic cells. For further investigation, TPM data of RNA-seq was converted from FPKM data and used for estimating the abundance of different immune cell types in tumor microenvironment by CIBERSORT (https://cibersort.stanford.edu/). RNA-seq data lung cancer samples were divided into two groups: Low SLC16A3 expression and High SLC16A3 expression, according to the expression level of SLC16A3.

The correlation of SLC16A3 and checkpoints were displayed with heat map. The horizontal and vertical coordinates represent genes, and different colors represent correlation coefficients (in the diagram, blue represents negative correlation, red represents positive correlation). Spearman or Pearson correlation analysis was further used to between SLC16A3 and genes of significance. Cancer Treatment Response gene signature DataBase (CTR-DB, http://ctrdb.ncpsb.org.cn/home) was used for the analysis of immunotherapy efficacy.

Cell Preparation and Lentivirus Transfection

A549 cells are lung adenocarcinoma cell line and obtained from ATCC. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, Grand island, NY, USA) 1% penicillin and streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

Lentiviruses were produced using previously established method.[26] Briefly, SLC16A3 shRNA plasmids (GenePharma, Shanghai, China) were packaged into lentiviral vectors by cotransfection into A549 cells with plasmids containing Gag/Pol, REV and VSV-G. Viral supernatant was harvested 24, 48, 72 and 96 h post transfection and filtered through 0.45-mm low protein-binding syringe filters. After 1 week, cells were put under selection with 1–5 mg/mL of puromycin for at least a week until there were enough healthy cells to be used for experiments. Silencing of SLC16A3 was verified using quantitative real-time PCR.

Interleukin Detection

For the detection of interleukin concentration, cell culture supernatants were collected and stored at –80 °C until analysis. Interleukins were measured with human IL-1β, IL-6, IL-8 or IL-33 ELISA kit (Multisciences (LIANKE) BIOTECH CO., LTD, China) according to the manufacturer's instructions. The absorbance at 450 nm wavelength was measured using the microplate reader (SpetraMax iD3).

Flow Cytometry

To quantify HIF-1a expression, A549 cells were adjusted to 1×10^6 cells/mL in suspension, fixed with 4% paraformaldehyde and then permeabilized with 90% methanol according to the manufacturer's instructions. Then, cells were washed and incubated with Alexa Fluor 488-anti-HIF-1a (Biolegend, 359707, USA) for 15 min in the dark at RT. Cells were analyzed on a flow cytometer (FACSVia, Becton Dickinson, USA).

Statistical Analysis

Data collection and statistics were performed with Graph prism 6.0. For data with normal distribution, results were presented as the mean ± SD. Unpaired *t*-test or ANOVA was used for analyzing statistical assessments. For data not in accord with normal distribution, results were present with median (IQR) and Wilcoxon test was utilized for analysis. Statistically significant difference was considered when $P < 0.05$.

Results

Overexpression of SLC16A3 in Patients with LUAD and LUSC

First, single-cell RNA sequencing data from Human Cell Landscape showed SLC16A3 was mainly distributed at chorionic villus, placenta, cord blood, adipose and jejunum in normal body (Figure 1A). Then, differential expression of SLC16A3 was estimated in pancancer cells. We found that the expression of SLC16A3 was increased in most tumors compared with normal tissues, especially in lung cancer (Figure 1B). TCGA further showed high SLC16A3 level in both LUAD ($n = 483$, $p < 0.001$) and LUSC ($n = 486$, $p < 0.001$) (Figure 1C). IHC results from HPA clearly displayed little detection on alveolar cells but medium on macrophages in normal lung tissue while strong SLC16A3 staining in both LUAD and LUSC cells (Figure 1D).

Aberrant SLC16A3 is Correlated with Poor Prognosis in LUAD and LUSC

Kaplan-Meier plotter was utilized to analyze the prognostic values of SLC16A3 and we found patients with high SLC16A3 level had shorter OS, DFS and PFS compared with those with low-SLC16A3 ($p < 0.05$) in both LUAD (Figure 2A) and LUSC (Figure 2B). Concurrently, there was a positive correlation between SLC16A3 expression and TNM stage ($p < 0.05$, data not shown). These results suggest that increased SLC16A3 may act as a volunteer in the malignant progression of LUAD and LUSC.

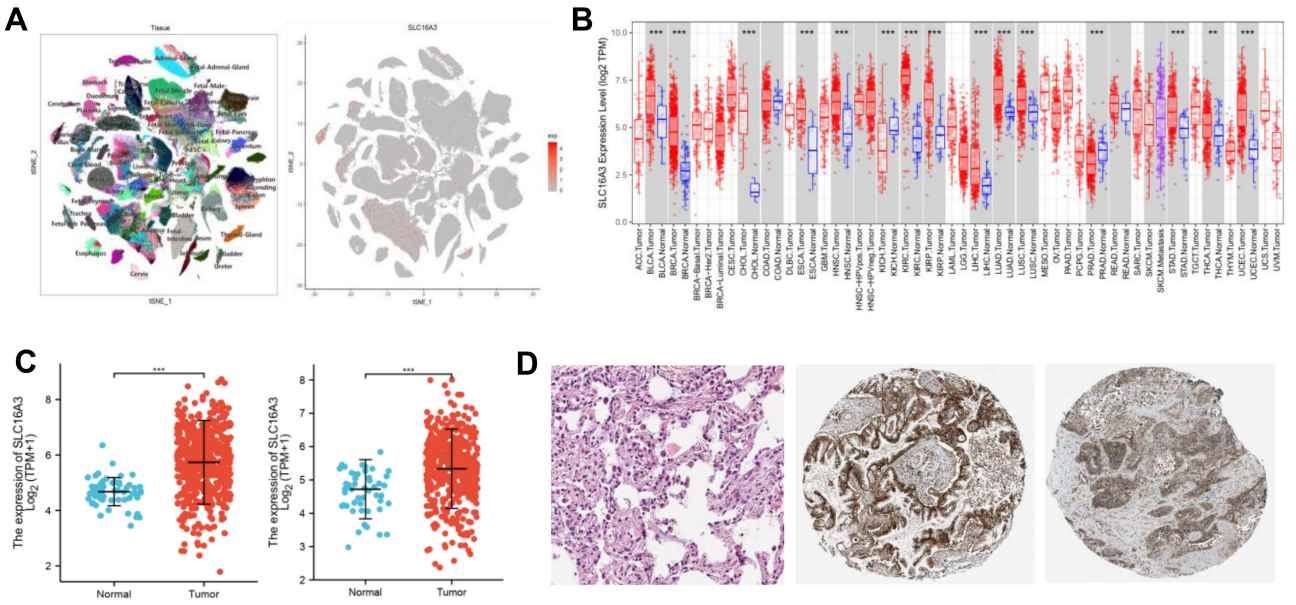

Figure 1 High SLC16A3 expression in lung cancers. (**A**) Single-cell RNA sequencing of SLC16A3 in normal tissues from Human Cell Landscape. (**B**) The expression of SLC16A3 in pan-cancers. (**C**) Significant increase of SLC16A3 was found in both LUAD (left) and LUSC (right). (**D**) Immunohistochemistry images (from Human Protein Atlas) of SLC16A3 expression in normal lung tissue (left), LUAD (middle) and LUSC (right). ***P < 0.000.

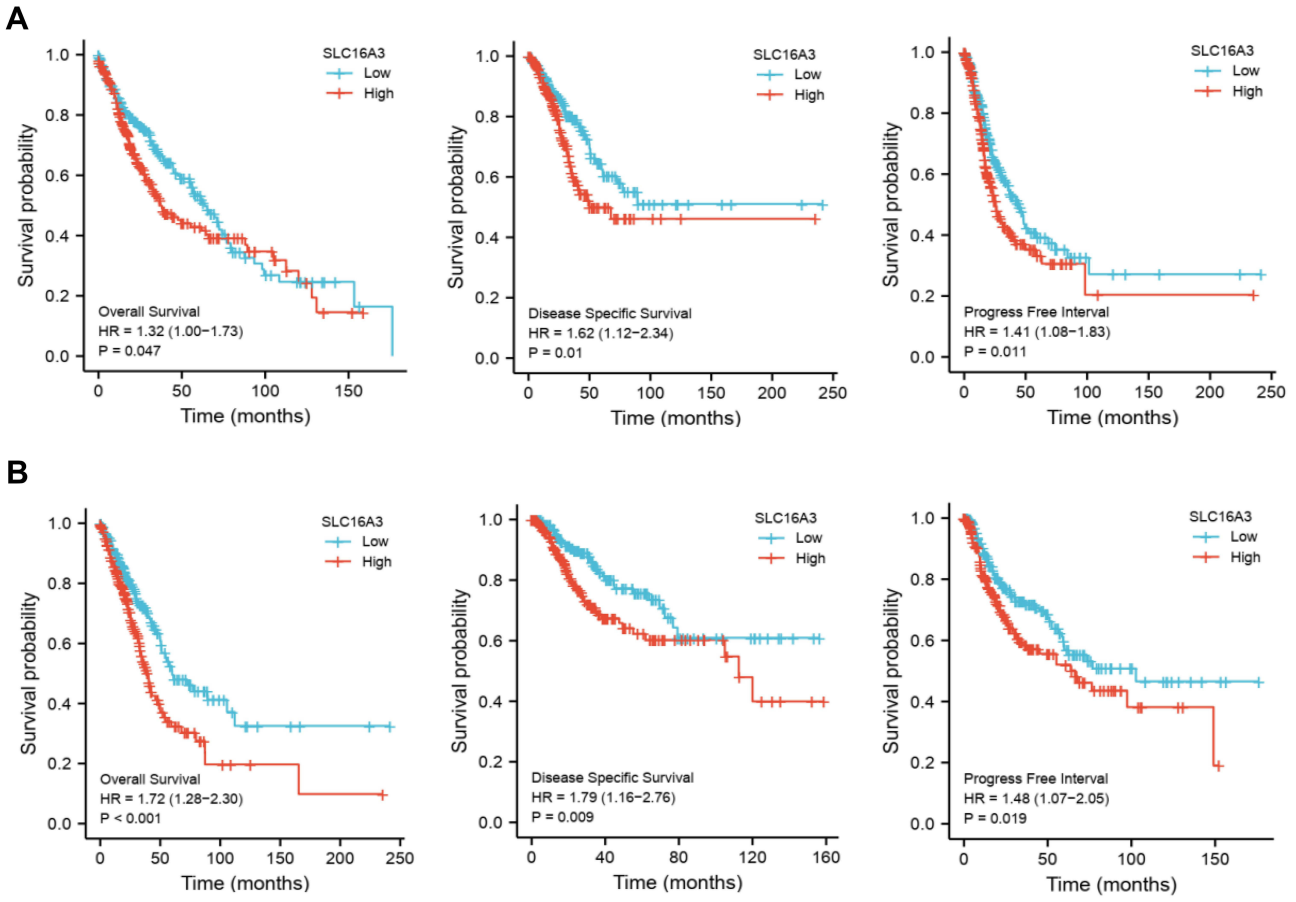

Figure 2 High SLC16A3 level is correlated with poor prognosis in lung cancers. Overall survival, disease specific survival and progression free survival of SLC16A3 in LUAD (**A**) and LUSC (**B**).

Enrichment Analysis Identifies SLC16A3-Related Signaling Pathways

To identify SLC16A3-related signaling pathways in lung cancer, SLC16A3-related genes were selected with GEPIA and for enrichment analysis in DAVID. The SLC16A3-related signaling pathways were measured with metascape. Results showed that SLC16A3 was involved in gluconeogenesis and HIF-1 signaling pathway in LUAD (Figure 3A) while extracellular matrix disassembly and Rap1 signaling pathway in LUSC (Figure 3B), indicating that function of SLC16A3 in lung cancers may be widely different not limited to glucose metabolism.

SLC16A3 is Associated with Negatively Regulated Immune Responses in LUAD

Here, we further explored the parts SLC16A3 plays in immune cell infiltration in both LUAD and LUSC. Results from TIMER indicated that SLC16A3 had positive correlation with DCs and neutrophils in LUAD ($p < 0.05$) (Figure 4A). Furthermore, SLC16A3 was estimated in immunosuppressive cells and it showed significant increase in enrichment score of Treg, Th2 and iDC in high-SLC16A3 LUAD (Figure 4B). We also displayed that common immune checkpoints (CTLA4, PD1, PD-L1, PD-L2, Tim-3, Lag3, ITGAM) and immune negatively regulated proteins (foxp3, TGF-β) had markedly differential expression in SLC16A3 low- and high-groups in LUAD not LUSC (Figure 4C). The correlation coefficient is shown in Figure 4D. CTR-DB further suggested that SLC16A3 was significantly negative with efficacy of anti-PD-1/PD-L1 therapy of lung cancer ($p = 0.0093$) (Table 1). These data suggest that SLC16A3 may be closely associated with immunosuppressive cells contributing to the immune evasion.

SLC16A3 Inhibition Attenuates IL-8 Section via HIF-1a

Tumor-derived interleukins are indicated to exert effects on immune checkpoints and recently IL-8 secreted from metastatic urothelial carcinoma cells has been associated with reduced clinical benefit of PD-L1 blockade.[26] Thus, we analyzed the correlation of SLC16A3 and IL-1β, IL-6, IL-8 and IL-33 that are reported tumor-associated. Results showed that SLC16A3 was positively relevant to IL-1β, IL-6, IL-8 but negatively to IL-33 in LUAD (Figure 5A).

On account of the prediction, we selected common LUAD cell line (A549) to establish SLC16A3 knockdown cells. The RNA level of SLC16A3 was significantly down-regulated in shSLC16A3 cells compared with control (data not shown). We found that only IL-8 expression was significantly decreased when SLC16A3 knocking down (Figure 5B).

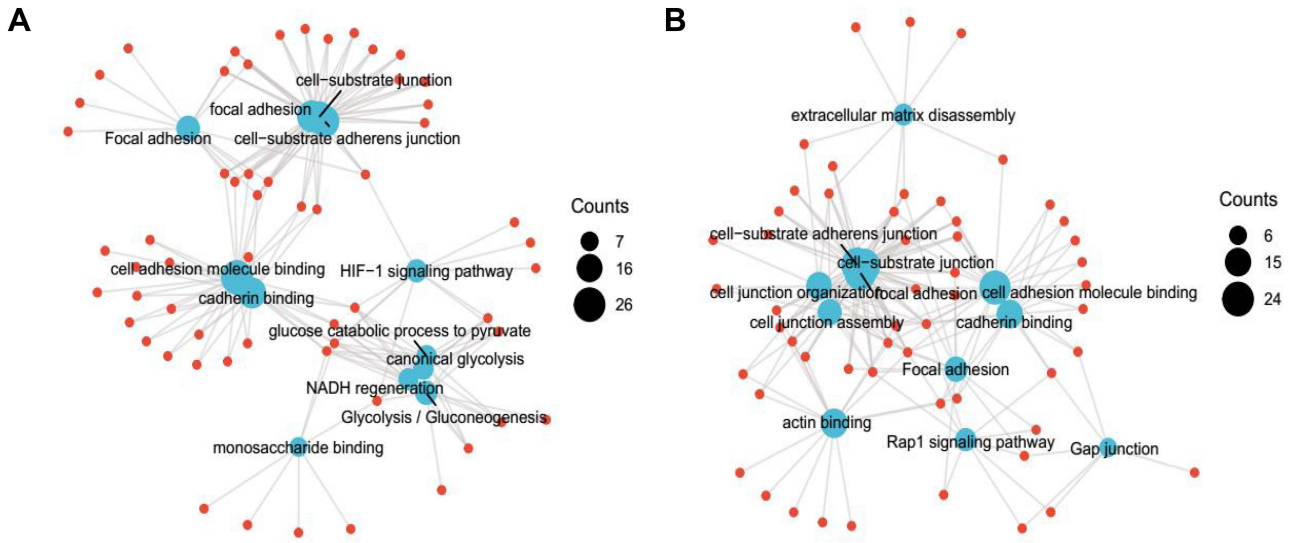

Figure 3 SLC16A3 is involved in different signaling pathways in lung cancers. GO enrichment analysis indicated different signal pathways of SLC16A3 in LUAD (**A**) and LUSC (**B**).

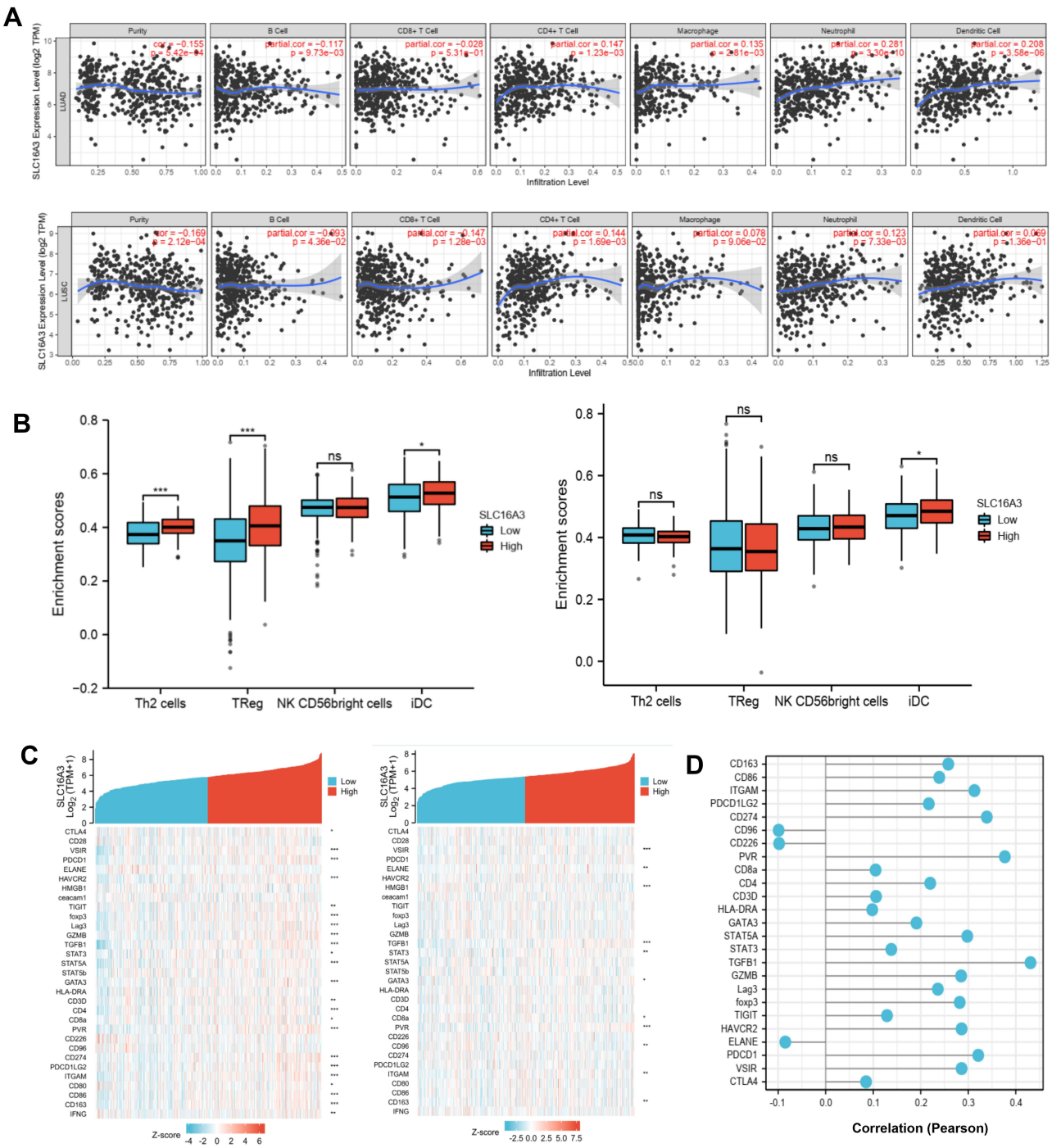

Figure 4 SLC16A3 is associated with immune suppressor cells and checkpoints in LUAD. (**A**) TIMER database showed SLC16A3 had positive correlation with DCs and neutrophils infiltration in LUAD (up) but not LUSC (below). (**B**) The correlation between SLC16A3 and immunosuppressive cells was detected in LUAD (left) and LUSC (right). Increased enrichment score of Treg, Th2 and iDC was seen in high-SLC16A3 LUAD. (**C**) The correlation between SLC16A3 and immune checkpoints was evaluated in LUAD (left) and LUSC (right). (**D**) SLC16A3 was correlated with various checkpoints in LUAD. * P < 0.05, ** P < 0.01, ***P < 0.000.

Previously SLC16A3 was predicted to be linked with HIF-1 signaling in LUAD (Figure 3A) and we also showed HIF-1a downregulation in SLC16A3 knockdown cells (Figure 5C). Moreover, DMOG could increase HIF-1a expression in SLC16A3 knockdown cells and simultaneously IL-8 secretion was also elevated compared to DMOG untreated (Figure 5D). These results suggest that SLC16A3 may promote immunosuppressive regulators by enhancing IL-8 secretion through HIF-1a signaling, which associates tumor cells and immune components in microenvironment.

Table 1 Indication of SLC16A3 in Therapeutic Effect of Cancers from CTR-DB

Cancer Type	Therapeutic Regimen	LogFC	P value
Colorectal cancer	Docetaxel+Gemcitabine	2.1	4.3e-9
Melanoma	Pembrolizumab	1.6	0.00062
Urinary bladder cancer	Carboplatin+Gemcitabine	1.6	0.0015
Colorectal cancer	2-Fluoropyrimidine	−1.4	0.0022
Lung cancer	Anti-PD-1/PD-L1	−1.2	0.0093
Breast cancer	Cyclophosphamide+Doxorubicin	−1.9	0.02

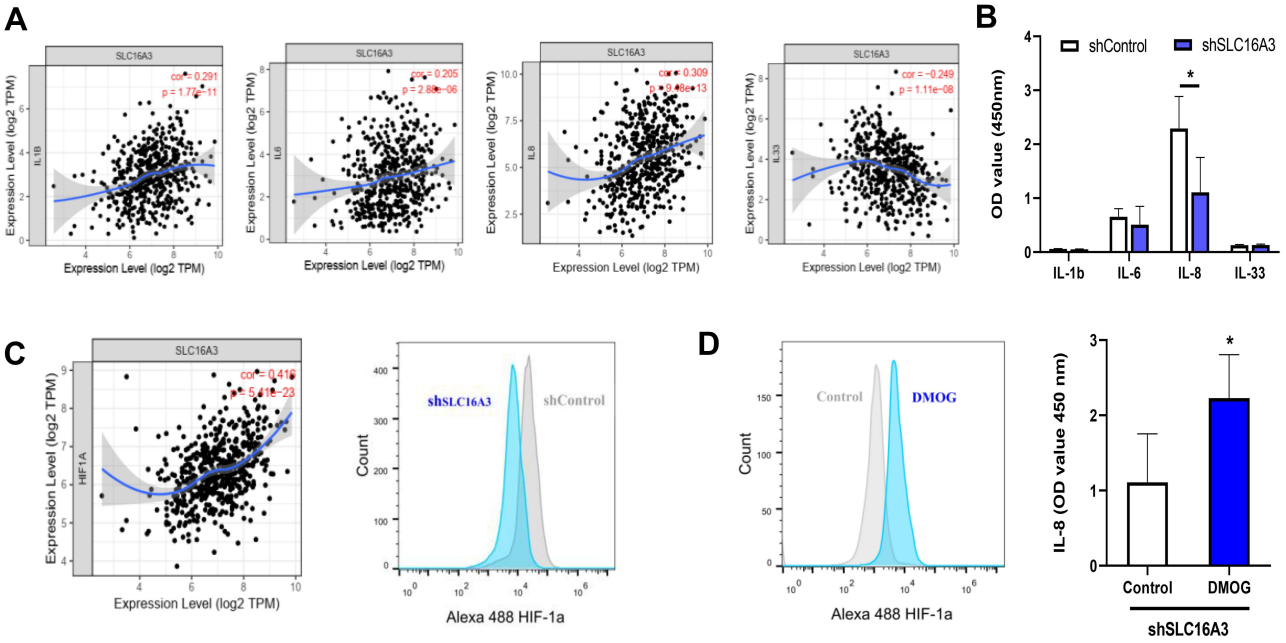

Figure 5 Knockdown of SLC16A3 attenuates IL-8 secretion via HIF-1a. (**A**) TIMER database showed SLC16A3 was positively relevant to IL-1β, IL-6, IL-8 but negatively to IL-33 in LUAD. (**B**) Interleukin level was detected with ELISA in A549 cells. SLC16A3 knockdown led to significant IL-8 secretion. (**C**) TIMER database indicated that SLC16A3 had positive relationship with HIF-1a and flow cytometry result showed SLC16A3 knockdown resulted in decreased HIF-1a expression. (**D**) DMOG stimulated HIF-1a expression and restored IL-8 level in SLC16A3 downregulated cells. Data were present with mean± SD, * P < 0.05.

Discussion

Although the differential spatial distribution and prognostic value in LUAD and LUSC have been reported, the role SLC16A3 plays in immune infiltration and suppression is not fully known. Here, we for the first time showed SLC16A3 is dynamic in diverse signal pathways in LUAD and LUSC, and more importantly it is correlated with immune suppressor cells (Th2 and Treg) and popular checkpoints (PD1, PD-L1, Tim-3, ITGAM) in LUAD but not LUSC. SLC16A3 may facilitate microenvironment immunosuppression via the increase of IL-8 secretion. Our results indicate that SLC16A3 functions not only as lactate transporter in glycolysis but as mediator in immune regulation in LUAD.

It showed that in normal body, tissues rich for SLC16A3 are mainly fetal like chorionic villus, placenta and cord blood but not mature. However, in multi-organ tumors, SLC16A3 has a general increase, which could be attributed to enhanced demand for glucose fuel and hyperactive lactate flux in the process of malignancy. Moreover, elevated SLC16A3 is found involved in biological behaviors of various tumors contributing to the poor prognosis.[16–21] Recently Xue et al found that the prognosis of LUAD patients with high SLC16A3 expression was worse than those with low SLC16A3 expression.[25] Here, we further added the evidence that high level of SLC16A3 also results in shorter OS and PFS in LUSC. Simultaneously, it was found different molecular functions and signaling pathways SLC16A3 participates in LUAD and LUSC not limited to carbon metabolism. These results suggest that SLC16A3 may play

different roles in LUAD and LUSC, and the mechanism of SLC16A3 involved in various signaling pathways needs further investigation.

Immune checkpoint inhibitors (ICIs) have made an indelible mark in the field of cancer immunotherapy, demonstrating unprecedented extension of patient survival. However, despite the success of ICIs, resistance to these agents restricts the number of patients able to achieve durable responses, and immune-related adverse events complicate treatment. Thus, a better understanding of the requirements for an effective and safe antitumor immune response following ICI therapy is needed. In this study, we for the first time showed high SLC16A3 level is accompanied by enrichment of Treg, Th2 and iDC in LUAD not LUSC. Concurrently, SLC16A3 is positively associated with significant immune checkpoints and immunosuppressive factors in LUAD.

Interleukin-8 is a well-known pro-inflammatory and proangiogenic chemokine that is prominently expressed in immune, endothelial, and tumor cells.[26] Recently, it has been reported that IL-8 could contribute to the immunosuppressive tumor microenvironment.[27,28] Moreover, IL-8 is capable to increase PD-L1 exposure and reduce the benefits of immune checkpoint inhibitors.[29,30] In this study, TIMER prediction data indicated that SLC16A3 was positively relevant to IL-8 in LUAD. Considering the limitation of our work, we can only detect the level of IL-8 with tumor cells and suggest that knockdown of SLC16A3 led to reduced IL-8 section in A549 cells. SLC16A3-induced IL-8 may account for the immunosuppressive cell infiltration and checkpoint exposure in LUAD, which could add new targets to improve the efficacy of ICI. Based on the evidence we provide, further studies would stress on the clinical significance of SLC16A3 as an immunotherapy biomarker.

Conclusion

Taken together, we come to the conclusion that SLC16A3 may facilitate immune tolerance in LUAD by regulating immunosuppressive cells and elevating checkpoints via HIF-1a-IL-8 axis. Inhibition of SLC16A3 may enhance efficacy of immunotherapy in combination with ICIs. Our results not only provide evidence of SLC16A3 as a promising therapeutic target but also reveal a novel mechanism for immune evasion in lung cancer.

Data Sharing Statement

The RNA-Seq data of SLC16A3 were from the Human Cell Landscape (http://bis.zju.edu.cn/HCL/). The differential expression of SLC16A3 of pan/lung cancers and adjacent normal tissues was obtained from the data of TCGA (https://cancergenome.nih.gov/) and analyzed with UCSC Xena database (https://xena.ucsc.edu/). The prediction pathway of SLC16A3 and its associated proteins via Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) on metascape (https://metascape.org). Immunohistochemistry images of SLCI6A3 protein expression were downloaded from the Human Protein Atlas (HPA) (http://www.proteinatlas.org/). All the datasets were open access datasets. The infiltrating level of different subsets of immune cells and SLC16A3 were from TIMER (https://cistrome.shinyapps.io/timer). The abundance of different immune cell types in tumor microenvironment was estimated by CIBERSORT (https://cibersort.stanford.edu/). CTR-DB (http://ctrdb.ncpsb.org.cn/home) was used for the analysis of immunotherapy efficacy.

Ethic Approval

This study was approved by the Ethics Committee of Harbin Medical University and conducted according to all current ethics guidelines.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published and agreed to be accountable for all aspects of the work.

Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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