**Name of Journal:** World Journal of Clinical Cases

**Manuscript NO:** 67321

**Manuscript Type:** ORIGINAL ARTICLE

**Basic Study**

**Solute carrier family 2 members 1 and 2 as prognostic biomarkers in hepatocellular carcinoma associated with immune infiltration**

Qing Peng, Li-Yuan Hao, Ying-Lin Guo, Zhi-Qin Zhang, Jing-Min Ji, Yu Xue, Yi-Wei Liu, Jun-Lan Lu, Cai-Ge Li, Xin-Li Shi

**Abstract**

**BACKGROUND**
Metabolic reprogramming has been identified as a core hallmark of cancer. Solute carrier family 2 was a major glucose carrier family. It consists of 14 members, and we mainly study solute carrier family 2 member 1 (SLC2A1) and solute carrier family 2 member 2 (SLC2A2) here. SLC2A1, mainly existing in human erythrocytes, brain endothelial cells, and a normal placenta, was found to be increased in hepatocellular carcinoma (HCC). While SLC2A2, the major transporter of the normal liver, decreased in HCC.

**AIM**
To identify SLC2A1 and SLC2A2 were associated with immune infiltration in addition to participating in the metabolic reprogramming in HCC.

**METHODS**
The expression levels of SLC2A1 and SLC2A2 were tested in HepG2 cells, HepG215 cells, and multiple databases. The clinical characteristics and survival data of SLC2A1 and SLC2A2 were examined by multiple databases. The correlation between SLC2A1
and SLC2A2 was analyzed by multiple databases. The functions and pathways in which SLC2A1, SLC2A2, and frequently altered neighbor genes were involved were discussed in String. Immune infiltration levels and immune marker genes associated with SLC2A1 and SLC2A2 were discussed from multiple databases.

RESULTS
The expression level of SLC2A1 was up-regulated, but the expression level SLC2A2 was down-regulated in HepG2 cells, HepG215 cells, and liver cancer patients. And then, the expression levels of SLC2A1 and SLC2A2 were related to tumor volume, grade, and stage in HCC. Interestingly, the expression levels of SLC2A1 and SLC2A2 were negatively correlated. Further, high SLC2A1 expression and low SLC2A2 expression were linked to poor overall survival and relapse-free survival. Moreover, SLC2A1, SLC2A2, and frequently altered neighbor genes played a major role in the occurrence and development of tumors. Notably, SLC2A1 was positively correlated with tumor immune infiltration, while SLC2A2 was negatively correlated with tumor immune infiltration. Particularly, SLC2A2 methylation was positively correlated with lymphocytes.

CONCLUSION
SLC2A1 and SLC2A2 were independent therapeutic targets for HCC. And then, they were quintessential marker molecules for predicting and regulating the number and status of immune cells in HCC.

**Key Words:** Hepatocellular carcinoma; Solute carrier family 2 member 1; Solute carrier family 2 member 2; Prognostic; Immune infiltration

Core Tip: We performed an integrated bioinformatics analysis to assess the influence of solute carrier family 2 member 1 (SLC2A1) and solute carrier family 2 member 2 (SLC2A2) in hepatocellular carcinoma (HCC). This study revealed that SLC2A1 and SLC2A2 were independent therapeutic targets in HCC and they were correlated with immune infiltration in addition to being involved in the metabolic reprogramming in HCC.
INTRODUCTION

Hepatocellular carcinoma (HCC) was the third most common cancer in the world\textsuperscript{[1]} among which 55% of cases came from China and 80% of HCC cases were caused by HBV infection\textsuperscript{[2]}. HCC was an inflammation-induced tumor. Immune escape was one of the characteristics\textsuperscript{[3]}. Immune cells and cytokines in the immune microenvironment played an important role in the occurrence and development of HCC\textsuperscript{[4]}. Based on this, programmed cell death 1 (PD-1), CD274 molecule (PD-L1), and cytotoxic T-lymphocyte associated protein 4 (CTLA4) monoclonal antibodies prevented T cells from failing and thus activated the anti-cancer immunity\textsuperscript{[5]}. However, clinical trials showed that PD-1 antibodies (nivolumab\textsuperscript{[6]} and pembrolizumab\textsuperscript{[7]}), and CTLA4 antibodies (tremelimunab\textsuperscript{[8]}) improved clinical outcomes in a few patients or were not effective at all. Therefore, recognition molecules representing the patient’s immune status will help identify subgroups sensitive to immunomodulatory drugs. Moreover, some independent target molecules associated with the tumor immune microenvironment are worth exploring in HCC.

Metabolic reprogramming was recognized as a core hallmark of cancer\textsuperscript{[9]}. The solute carrier family 2 was the important carrier for glucose to enter target cells, and its ability to transport glucose was the first rate-determining step in tumor metabolic reprogramming\textsuperscript{[10]}. Solute carrier family 2 member 1 (SLC2A1), one of the members of the solute carrier family 2, mainly existed in human erythrocytes, brain endothelial cells, and a normal placenta\textsuperscript{[11]}. It was highly expressed in HCC\textsuperscript{[12]}. Interestingly, solute carrier family 2 member 2 (SLC2A2), the major transporter of the normal liver\textsuperscript{[13]}, was low expressed when HCC occurred\textsuperscript{[14]}. The lactic acid produced and PH changed by the Warburg effect which was part of metabolic reprogramming affected immune cells in the tumor immune microenvironment\textsuperscript{[15]}. A previous study showed that overexpression of SLC2A1 was correlated with suppressing CD8+ T cells and B cells in gastric cancer\textsuperscript{[16]}. Nevertheless, the potential functions and mechanisms of SLC2A2 in HCC are still unclear.
In this study, multiple databases were used to estimate the expression levels of SLC2A1 and SLC2A2 in HCC. Then, expressions of SLC2A1 and SLC2A2 were correlated with the clinical characteristics and prognosis of patients. In addition, we also evaluated the pathways in which SLC2A1, SLC2A2, and frequently altered neighbor genes participated were involved in the occurrence and development of tumors. Notably, we analyzed the correlation between SLC2A1 and SLC2A2 and immune cells in the HCC microenvironment. Anyway, this study clarified the crucial role of SLC2A1 and SLC2A2, and it was first proposed that SLC2A1 and SLC2A2 are related to immune cells in HCC.

**MATERIALS AND METHODS**

*National center for biotechnology information's gene*


*Gene expression series 121248 (GSE121248) in gene expression omnibus*

Gene expression omnibus (www.ncbi.nlm.nih.gov/geo/)\(^{[18]}\) was an international public repository of microarray chips, second-generation sequencing, and other high-throughput genetic data uploaded by researchers around the world. GSE121248\(^{[19]}\), a cohort of HCC patients, demonstrated the expression levels of SLC2A1 and SLC2A2 compared with normal samples and the correlation between SLC2A1 and SLC2A2 expression in HCC.

*Cancer genome atlas*

Cancer genome atlas (portal.gdc.cancer.gov/)\(^{[20]}\) was one of the most ambitious and successful cancer genomics programs to date. Raw counts of RNA sequencing data,
corresponding clinical information from 371 HCC and normal tissue samples were available from TCGA. Clinical information of these patients was shown in Table 1.

**Human protein atlas**

Human protein atlas (www.proteinatlas.org)\(^{[21]}\) provided information on the tissue and cellular distribution of 24,000 human proteins. SLC2A1 and SLC2A2 immunofluorescence images of HepG2 cells were obtained. Next, immunohistochemistry images showed SLC2A1 and SLC2A2 in liver cancer patients and normal tissues.

**Tumor immune estimation resource**

Tumor immune estimation resource (cistrome.shinyapps.io/timer/)\(^{[22]}\) was a comprehensive resource for the systematic analysis of immune infiltrates across diverse cancer types. It revealed the expression levels of SLC2A1 and SLC2A2 in different cancers. Furthermore, the expression of SLC2A1 and SLC2A2 and the level of immune cells (B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells) were analyzed. Moreover, the correlation between SLC2A1 and SLC2A2 expression levels and immune cell markers [markers of CD8+ T cells, T cells (general), B cells, monocytes, tumor-associated macrophage (TAM), M1 macrophages, M2 macrophages, neutrophils, natural killer cells, dendritic cells, Th1 cells, Th2 cells, Tfh cells, Th17 cells, T cell regulatory (Tregs) and T cell exhaustion] were analyzed in details.

**ONCOMINE**

ONCOMINE (www.oncomine.org)\(^{[23]}\) was an online cancer microarray database. It was used to analyze the expression levels of SLC2A1 and SLC2A2 in different cancers.

**UALCAN**
UALCAN (ualcan.path.uab.edu/index.html)[24] was a web resource for analyzing cancer omics data. Expressions of SLC2A1 and SLC2A2 in different tumor grades and stages of HCC were analyzed by UALCAN.

**Gene expression profiling interactive analysis**
Gene expression profiling interactive analysis (gepia.cancer-pku.cn)[25] was a developed interactive website server for analyzing the RNA sequencing expression data from the TCGA and the Genotype-Tissue Expression projects. The correlation between SLC2A1 and SLC2A2 and immune marker genes of different immune cells was analyzed.

**Kaplan-Meier plotter**
The free online database Kaplan-Meier plotter (kmplot.com)[26] which included gene expression data and survival information of 364 clinical HCC patients was utilized to predict overall survival (OS) and relapse-free survival (RFS) of HCC patients. We assessed factors such as gender, race, disease stage, disease grade, American Joint Committee on Cancer system for tumor staging (AJCC-T), vascular infiltration, treatment with sorafenib, alcohol consumption, and HBV infection associated with survival.

**String**
Protein-protein interaction (PPI) networks of SLC2A1, SLC2A2, and their neighbors were constructed. And then, the Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were obtained by String (string-db.org)[27]. Three aspects were contained in GO enrichment analysis: biological process (BP), molecular function (MF), and cellular component (CC). GO and KEGG enrichment analysis results were selected by P values less than 0.05 as the critical criterion.

**Cytoscape**
PPI of SLC2A1, SLC2A2, and neighbor genes from the String database was reconstructed by Cytoscape (v.3.6.1, cytoscape.org/)[28].

**Tumor and immune system interaction database**

Tumor and immune system interaction database (cis.hku.hk/TISIDB/)\(^{29}\) was a web portal for tumor and immune system interaction, which integrated multiple heterogeneous data types. Immune infiltration associated with SLC2A2 methylation was searched to elucidate the tumor-immune system interaction.

**R project**

The RV4.0.3 project (www.r-project.org/) was used to visualize the top twenty GO and KEGG data in the String database. And meanwhile, data from TCGA were also processed and visualized.

**Cell culture and drug treatment**

Human hepatoma cell lines HepG2 cells and HepG2215 cells were acquired from the American Type Culture Collection (Manassas, VA, United States). They were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, United States) supplemented with 10% fetal bovine serum (Gibco, United States), 100 U/mL penicillin, and 100 μg/mL streptomycin. They were maintained in an atmosphere of 5% CO\(_2\) at 37°C.

**RNA sequencing**

Total RNA was extracted from the sample and the quality was tested. Then mRNA was extracted from total RNA by using poly-t oligo attached magnetic beads and was disrupted to about 300 bp. The first strand of cDNA was synthesized with random primers and reverse transcriptase by using RNA as the template, and then the second strand of cDNA was synthesized by using the first strand of cDNA as the template. PCR was used to amplify DNA and 450 bp fragments were screened out to form the libraries.
The total and effective concentrations were tested after libraries were inspected by using Agilent 2100 Bioanalyzer. Libraries containing different index sequences were mixed proportionally according to the effective concentration and the amount of data required by libraries. Mixed libraries were uniformly diluted to 2 nmol/L, and single-chain libraries were formed by alkali denaturation. These libraries were paired-end (PE) sequenced by the Next-Generation Sequencing (NGS) based on the Illumina HiSeq platform. (This work was entrusted to Nanjing Personal Gene Technology co., LTD.)

Statistical analysis
Statistical analyses were performed by the Statistical Product and Service Solutions (SPSS) 23.0 statistics software (SPSS, Chicago, IL), GraphPad Prism 8 software, and Rv4.0.3 software. Each sample was repeated at least three times. If data were normally distributed, they are represented as mean ± SD. When more than two groups were included, a one-way analysis of variance would be utilized. Differences were considered statistically when P-value was less than 0.05.

RESULTS
SLC2A1 expression was increased, whereas SLC2A2 expression was decreased in liver cancer
We investigated the expression of solute carrier family 2 in normal liver tissues by the NCBI gene. And we found that SLC2A2, as the normal liver transporter, was highly expressed compared with other members of the family (Figure 1A). Next, we compared the mRNA level of solute carrier family 2 in HepG2 and HepG2215 cells. Interestingly, we found that SLC2A2 expression was less, but SLC2A1 was elevated in these two cell lines (Figure 1B and C). Similarly, cohort (GSE121248) of HCC patients showed that SLC2A1 expression was elevated, while SLC2A2 expression was reduced compared with the normal samples (Figure 1D). Consistently, TCGA data also indicated that SLC2A1 was up-regulated and SLC2A2 was down-regulated in HCC patients (Figure 1E). Notably, the HPA database revealed that SLC2A1 protein expression was
expressed and SLC2A2 protein expression was weakly expressed in HepG2 cells (Figure 1F). As expected, SLC2A1 protein expression (Figure 1G) was increased and SLC2A2 protein expression (Figure 1H) was decreased in liver cancer in the HPA database. Taken together, SLC2A1 was up-regulated while SLC2A2 was down-regulated in liver cancer patients.

**SLC2A1 and SLC2A2 mRNA expression levels in different human cancers were changed by using TIMER and ONCOMINE databases**

To see if this phenomenon was universal, the mRNA expressions of SLC2A1 and SLC2A2 in different human cancers were investigated by using the TIMER database. This analysis revealed that the SLC2A1 was higher in BRCA (breast invasive carcinoma), CESC (cervical squamous cell carcinoma and endometrial adenocarcinoma), CHOL (cholangiocarcinoma), COAD (colon adenocarcinoma), ESCA (esophageal carcinoma), HNSC (head and neck cancer), KIRC (kidney renal clear cell carcinoma), LIHC (liver hepatocellular carcinoma), LUAD (lung adenocarcinoma), LUSC (lung squamous cell carcinoma), READ (rectum adenocarcinoma), STAD (stomach adenocarcinoma), THCA (thyroid carcinoma) and UCEC (uterine corpus endometrial carcinoma) (Figure 2A). But it was lower in KICH (kidney chromophobe), KIRP (kidney renal papillary cell carcinoma), and PRAD (prostate adenocarcinoma) (Figure 2A). Similarly, SLC2A2 was lower in CHOL (cholangitis carcinoma), KICH (kidney chromophobe), and LIHC (liver hepatocellular carcinoma) (Figure 2A). But it was higher in LUAD (lung adenocarcinoma), BLCA (bladder urothelial carcinoma), BRCA (breast invasive carcinoma), ESCA (esophageal carcinoma), GBM (glioblastoma multiforme) (Figure 2A). Although SLC2A2 was increased in these cancers, it was expressed at low or absent levels in these cancers and the corresponding normal samples.

To further verify expressions of SLC2A2 and SLC2A1 in cancer, we examined their expressions in the ONCOMINE database. The result showed that SLC2A1 was upregulated in most cancers, and SLC2A2 was downregulated in most cancers in most
datasets. SLC2A1 has been observed to be upregulated in bladder cancer, breast cancer, cervical cancer, colorectal cancer, esophageal cancer, gastric cancer, head and neck cancer, kidney cancer, leukemia, liver cancer, lung cancer, lymphoma, myeloma, ovarian cancer, pancreatic cancer, and prostate cancer. However, some datasets showed that SLC2A1 was downregulated in brain and CNS cancer, breast cancer, colorectal cancer, esophageal cancer, kidney cancer, leukemia, lung cancer, lymphoma, melanoma, and sarcoma (Figure 2B). Similarly, the result showed the expression of SLC2A2 was reduced in brain and CNS cancer, breast cancer, cervical cancer, colorectal cancer, esophageal cancer, gastric cancer, kidney cancer, leukemia, liver cancer, lymphoma, pancreatic cancer, prostate cancer, and sarcoma. In other datasets, SLC2A2 was upregulated in breast cancer, colorectal cancer, head and neck cancer, leukemia, and pancreatic cancer (Figure 2B). In addition to HCC, SLC2A1 elevation and SLC2A2 reduction have also been observed in several other cancers. SLC2A2, as key transporter in normal liver tissue, was decreased in the occurrence of HCC, while SLC2A1 expression was increased. So we still discuss relevant issues of SLC2A1 and SC2A2 in HCC.

**SLC2A1 and SLC2A2 transcription levels in HCC patients were correlated with tumor volume, grade, and stage**

Next, we investigated the correlation between SLC2A1 and SLC2A2 expression levels and tumor volume, grade, and stage in HCC patients in TCGA data and UALCAN database. The results showed that the SLC2A1 level gradually increased, while the SLC2A2 level gradually decreased with the process of primary tumor volume enlargement in TCGA data (Figure 3A). Consistently, highly differentiated HCC indicated lower SLC2A1 and higher SLC2A2 compared with poorly differentiated HCC in TCGA data and UALCAN database (Figure 3B and C). Notably, the expression of SLC2A1 was higher, while the expression of SLC2A2 was lower with the progression of HCC grade (Figure 3D) in UALCAN database. Collectively, with the development of
HCC, the expression of SLC2A1 was increased, while the expression of SLC2A2 was decreased.

**SLC2A1 and SLC2A2 mRNA levels were correlated with survival rates of HCC patients in TCGA data and the Kaplan-Meier Plotter database**

To clarify the impact of the expression levels of SLC2A1 and SLC2A2 on patient prognosis, survival analysis was utilized to analyze the correlation between these expression levels and the survival rates of HCC patients.

Patients with high expression of SLC2A1 had poor OS, which suggested that high SLC2A1 expression posed a risk factor for HCC patients (Figure 4A and B). Consistently, higher expression of SLC2A2 patients had a better OS, which suggested high SLC2A2 expression was a protective factor in HCC (Figure 4D and E). One-year, three-year, and five-year areas under the curve of SLC2A1 and SLC2A2 were all greater than 0.6, which suggested the prediction result was reliable (Figure 4C and F). In addition, low expression SLC2A1 and high expression SLC2A2 have better OS and RFS in the Kaplan-Meier Plotter database (Figure 4G and H).

Next, we analyzed the correlation between SLC2A1 and SLC2A2 expression and clinical characteristics. These clinical characteristics included gender, race, stage, grade, AJCC-T, and whether the patients received sorafenib treatment, were infected with HBV, consumed alcohol, and had vascular invasion (Table 2). These results suggested that high SLC2A1 and low SLC2A2 were independent risk factors for HCC, independent of these factors. Notably, prominent expression of SLC2A1 was more dangerous for males than females, Asians than whites, and those who were infected with HBV than uninfected. Confusingly, high expression of SLC2A1 was more dangerous in non-alcoholics than in alcoholics. Notably, with the progression of the stage, the grade, and AJCC-T of HCC, the high expression of SLC2A1 and low expression of SLC2A2 became worse for patients.

Together, both the high expression of SLC2A1 and the low expression of SLC2A2 were independent risk factors for HCC patients and led to poor prognosis.
**SLC2A1 and SLC2A2 showed negative correlations in HCC patients in GSE121248, TCGA, TIMER database, and GEPIA database**

To investigate whether the opposite trend of SLC2A1 and SLC2A2 expression was accidental, we proceeded to correlation analysis of the expression levels. The results showed that there were always negative correlations between SLC2A1 and SLC2A2 expression levels by the cohort GSE121248 \( (r = -0.4756) \) (Figure 5A), TCGA data \( (r = -0.28) \) (Figure 5B), TIMER database \( (r = -0.252) \) (Figure 5C) and GEPIA \( (r = -0.19) \) (Figure 5D) database. It was inevitable that SLC2A1 and SLC2A2 had an opposite expression tendency in HCC, because they may be involved in some biological processes besides serving as glucose transporters together.

*String database was used to predict the effects of SLC2A1, SLC2A2, and frequently altered adjacent genes expression changes on functions and pathways*

The expression levels of SLC2A1 and SLC2A2 were modified in HCC, and their expressions were negatively correlated. Therefore, we explored the influence of changes of them and their neighbor genes on the functions and pathways in the String database. We structured the PPI network of SLC2A1, SLC2A2, and their neighbor genes in the String database, which contained 22 nodes and 107 edges (Figure 6A). In this network diagram, genes tumor protein p53 (TP53), ATM serine/threonine kinase (ATM), MDM2 proto-oncogene (MDM2), protein kinase-B (AKT1), hypoxia inducible factor 1 subunit alpha (HIF1A), binding protein p300 (EP300), insulin (INS), glucokinase(GCK), and hexokinase 2 (HK2) were included.

Next, we carried out GO analysis on these genes in the database, and GO analysis results included three different levels of biological process (BP), cellular component (CC), and molecular function (MF). In Figures 6C, D and E, we selected and showed the first 20 p-values from small to large. The results indicated that cellular response to hypoxia (GO:0001666), cellular response to stress (GO:0033554), regulation of cell communication (GO:0010646), regulation of transcription from RNA polymerase II
promoter in response to stress (GO:0043618), glucose homeostasis (GO:0042593), negative regulation of cellular metabolic process (GO:0031324), negative regulation of cellular metabolic process (GO:0031324), positive regulation of cellular catabolic process (GO:0045787), regulation of apoptotic signaling pathway (GO:2001233) and others were involved.

And then, to explore which pathways these genes influenced together, we performed KEGG analysis and showed the top 20 from small to large according to the p-value. KEGG pathway analysis of these genes presented Hypoxia-inducible factor 1 (HIF-1) signaling pathway (hsa04066), central carbon metabolism in cancer (hsa05230), pathways in cancer (hsa05200), Forkhead box O (FOXO) signaling pathway (hsa04068), phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway (hsa04151), cell cycle (hsa04110), insulin resistance (hsa04931), glucagon signaling pathway (hsa04922), insulin signaling pathway (hsa04910) and others were involved (Figure 6B).

These complex regulatory relationships indicated that SLC2A1 and SLC2A2 were not only directly involved in the metabolism of glucose, but also participated in many important pathways together with other neighbor genes. Although GO and KEGG have proved that SLC2A1, SLC2A2, and their neighbor genes are involved in the occurrence and development of tumors, they have not proved the influence on the immune microenvironment. These results were based on previously discovered interrelationships and existing, but did not include what has not been discovered.

*Landscape of infiltrating immune cells in HCC was different from that in normal liver tissues and expressions of SLC2A1 and SLC2A2 correlated with immune infiltration levels in HCC*

It has been noted that the lactic acid microenvironment associated with the Warburg effect always affected the number and function of some immune cells[30]. Some studies have also found that SLC2A1 expression levels were correlated with immune cells in some tumors[16,31]. However, the relationship between glucose transporters SLC2A1 and SLC2A2 and the immune microenvironment was unclear in HCC.
The cell type identification by estimating relative RNA transcript subsets (CIBERSORT) was performed firstly. The results showed that HCC had different levels of immune invasion compared to normal tissues. In comparison to normal tissues, the proportions of B cell memory, Tregs, T cell gamma delta, monocyte, macrophage M0, myeloid dendritic cell resting, mast cell activated, mast cell resting, and neutrophil had changed in HCC (Figure 7A and B).

Next, SLC2A1 and SLC2A2 were investigated to determine whether their expressions were correlated to the level of immune cells with infiltration by TIMER database. The result showed that SLC2A1 expression had a weak negative correlation with tumor purity. But the SLC2A1 expression level had a positive correlation with infiltrating levels of B cells (r = 0.291), CD8+ T cells (r = 0.296), CD4+ T cells (r = 0.238), macrophages (r = 0.425), neutrophils (r = 0.424) and dendritic cells (r = 0.368) (Figure 7C). Conversely, SLC2A2 expression showed a weak positive correlation with tumor purity. But the SLC2A2 expression level had a negative correlation with infiltrating levels of B cells (r = -0.188), CD8+ T cells (r = -0.188), macrophages (r = -0.148), neutrophils (r = -0.115) and dendritic cells (r = -0.17) (Figure 7D).

**SLC2A1 and SLC2A2 expressions correlated with immune marker genes in HCC by using TIMER and GEPIA databases**

To further understand the relationship between SLC2A1 and SLC2A2 and various immune cells in HCC, we studied the correlation between SLC2A1 and SLC2A2 and immune marker genes of different immune cells in TIMER and GEPIA databases. These immune marker genes included CD8+ T cells, T cells (general), B cells, monocytes, TAM, M1 macrophages, M2 macrophages, neutrophils, natural killer cells, and dendritic cells. We also analyzed the different functional T cells, such as Tregs, Th1 cells, Th2 cells, Th17 cells, Th17 cells, and T cell exhaustion. After the correlation adjustment by purity, the result showed that the SLC2A1 expression was positively correlated with most immune marker genes. On the contrary, SLC2A2 was negatively correlated with these immune cells markers.
Notably, these results showed that SLC2A1 had a strong positive correlation with the immune markers of CD8+ T cells, T cells (general), and B cells, while SLC2A2 had a weak negative correlation with these markers in TIMER (Table 3) and GEPIA (Table 4) databases. These markers included CD8A, CD8B, CD3D, CD3E, CD2, CD19 and CD79A.

Next, SLC2A1 had a strong positive correlation with monocytes, TAM, M1, and M2 macrophages. And the correlation with M2 macrophage makers was stronger than that of M1 macrophage. Whereas SLC2A2 presented a negative correlation with CD86 and CD115 of monocytes and C-C motif chemokine ligand 2 (CCL2), CD68 and interleukin 10 (IL10) of TAM, and only presented a positive correlation with nitric oxide synthase 2 (iNOS) of M1 macrophage, but had no obvious or negative correlation with M2 macrophage. (Figure 8A, B, Table 3 and Table 4).

Furthermore, high expression of SLC2A1 was always accompanied by high dendritic cell marker histocompatibility complex, class II, DP beta 1 (HLA-DPB1), major histocompatibility complex, class II, DQ beta 1 (HLA-DQB1), major histocompatibility complex, class II, DR alpha (HLA-DRA), major histocompatibility complex, class II, DP alpha 1 (HLA-DPA1), neuropilin 1 (BDCA-4) and integrin subunit alpha X (CD11c), Tfh (BCL6 transcription repressor (BCL6) and interleukin 21 (IL21)), Th1 (signal transducer and activator of transcription 1 (STAT1), interferon gamma (IFN-γ) and tumor necrosis factor (TNF-α)) and Th2 (GATA binding protein 3 (GATA3), signal transducer and activator of transcription 6 (STAT6) and signal transducer and activator of transcription 5A (STAT5A)) infiltration. However, high expression SLC2A2 was always accompanied by low dendritic cell (HLA-DPB1, HLA-DQB1, HLA-DPA1, BDCA-4, and CD11c), Th1 (IFN-γ and TNF-α), and Th2 (GATA3) infiltration. (Figure 8C, D, Table 3 and Table 4).

Moreover, we also found that SLC2A1 showed a strong positive correlation with many markers of T cells exhaustion, while SLC2A2 showed a strong negative correlation with these markers. These biomarkers included PD-1, CTLA4, lymphocyte activating 3 (LAG3), hepatitis A virus cellular receptor 2 (TIM-3), and granzyme B.
(GZMB). Notably, SLC2A1 was always positively correlated with C-C motif chemokine receptor 8 (CCR8), signal transducer and activator of transcription 5B (STAT5B), and transforming growth factor beta 1 (TGF-β1) markers of Tregs, while SLC2A2 was positively correlated with forkhead box P3 (FOXP3) and signal transducer and activator of transcription 5B (STAT5B) markers of Tregs but negatively correlated with TGF-β. (Figure 8E, F, Table 3 and Table 4)

**SLC2A2 methylation correlated with immune cells in HCC by using the TISIDB database**

Previous studies have revealed that the reduced expression of SLC2A2 in HCC was due to the methylation of SLC2A2[32]. Therefore, we assessed the correlation between SLC2A2 methylation and immune cells.

These results suggested that SLC2A2 methylation was positively correlated with lymphocytes (Act CD8, Tcm CD8, Tem CD8, Act CD4, Tcm CD4, Tem CD4, Tfh, Tgd, Th1, Th17, Th2, Act B, Imm B, Mem B, NK, CD56<sup>bright</sup>, CD56<sup>dim</sup>, MDSC, NKT, Act DC, pDC, iDC, macrophage, eosinophil, mast, monocyte, neutrophil) (Figure 9A and 9B). These results were consistent with the correlation between lower SLC2A2 expression and positive immune infiltration in HCC. In conclusion, both elevated SLC2A1 and decreased SLC2A2 indicated high immune infiltration in HCC patients.

**DISCUSSION**

Several immune checkpoint inhibitors have been approved for in clinical trials in HCC. However, they were effective for only a small percentage of patients or were not effective at all[6-8]. Therefore, it is great to identify sensitive populations with immunotherapeutic drugs or search for more effective immunotherapeutic drugs to improve the survival time in HCC. In other words, knowing the immune status of patients in advance and selecting appropriate immunotherapeutic drugs was one of the methods to improve the survival rate of patients. A previous study found that lactic acid and other related substances which were produced by the Warburg effect affected
the number and function of immune cells in the tumor microenvironment\cite{15}, SLC2A1 and SLC2A2 have been confirmed to be involved in the Warburg effect of tumors as glucose transporters\cite{33}, but whether they are related to immune cells has not been reported in HCC in detail. In our study, we identified that SLC2A1 and SLC2A2 were independent target molecules for HCC, but their expressions were negatively correlated. Secondly, both high expression SLC2A1 and low expression SLC2A2 were correlated with higher levels of immune cells. Thirdly, SLC2A2 methylation was positively correlated with the levels of immune infiltration. Anyway, this study clarified that SLC2A1 and SLC2A2 were important target molecules, and it was first proposed that SLC2A1 and SLC2A2 were related to immune cells in HCC.

In our study, SLC2A1 was overexpressed while SLC2A2 was low expressed in HepG2, HepG2215 cells, and liver cancer patients. A previous study has shown that SLC2A1 was highly expressed in HepG2, Hep3B, and SK-HEP1 cells compared with normal hepatocytes\cite{34}. Another study also confirmed that SLC2A1 protein expression increased, while SLC2A2 protein expression decreased with the progression of HCC\cite{35}. In addition, the trend was becoming worse and worse with the progress of tumor volume, grade, and stage of HCC. We also analyzed the expression of SLC2A1 and SLC2A2 in other cancers, and the results showed that SLC2A1 and SLC2A2 were increased in some cancers but decreased in others compared with normal samples. This may be related to tumor heterogeneity or the different collection criteria and calculation methods in different data sets.

Interestingly, our study found that the expression levels of SLC2A1 and SLC2A2 were negatively correlated in HCC. Another study has also found that their expressions were negatively correlated at the protein level\cite{38}. One possible reason was that the SLC2A2 glucose transporter was highly reversible whereas the SLC2A1 glucose transporter was not. They had this mode of expression ensured higher glucose flux in cancer cells\cite{9}. It has been reported that cancer cells expressing oncogene KRAS proto-oncogene, GTPase (KRAS) or oncogene B-Raf proto-oncogene, serine/threonine kinase (BFAF) required SLC2A1\cite{36}, which may be one of the reasons for the SLC2A1
overexpression in HCC. And other studies have found that activation of pregnane X receptor (PXR) dysregulated SLC2A2 expression and subcellular localization in the liver\textsuperscript{[37]}. However, other reasons for increased SLC2A1 expression and decreased SLC2A2 expression in HCC are still worth exploring.

Meanwhile, we showed that high SLC2A1 and low SLC2A2 expression were positively correlated with the poor OS and RFS in HCC. Notably, high expression of SLC2A1 and low expression of SLC2A2 were more dangerous for males, Asians, non-alcoholics, infected by HBV, high stage, high grade, and high AJCC-T than that in the control group. When primary rat hepatocytes were infected with an adenoviral vector expressing portions of the HBV genome, upregulation of SLC2A1 and downregulation of SLC2A2 were observed\textsuperscript{[38]}. And Teng \textit{et al}\textsuperscript{[39]} have demonstrated that HBV Pre-S2 mutant upregulated SLC2A1 expression through the mammalian target of rapamycin/YY1 transcription factor/ MYC proto-oncogene signaling. Besides, downregulation of SLC2A2 has also been revealed in HBV replicon transfected Huh-7 cells\textsuperscript{[40]}. Other studies have also found that high SLC2A1 expression level was associated with advanced tumor stage, high tumor grade, depth of the invasion, and poor differentiation in various cancers\textsuperscript{[41-43]}. The study showed that SLC2A2 protein expression was decreased following the malignant progression of HCC\textsuperscript{[35]}. Therefore, we suggested that SLC2A1 and SLC2A2 were independent prognostic factors for HCC.

Next, we discussed that SLC2A1 and SLC2A2 and their neighbor genes were involved in pathways in the String database. Notably, genes TP53, ATM, MDM2, AKT1, HIF1A, EP300, INS, GCK, and HK2 were included. Studies have demonstrated that tumor-associated mutation P53 stimulated the Warburg effect by promoting SLC2A1 translocation to the plasma membrane\textsuperscript{[44]}. HIF1A promoted the Warburg effect of various cancers by increasing the expression of multiple glycolysis genes (such as SLC2A1 and carbonic anhydrase 9)\textsuperscript{[45]}. When these oncogenes or tumor suppressor genes functioned, they all seemed to be associated with the SLC2A1-induced Warburg effect. The study indicated that SLC2A1, SLC2A2, and their neighbors were involved in the central carbon metabolism in cancer (hsa05230), pathways in cancer (hsa 05200),
FOXO signaling pathway (hsa04068) and PI3K-Akt signaling pathway (hsa 04151), and so on. FOXOs were involved in cellular differentiation, apoptosis, cell proliferation, DNA damage and repair, and as mediators of oxidative stress\textsuperscript{[46]}. Studies reported that Akt activation, caused by disturbances of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha or phosphatase and tensin homolog, mediated increased glucose uptake and solute carrier family 2 overexpression\textsuperscript{[33]}. In conclusion, SLC2A1, SLC2A2, and their neighbor genes played an important role in the occurrence and development of tumors. Regrettably, we had not enriched some pathways associated with immunity in the String database. This may be attributed to the lack of database algorithms or the lack of studies on the relationship between glucose transporters and immune cells.

A previous study found that lactic acid and other related substances produced from the Warburg effect affected the number and function of immune cells in the tumor microenvironment\textsuperscript{[15]}. High expression of SLC2A1 in gastric cancer cells was associated with suppressing CD8\textsuperscript{+} T cells and B cells\textsuperscript{[16]}. And an inverse correlation between SLC2A1 expression and the number of CD8\textsuperscript{+} T cells in renal cell carcinoma\textsuperscript{[31]} was showed. Other studies reported that T cells, CD8\textsuperscript{+} T cells, and B cells reduced in the SLC2A1 express group of human papillomavirus type 16-positive cervical cancer\textsuperscript{[47]}. However, the relationship between SLC2A2 and immunity in tumors has rarely been reported, which may be due to its low or no expression in various cancers. These results suggested that SLC2A1 and SLC2A2 affected the number and function of immune cells in the tumor microenvironment.

In this study, the results suggested that HCC had different levels of immune invasion compared to normal tissues. Of course, immune dysregulation in HCC has long been recognized\textsuperscript{[48]}. Notably, it was presented for the first time that expressions of SLC2A1 and SLC2A2 were correlated with the infiltration levels of various immune cells and immune marker genes in HCC. Furthermore, both high SLC2A1 and low SLC2A2 expressions, as independent risk factors for HCC, pointed to a common outcome of high levels of immune cell infiltration. These results suggested that the
expression of SLC2A1 and SLC2A2 played an important role in the number and function of immune cells in HCC.

Firstly, high expression of SLC2A1 and low expression of SLC2A2 had positive correlations with infiltrating levels of B cells, CD8\(^+\) T cells, CD4\(^+\) T cells, macrophages, neutrophils in HCC.

Secondly, the expression of SLC2A1 has correlated with the expressions of monocytes markers (CD86 and CD115), TAM markers (CCL2, CD68, and IL10), M1 macrophage [interferon regulatory factor 5 (IRF5), and cytochrome c oxidase subunit II (COX2)] and M2 macrophage [CD163, V-set and immunoglobulin domain containing 4 (Vsig4) and membrane spanning 4-domains A4A (MS4A4A)]. Monocytes and macrophages were involved in tumor initiation, growth, migration, vascularization, invasion, and metastasis\(^{[49]}\). Studies have found that lactic acid produced by the Warburg effect could promote T-cell apoptosis by activating M2-TAM and regulating PD-1/PD-L1 signals\(^{[50]}\). These results indicated that SLC2A1 had potential regulatory effects on tumor-associated macrophages.

Thirdly, SLC2A1 and SLC2A2 were correlated with several dendritic cell markers, indicating that they had an important relationship with the normal function of dendritic cells. And tumor-derived lactic acid regulated the activation and antigen expression of dendritic cells\(^{[51]}\). Therefore, one of the reasons why SLC2A1 and SLC2A2 affected dendritic cell function was that they could affect cell function through the lactic acid produced by the mediated Warburg effect.

Fourthly, the expression level of SLC2A1 was also correlated with the expressions of several markers of T helper cells. They included Th1 cell makers (STAT1, IFN-\(\gamma\), and TNF-\(\alpha\)), Th2 cell markers (GATA3, STAT6, and STAT5A), and Tfh cell makers (BCL6 and IL21). These results indicated that SLC2A1 expression level played an important role in the function of Th1 cells, Th2 cells, and Tfh cells.

Notably, the results revealed that high expression of SLC2A1 and low expression of SLC2A2 had positive correlations with Treg marker transforming growth factor-beta (TGF\(\beta\)) and T cell exhaustion markers (PD-1, CTLA4, LAG3, TIM-3, and GZMB).
Furthermore, high expression of SLC2A1 and low expression of SLC2A2 had the potential to induce T cell exhaustion. The PD-1/PD-L1 axis inhibited T cell activation, proliferation, and survival, and cytotoxic secretion in tumors\cite{52}. CTLA4 interacted with its ligand to inhibit T cell activation, but the precise mechanism was not fully understood\cite{53}. Increased TGFβ caused T cell exhaustion by upregulation of PD-1, and inhibiting TGFβ might directly enhance antitumor immunity in HCC\cite{54}. High expression of TIM-3 indicated T cell exhaustion\cite{55}, which together with Tregs supported exhausted CD8+ T cell development and limiting the expansion of CD4+ and CD8+ T cells\cite{56}. TIM-3 expression inhibited glucose uptake, glucose consumption, and lactic acid release. Concomitantly, TIM-3 expression inhibited SLC2A1 expression but not SLC2A2 expression in Jurkat T cells\cite{57}.

Notably, previous studies have found that the reduced expression of SLC2A2 in HCC was due to the methylation of SLC2A2\cite{52} and we analyzed the correlation between SLC2A2 methylation and immune cells. These results suggested that SLC2A2 methylation or low expression SLC2A2 was positively correlated with immune cells. However, larger studies are required to further elucidate the relationship between expressions of SLC2A1 and SLC2A2 and the immune microenvironment.

**CONCLUSION**

Collectively, SLC2A1 and SLC2A2 were independent therapeutic targets for HCC. Then, both high SLC2A1 and low SLC2A2 expression pointed to a common outcome of high levels of immune cell infiltration. However, larger studies are required to clarify the preliminary findings.
<table>
<thead>
<tr>
<th>#</th>
<th>Source</th>
<th>Words</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>res.mdpi.com</td>
<td>96</td>
<td>2%</td>
</tr>
<tr>
<td>2</td>
<td>downloads.hindawi.com</td>
<td>82</td>
<td>1%</td>
</tr>
<tr>
<td>3</td>
<td>Yue-tao Zhou, Hong Chen, Min Ai, Shuang-shuang Li, Bo-yan Li, Ye Zhao, Wei-wei Cai, Bao Hou, Lu-lu Ni, Fei Xu, Li-ying Qiu. &quot;Type-1 Na+/H+ exchanger is a prognostic factor and associate with immune infiltration in liver hepatocellular carcinoma&quot;, Life Sciences, 2021</td>
<td>60</td>
<td>1%</td>
</tr>
<tr>
<td>4</td>
<td>opastonline.com</td>
<td>42</td>
<td>1%</td>
</tr>
<tr>
<td>5</td>
<td><a href="http://www.researchsquare.com">www.researchsquare.com</a></td>
<td>41</td>
<td>1%</td>
</tr>
<tr>
<td>6</td>
<td>assets.researchsquare.com</td>
<td>35</td>
<td>1%</td>
</tr>
<tr>
<td>7</td>
<td><a href="http://www.ncbi.nlm.nih.gov">www.ncbi.nlm.nih.gov</a></td>
<td>33</td>
<td>1%</td>
</tr>
</tbody>
</table>