

Role of SLC39A6 in the development and progression of liver cancer

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Abstract. Liver cancer is one of the most common malignant solid tumor types worldwide. The solute carrier (SLC)39A family is a main member of the SLC group of membrane transport proteins, which transfer zinc to the cytoplasm when cells are depleted of zinc; thus, it may provide a novel therapeutic target for human cancer. However, the prognostic value of SLC39A genes in patients with liver cancer has remained elusive. Therefore, the present study aimed to explore whether SLC39A family genes are associated with the survival rate of patients with liver cancer and to investigate the role of key genes of the SLC39A family in liver cancer. The mRNA expression of the SLC39A family in liver cancer was obtained from the UALCAN database. Survival curve analysis was performed to investigate the prognostic value of SLC39A family genes in the overall survival of patients with liver cancer. In addition to the bioinformatics analysis, SLC39A6 was knocked down in HepG2 and Hep3B cells to examine the effect on the proliferation, migration and invasion of liver cancer cells. The results suggested that SLC39A6 was significantly upregulated in liver cancer tissues compared with normal liver tissues. High expression of SLC39A6 was significantly associated with poor overall survival of patients with liver cancer. Furthermore, knockdown of SLC39A6 inhibited the proliferation, migration and invasion of liver cancer cells *in vitro* and *in vivo*. Collectively, the results of the present study suggested that SLC39A6 may be a promising prognostic biomarker for liver cancer and is associated with the proliferation, migration and invasion of liver cancer.

Introduction

Liver cancer is the fourth leading cause of cancer-related mortality and is ranked sixth in terms of cancer incidence; it is estimated that over one million individuals will succumb to the disease by 2030 (1). In the US, the 5-year survival rate for patients with liver cancer is 18.1% (2), while in China, it is only 12.1% (3). The majority of primary liver cancers are pathologically diagnosed as hepatocellular carcinoma, accounting for 70-85% of cases (4). Previous studies have reported that the majority of liver cancers occur in patients with chronic liver diseases, including hepatitis B virus or hepatitis C virus infection (5), non-alcoholic fatty liver disease (6) and alcohol abuse (7). To date, surgical excision, radiofrequency ablation, interventional embolization, liver transplantation, as well as targeted and systemic therapies have been applied in the treatment of liver cancer, which have improved the prognosis of those patients to a certain extent (8). Thus, further elucidation of the molecular mechanisms involved in the occurrence and development of liver cancer is warranted, as this may contribute to the development of novel preventive, diagnostic and therapeutic strategies for liver cancer.

Previous studies have indicated that zinc, an important nutrient (9), has a key role in organisms, including growth, development, reproduction, enzyme function, immune function, DNA repair, gene expression and endocrine function, as well as cancer biology (9-12). Under normal conditions, 98% of zinc is localized in the intracellular compartment (11). The balance of intracellular and extracellular zinc is regulated by the Zrt- and Irt-like proteins [ZIP/solute carrier (SLC)39 family, SLC39A] and the SLC30 family (SLC30A/ZnT) (13,14). The ZnT family member functions to move zinc from the inside of the cell to the outside (13), while the ZIP family member (SLC39A1-14) serves to pass zinc into the cytoplasm (14). An imbalance of zinc homeostasis may be associated with the development of a number of diseases. It has been demonstrated that zinc levels in prostate cancer cells are significantly reduced and are associated with metabolic reprogramming (15). However, increased zinc levels may also be associated with tumorigenesis and progression; for instance, SLC39A8 expression has been determined to be upregulated in early renal clear cell carcinoma (16), and SLC39A7 has been reported to have a key role in the growth and survival of breast cancer cells (17). To the best of our knowledge, the role of the SLC39A family

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in liver cancer has remained elusive. The present study performed an integrated bioinformatics analysis of several independent studies to explore the role of the SLC39A family in the development and progression of liver cancer. In addition, the function of SLC39A6, a key gene of the SLC39A family, in liver cancer was also investigated.

Materials and methods

UALCAN database. UALCAN is a public web portal that provides comprehensive tumor transcriptome data from The Cancer Genome Atlas (TCGA) (<http://ualcan.path.uab.edu/>). The relative mRNA expression of SLC39A in liver cancer and corresponding normal tissues was detected using the UALCAN database.

Gene expression profiling interactive analysis (GEPIA) database. The mRNA expression data of SLC39A family genes in liver cancer tissues, as well as the clinicopathological and survival data of patients with liver cancer were downloaded from the University of California Santa Cruz (UCSC) Xena browser (<https://xenabrowser.net/>). To determine the potential prognostic values of SLC39A family genes in patients with liver cancer, the association between SLC39A mRNA expression and the overall survival (OS) of patients with liver cancer was analyzed using the GEPIA database (<http://gepia.cancer-pku.cn/>) or by GraphPad Prism 6.0 (GraphPad Software, Inc.). The association of SLC39A family genes with disease-free survival (DFS) was also analyzed. Furthermore, the prognostic value of SLC39A6 mRNA expression in patients with different disease grades of liver cancer was also analyzed.

Oncomine database. Oncomine is a cancer microarray database to facilitate discovery from genome-wide expression analysis data (<https://www.oncomine.org/resource/main.html>). The relative expression of SLC39A6 in the Mas (18), Chen (19) and Roessler (20) liver cancer and normal samples were downloaded from the Oncomine database.

Gene set enrichment analysis (GSEA). To identify the hallmark effect gene sets associated with SLC39A6 mRNA expression in the TCGA-Liver Hepatocellular Carcinoma dataset, GSEA was performed using GSEA software (v. 4.1.0; <http://www.gsea-msigdb.org/gsea/login.jsp>). For enriched genomes, pathways with false discovery rate values <0.25 and $P < 0.05$ were considered after 1,000 permutations as significantly enriched pathways.

Tissue samples. The study population included 12 patients who had been diagnosed with liver cancer (T3/T4) composed of 6 males and 6 females with a median age of 63.93 years (range, 56-78 years) between March 2021 and June 2021 at the Department of General Surgery, The First Affiliated Hospital of Nanchang University (Nanchang, China). Patients or their families who refused to provide liver cancer specimens were excluded. The present study was approved by the Human Research Ethics Committee of Nanchang University (no. 2021-027) and all the patients were fully informed of the study procedures orally and signed an informed consent form. The tumor tissues and normal tissues (~5 cm away from tumor

tissue) were collected and then either immediately frozen in liquid nitrogen or fixed in formalin after the surgery.

Histological analysis. Three pairs of tumors and healthy tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. The sections were immunohistochemically (IHC) stained with SLC39A6 rabbit polyclonal antibody (cat. no. 14236-1-AP; 1:200 dilution; Proteintech Group, Inc.) and observed using microscopy (IX71; Olympus Corporation). IHC scores (21) were used to evaluate the expression level of SLC39A6. SLC39A6 staining intensity was scored as 0-3 points (0, negative; 1, weak; 2, mild; 3, strong). The percentage score of SLC39A6-positive cells was as follows: 0, unstained; 1, 1-25; 2, 26-50; 3, 51-75; 4, 76-100% stained cells. The staining intensity score was then multiplied with the positive cell proportion score to obtain the final score.

Cells and cell culture. The human HepG2 and Hep3B liver cancer cell lines were obtained from Procell Life Science & Technology Co., Ltd., and cultured in high-glucose DMEM (cat. no. SH30022.01) containing 10% FBS (cat. no. SH30084.03) and 1% penicillin-streptomycin (cat. no. SV30010; all from HyClone; Cytiva) in an incubator with 5% CO₂ at 37°C. The authenticity of the cell lines was confirmed using short tandem repeat methods.

Cell transfection. The human SLC39A6 small interfering (si) RNA expression vector (si-SLC39A6) and corresponding negative control siRNA (si-NC) were synthesized and provided by Vigene Biosciences, Inc. The HepG2 and Hep3B cells were transfected with si-SLC39A6 and si-NC using Lipofectamine 2000[®] according to the manufacturer's protocol. The cells were collected at 48 h following transfection for use in subsequent experiments. Short hairpin (sh)RNA for SLC39A6 (Shanghai Genechem Co., Ltd.) and the corresponding control vector (Shanghai Genechem Co., Ltd.) were transfected into the HepG2 cells at a multiplicity of infection of 20 using enhanced infection solution (Shanghai Genechem Co., Ltd.) according to the manufacturer's protocol. The plasmid backbone (GV493) for shRNA-containing plasmid was provided by Shanghai Genechem Co., Ltd. Protein lysates and total RNA were collected 96 h following transfection to verify the transfection efficiency and for use in subsequent animal experiments. The sequences were as follows: si-SLC39A6, 5'-UUGCAUUGCUGGUUCUUCUAUGGCUA-3' and si-NC, 5'-CGCTTCGCGGCCCGTTCAA-3'; shRNA for SLC39A6, 5'-UUCCAUUGCUGGUUCUUCUAUGGCUA-3' and the respective NC-shRNA, 5'-CGCTTCCGCGGCCCGTTCAA-3'.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from 12 pairs of tumor and normal tissue samples from patients with liver cancer and liver cancer cell lines (HepG2 and Hep3B) using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse-transcribed into cDNA according to the manufacturer's protocol using an RT kit (Vazyme Biotech Co., Ltd.) at 95°C for 5 min and 65°C for 60 min, and then kept at 4°C for storage. qPCR was performed using SYBR-Green qPCR Master Mix (Vazyme Biotech Co., Ltd.). The qPCR conditions were used as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 sec,

annealing at 52°C for 30 sec and extension at 72°C for 30 sec. Each sample was repeated in triplicate in qPCR step. The relative mRNA levels were normalized to β -actin. The primer sequences were as follows: SLC39A6 forward, 5'-GCCGCGGAGACTGTTTCAAT-3' and reverse, 3'-TGTTGCATTCAGCGGAACCT-5'; and β -actin forward, 5'-GGCTCTTTTCCAGCCTTCCT-3' and reverse, 3'-AATGCCAGGGTACATGGTGG-5'. The relative gene expression level was analyzed by the $2^{-\Delta\Delta C_q}$ method (22).

Western blot analysis. Proteins were extracted from tissues from patients with liver cancer and cell lines (HepG2 and Hep3B) using radioimmunoprecipitation assay buffer containing protease inhibitor. The protein concentration was determined using a bicinchoninic acid assay. For western blot analysis, 50 μ g of protein per lane was separated by 15% SDS-PAGE and transferred onto nitrocellulose membranes (cat. no. FFN08; Beyotime Institute of Biotechnology). The membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated with polyclonal primary antibodies [SLC39A6 rabbit polyclonal antibody (cat. no. 14236-1-AP; 1:1,000 diluted; Proteintech Group, Inc.) and β -actin mouse monoclonal antibody (cat. no. 66009-1-Ig; 1:5,000 diluted; Proteintech Group, Inc.)] in Tris-buffered saline containing Tween-20 (TBST) buffer overnight at 4°C. The membranes were washed with TBST solution three times and incubated with secondary antibody [HRP-conjugated Affinipure goat anti-rabbit IgG(H+L); cat. no. SA00001-2; 1:5,000 diluted; Proteintech Group, Inc.] for 1 h at room temperature. The target protein was visualized using enhanced chemiluminescence reagent (cat. no. 21050; Thermo Fisher Scientific, Inc.).

Colony formation assay. The HepG2 and Hep3B cells transfected with si-SLC39A6 or si-NC were seeded in 6-well plates at a concentration of 1,000 cells per well in 3 ml complete medium. Following incubation at 37°C for 9 days, the cell colonies were fixed with 4% paraformaldehyde for 15 min, stained with 0.04% crystal violet for 20 min at room temperature and images were acquired.

Cell proliferation assay. Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8) assay (cat. no. HY-K030; MedChemExpress). HepG2 and Hep3B cells transfected with si-SLC39A6 or si-NC were seeded in 96-well plates at a concentration of 1,000 cells per well in 100 μ l complete medium. After culturing the cells for 1-4 days, the medium was removed and 10 μ l CCK-8 stain was added per well. The absorbance at a wavelength of 450 nm was measured following incubation for 1 h using an automated microplate reader (Infinite F50; Tecan Group).

In vitro migration and invasion assays. *In vitro* migration and invasion assays were performed using 24-well Transwell chambers with 8- μ m pore size membranes. For the migration assay, the HepG2 and Hep3B cells transfected with si-SLC39A6 and si-NC were seeded in the upper chamber at a concentration of 2×10^4 cells per well in 200 μ l FBS-free medium, while 500 μ l DMEM with 10% FBS was added to the lower chamber. Following incubation at 37°C for 36 h, the cells on the upper surface of the filters were removed and the cells that had migrated to the lower surface were fixed in 4% paraformaldehyde, subsequently stained with

0.04% crystal violet for 20 min at room temperature and counted under a microscope (IX71; Olympus Corporation). For the invasion assay, the procedure was repeated as described above, but the membrane was initially coated with Matrigel® at 4°C.

Animal experiment. A total of 2×10^6 HepG2 cells transfected with shRNA for SLC39A6 or the corresponding control vector were subcutaneously injected into the right infra-axillary dermis of 8-week-old female nude mice (n=5 per group; the total number of the animals was 10 and the weight was 20.23 ± 1.13 g). All mice were provided by GemPharmatech Co., Ltd., and housed in a specific pathogen-free facility (temperature, 18-29°C; relative humidity, 50-80%). Tumor volumes were measured using a digital caliper every 3 days to access tumor growth and the tumor weight was measured when the mice were sacrificed on day 30 following cell implantation. The mice were anesthetized with 1% pentobarbital sodium at 50 mg/kg body weight by intraperitoneal injection and sacrificed by cervical dislocation. Tumor sizes were measured every 3 days, commencing at 6 days after the cells were implanted, for a total of nine times. According to previous authoritative studies (23-26), when the tumor volumes reached 2,000 mm³, the mice underwent euthanasia as the humane end-point of the study. The mean diameter did not exceed 1.5 cm. In addition, if body weight loss exceeded 20%, the animals became moribund or the subcutaneous tumor reached a volume of 2,000 mm³ or became necrotic, humane endpoints were considered to have been reached in the present study (25-28). All the animal experiments were performed in accordance with the guidelines provided by the UK Animals (Scientific Procedures) Act, 1986, the EU Directive 2010/63/EU and approved by the Ethics Committee for Animal Experiments of Nanchang Royo Biotech Co. Ltd., (no. RYE2021041402).

Statistical analysis. Statistical analysis was performed using SPSS 21.0 software (IBM Corp.) and GraphPad Prism 6.0 (GraphPad Software, Inc.). Values are expressed as the mean \pm standard deviation. All comparisons between the two groups were performed using unpaired Student's t-tests. For the two paired-group comparisons, the paired Student's t-test was used. The survival curves were compared by the log-rank test, while two-stage log-rank analysis (29) was used for those survival plots where late-stage crossover between the groups was present. Based on literature reports (29), P1 represents the P-value prior to curve crossing, P2 represents the P-value after curve crossing and P represents the P-value for the whole. Univariate and multivariate analyses of OS and DFS according to prognostic factors were analyzed using Cox regression analysis. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Relative mRNA expression of SLC39A family genes in liver cancer. To examine the expression levels of SLC39A family genes in liver cancer, the expression data of 14 SLC39A genes in patients with liver cancer from the TCGA database were analyzed using the UALCAN database. As presented in Fig. 1A-N, the expression of SLC39A1, SLC39A3,

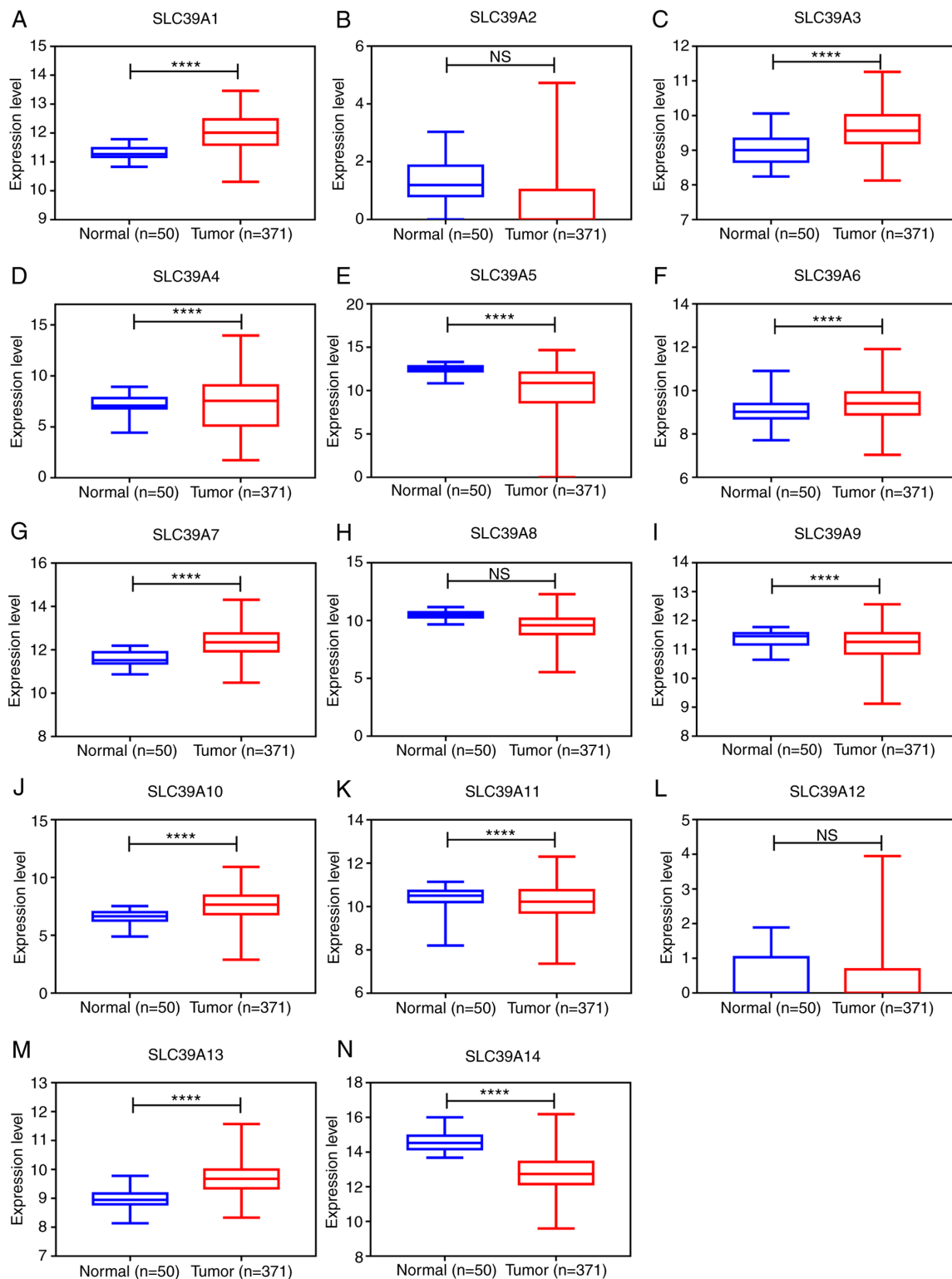


Figure 1. mRNA expression of SLC39A family genes in primary tumor and corresponding normal tissues in patients with liver cancer using the dataset from the UALCAN database. (A) SLC39A1, (B) SLC39A2, (C) SLC39A3, (D) SLC39A4, (E) SLC39A5, (F) SLC39A6, (G) SLC39A7, (H) SLC39A8, (I) SLC39A9, (J) SLC39A10, (K) SLC39A11, (L) SLC39A12, (M) SLC39A13 and (N) SLC39A14. **** $P < 0.0001$. SLC, solute carrier; NS, no significance.

SLC39A4, SLC39A6, SLC39A7, SLC39A10 and SLC39A13 in the cancer tissues was significantly higher than that in healthy tissues; however, SLC39A5, SLC39A9, SLC39A11 and SLC39A14 were expressed at markedly lower levels in the tumor tissues compared with the normal tissues. The expression of SLC39A2, SLC39A8 and SLC39A12

exhibited no significant difference between cancer and normal tissues.

SLC39A genes have a high prognostic value in patients with liver cancer in the GEPIA dataset. The potential prognostic values of SLC39A family genes in liver cancer were investigated

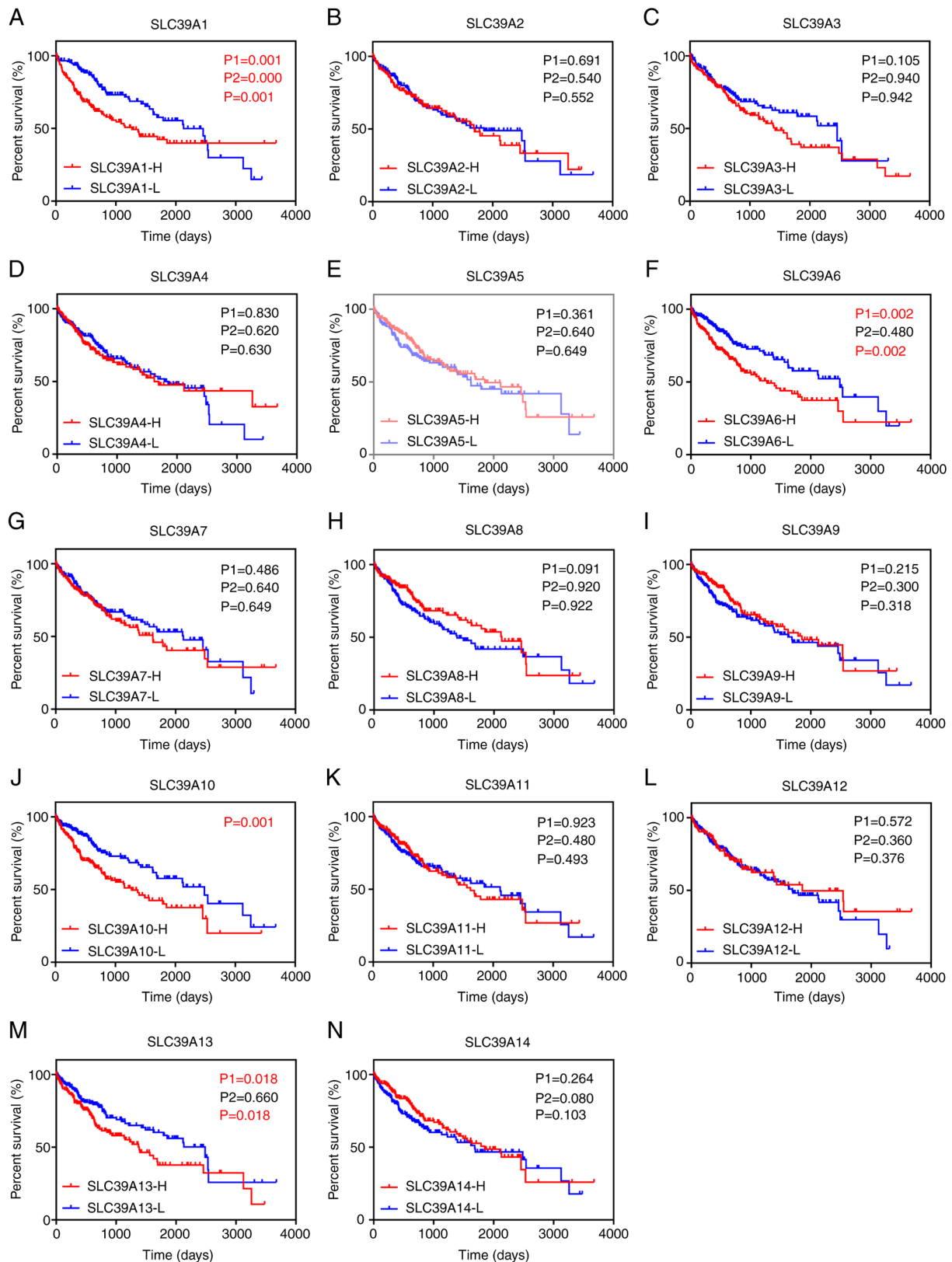


Figure 2. Association of SLC39A family genes with overall survival of patients with liver cancer based on data from the Gene Expression Profiling Interactive Analysis database. (A) SLC39A1, (B) SLC39A2, (C) SLC39A3, (D) SLC39A4, (E) SLC39A5, (F) SLC39A6, (G) SLC39A7, (H) SLC39A8, (I) SLC39A9, (J) SLC39A10, (K) SLC39A11, (L) SLC39A12, (M) SLC39A13 and (N) SLC39A14. P1 and P2 represent the P-value prior to and after curve crossing, respectively, and P represents the P-value for the whole graphs. P<0.05 was considered to indicate statistical significance. SLC, solute carrier; H, high; L, low.

using the GEPIA database. As presented in Fig. 2A-N, positive associations were observed between high expression of several genes and a significantly unfavorable OS in patients with

liver cancer, including SLC39A1, SLC39A6, SLC39A10 and SLC39A13. However, no positive associations were observed between high expression of genes and a significantly poorer

Table I. Univariate and multivariate analyses of the influence of SLC39A mRNA levels and other factors on patient overall survival.

Variable	Univariate analysis			Multivariate analysis		
	HR	95%CI	P-value	HR	95%CI	P-value
SLC39A1	1.799	1.266-2.556	0.001	1.462	1.011-2.112	0.043
SLC39A2	1.072	0.760-1.514	0.691			
SLC39A3	1.334	0.940-1.893	0.107			
SLC39A4	1.039	0.735-1.468	0.830			
SLC39A5	0.852	0.603-1.202	0.361			
SLC39A6	1.736	1.222-2.465	0.002	1.505	1.029-2.203	0.035
SLC39A7	1.130	0.801-1.595	0.487			
SLC39A8	0.742	0.525-1.050	0.092			
SLC39A9	0.216	1.568-1.136	0.216			
SLC39A10	1.801	1.265-2.565	0.001	1.341	0.908-1.983	0.141
SLC39A11	0.983	0.697-1.388	0.923			
SLC39A12	0.899	0.621-1.301	0.572			
SLC39A13	1.518	1.072-2.149	0.019	1.429	1.000-2.041	0.050
SLC39A14	0.822	0.582-1.161	0.265			
Age	1.186	0.836-1.683	0.339			
Sex	0.800	0.562-1.141	0.218			
T stage	1.682	1.403-2.017	<0.001	0.558	0.064-4.870	0.597
N stage	2.012	0.493-8.212	0.330			
M stage	1.055	1.274-12.906	0.018	1.475	0.281-7.740	0.646
TNM stage	1.670	1.361-2.048	<0.001	0.245	0.032-1.845	0.172
Grade	1.114	0.881-1.408	0.368			
Recurrence	1.610	1.099-2.357	0.014	0.640	0.392-1.043	0.073

Cutoff high, 50%; cutoff low, 50%. HR, hazard ratio; CI, confidence interval; TNM, Tumor Nodes Metastasis; SLC, solute carrier.

DFS (Fig. S1A-N). Furthermore, univariate and multivariate analyses of OS and DFS were performed to identify key genes of the SLC39A family in patients with liver cancer. The results suggested that SLC39A1, SLC39A6 or SLC39A13 overexpression was an influencing factor for poor prognosis of patients with liver cancer and may thus be a potential prognostic factor for liver cancer (Tables I and II). In this study, SLC39A6 was selected for follow-up experiments.

Association between the expression of SLC39A6 and the prognosis of patients with liver cancer. The Oncomine database and UCSC Xena browser were used to analyze the sequencing data of SLC39A6 in liver cancer. As presented in Fig. 3A-D, the mRNA expression levels of SLC39A6 were significantly higher in the tumor vs. normal tissues in the datasets by Mas *et al* (18), Chen *et al* (19) and Roessler *et al* (20). The expression levels of SLC39A6 in patients with different stages of liver cancer were also evaluated (Fig. 3E and F). The results revealed that increased expression of SLC39A6 mainly occurred in the late stages of liver cancer, while its expression level remained unaltered in patients with early-stage liver cancer.

Furthermore, survival curve analysis indicated that high expression of SLC39A6 was associated with markedly shorter OS and DFS of patients with different stages of liver cancer (Fig. 3G-L). These results suggested that the expression levels

of SLC39A6 had a good diagnostic value for patients with liver cancer.

Protein expression and biological function of SLC39A6 in patients with liver cancer. To investigate the role of SLC39A6 in the progression of liver cancer, the mRNA and protein expression of SLC39A6 in T3 liver cancer tissues was analyzed using RT-qPCR, western blot analysis and IHC. The results revealed that SLC39A6 mRNA and protein expression in the tumor tissues was markedly higher than that in the corresponding non-cancerous tissues, indicating that SLC39A6 is an important target for liver cancer (Figs. 3M-O, 4A and S2A). GSEA was performed to evaluate the Kyoto Encyclopedia of Genes and Genomes and Gene Ontology gene sets in patients with liver cancer (Fig. 4B). The results revealed that the group with high expression of SLC39A6 was significantly enriched in gene sets associated with liver cancer, tumor adhesion and the tumor metastasis pathway. Thus, these results demonstrated that the expression of SLC39A6 was significantly upregulated in liver cancer tumor tissues and that it was associated with the tumor metastasis pathway.

Downregulation of SLC39A6 suppresses liver cancer cell growth, migration and invasion. To analyze the function of SLC39A6 in the biological behavior of liver cancer cells, HepG2

Table II. Univariate and multivariate analyses of the influence of SLC39A mRNA levels and other factors on patient progression-free interval.

Variable	Univariate analysis			Multivariate analysis		
	HR	95%CI	P-value	HR	95%CI	P-value
SLC39A1	1.096	0.819-1.466	0.539			
SLC39A2	0.868	0.647-1.165	0.347			
SLC39A3	0.911	0.680-1.219	0.530			
SLC39A4	0.828	0.618-1.110	0.207			
SLC39A5	0.932	0.697-1.247	0.637			
SLC39A6	1.279	0.956-1.712	0.098			
SLC39A7	0.941	0.703-1.260	0.685			
SLC39A8	0.918	0.686-1.229	0.567			
SLC39A9	1.020	0.762-1.366	0.895			
SLC39A10	1.347	1.006-1.803	0.046	1.295	0.907-1.850	0.155
SLC39A11	0.859	0.641-1.151	0.309			
SLC39A12	0.837	0.611-1.145	0.264			
SLC39A13	1.083	0.809-1.449	0.593			
SLC39A14	0.875	0.654-1.170	0.368			
Age	1.011	0.755-1.354	0.941			
Sex	0.979	0.718-1.334	0.893			
T stage	1.622	1.391-1.892	<0.001	1.284	0.588-2.805	0.530
N stage	1.379	0.340-5.586	0.653			
M stage	3.460	1.086-11.025	0.036	1.094	0.315-3.795	0.888
TNM stage	1.660	1.397-1.973	<0.001	1.100	0.485-2.497	0.820
Grade	1.103	0.908-1.340	0.322			
Recurrence	23.799	12.902-43.900	<0.001	40.509	16.469-99.640	<0.001

Cutoff high, 50%; cutoff low, 50%. HR, hazard ratio; CI, confidence interval; TNM, Tumor Nodes Metastasis; SLC, solute carrier.

and Hep3B cells were transfected with si-SLC39A6 to down-regulate the expression of SLC39A6. HepG2 and Hep3B were the most commonly used cell lines to study liver cancer (30-34), so these cell lines were selected for the present study. In addition, the Cancer Cell Line Encyclopedia database (35-38) was checked and the results indicated that HepG2 and Hep3B were the liver cancer cell lines that expressed high SLC39A6. Thus, it was considered appropriate to select these two cell lines to study SLC39A6. As presented in Figs. 5A and S2B, the mRNA and protein expression of SLC39A6 was significantly decreased in the si-SLC39A6-transfected HepG2 and Hep3B cells. To determine whether SLC39A6 was essential for liver cancer cell proliferation, colony formation and CCK-8 assays were performed. In the colony formation assay, the colonies of the si-SLC39A6-transfected HepG2 and Hep3B cells were much smaller than those of the control cells (Figs. 5B and S2C). The results of the CCK-8 assay also demonstrated that the growth of the si-SLC39A6-transfected HepG2 and Hep3B cells was significantly inhibited compared with that of the control cells (Fig. 5C).

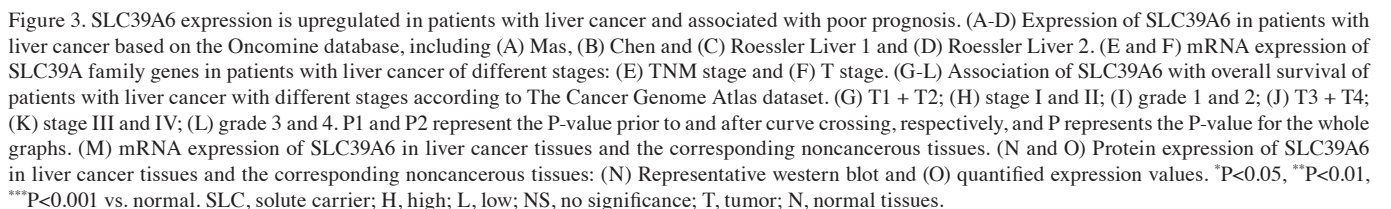
Furthermore, the effects of SLC39A6 on the migration and invasion of liver cancer cells were investigated. As presented in Fig. 5D, the migratory and invasive ability of the si-SLC39A6-transfected HepG2 and Hep3B cells was significantly decreased compared with that of the control

cells. These results revealed that knockdown of SLC39A6 inhibited the proliferation, migration and invasion of liver cancer cells.

Knockdown of SLC39A6 significantly suppresses liver cancer progression in vivo. In order to further verify the aforementioned observations, the role of SLC39A6 in the occurrence and development of liver cancer was investigated *in vivo*. As presented in Fig. S2D, the prognosis of patients with liver cancer was not significantly associated with sex. Furthermore, for the animal experiment, only female mice were available. HepG2 cells transfected with shRNA targeting SLC39A6 or the corresponding control vector were implanted subcutaneously into nude mice. The tumor size was strictly controlled to remain <2,000 mm³ and the maximum percentage of body weight loss was 8.65% (Figs. 6A and S2E), fully complying with the ethical animal standards. The maximum tumor size observed was 1,648.70 mm³. The present results demonstrated that knockdown of SLC39A6 significantly reduced both the volume and weight of the tumors *in vivo* (Fig. 6A-C).

Discussion

Liver cancer is one of the most common malignant solid tumor types worldwide, with >42,000 new cases and 30,000



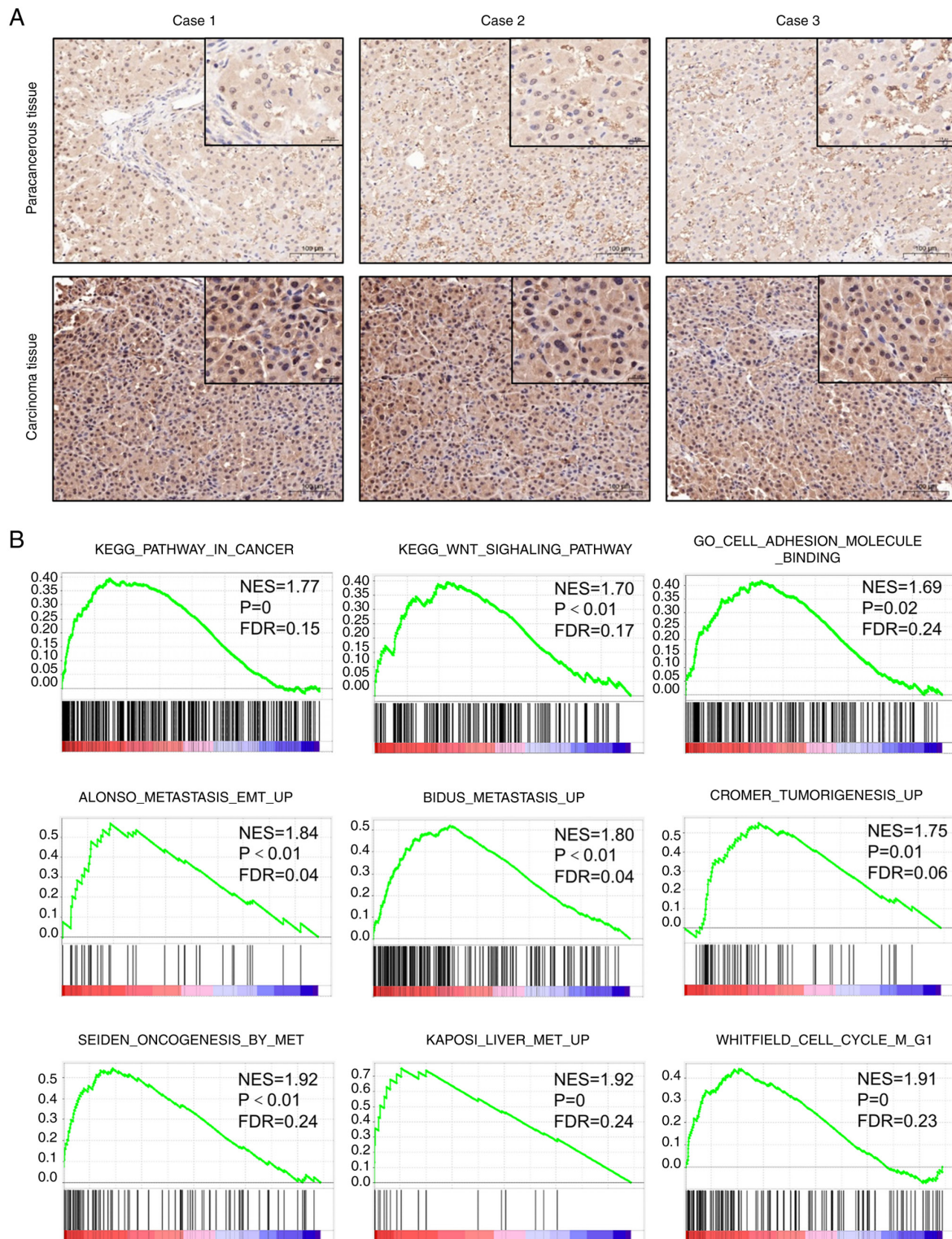


Figure 4. Expression and biological function of SLC39A6 in patients with liver cancer. (A) Typical immunohistochemical images of SLC39A6 expression in liver cancer tissues and the corresponding noncancerous tissues in three representative cases (magnification and scale bar, x100 and 100 μ m, or x400 and 20 μ m in the magnified windows, respectively). (B) The liver cancer, tumor adhesion and tumor metastasis pathway were highly enriched in patients with liver cancer with SLC39A6 high expression according to gene set enrichment analysis of The Cancer Genome Atlas Liver Hepatocellular Carcinoma dataset. SLC, solute carrier; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; EMT, epithelial-to-mesenchymal transition; NES, normalized enrichment score.

associated mortalities in 2020 (39). Among the primary cancers of the liver, hepatocellular carcinoma is the main histological subtype, accounting for 70–85% of the total cases (40). Although a variety of molecular changes have been detected

over the past years (41–44), to the best of our knowledge, no sensitive and specific biomarkers and no accurate indicators are yet available for the early diagnosis or for predicting the prognosis of patients with liver cancer. Therefore, the early

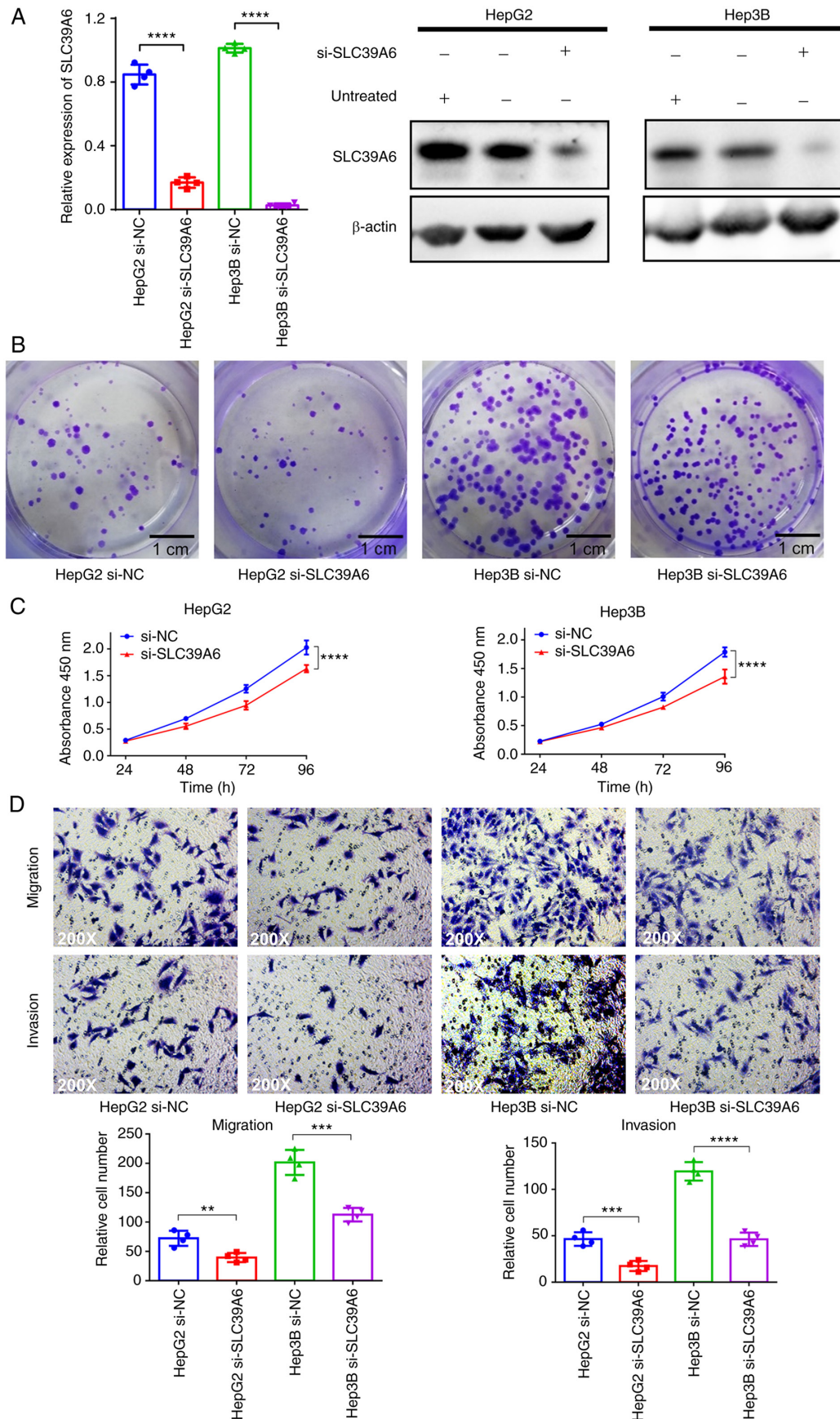


Figure 5. Knockdown of SLC39A6 inhibits liver cancer cell growth, migration and invasion *in vitro*. (A) Reverse transcription-quantitative PCR and western blot analyses of HepG2 and Hep3B cells transfected with si-NC and si-SLC39A6 were used to confirm knockdown. (B) Knockdown of SLC39A6 inhibited HepG2 and Hep3B cell growth in colony formation assays. Scale bar, 1 cm. (C) Knockdown of SLC39A6 inhibited HepG2 and Hep3B cell growth in Cell Counting Kit-8 assays. (D) Images of migration and invasion assays of SLC39A6 knockdown-HepG2 and Hep3B cells. Magnification, x200. The data in each group are presented as the mean \pm standard deviation. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. SLC, solute carrier; si-SLC39A6, small inhibitory RNA targeting SLC39A6; si-NC, negative control small inhibitory RNA.

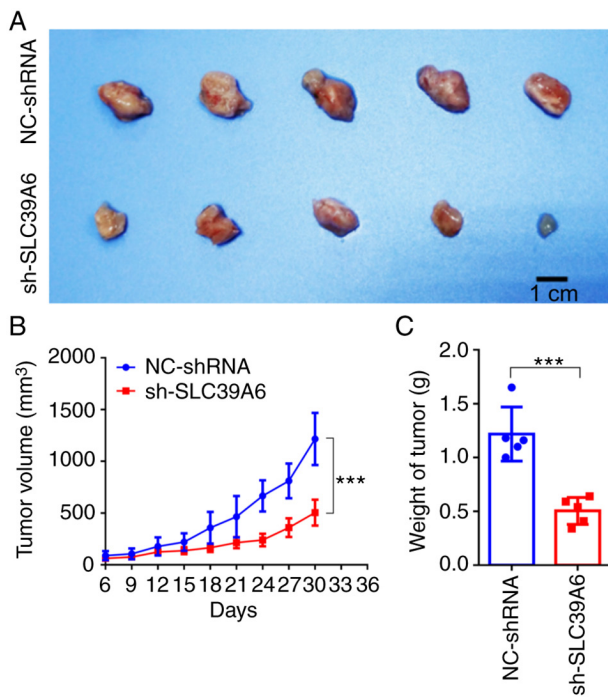


Figure 6. Knockdown of SLC39A6 significantly represses liver cancer growth in mice. (A) Images of tumors dissected from the mice. Scale bar, 1 cm. (B) Curve of tumor volume (mm³) over time. (C) Weight of tumors taken from the mice. Values are expressed as the mean \pm standard deviation for separate tumors of each group. *** $P < 0.001$ vs. NC-shRNA. SLC, solute carrier; sh-SLC39A6, short hairpin RNA targeting SLC39A6; NC, negative control.

detection of liver cancer is of utmost importance in order to improve the prognosis of patients with liver cancer.

Zinc usually has an important role in tumor events and low levels of zinc have been detected in serum and tumor tissues of patients with cancer (45,46). SLC39A6 is a subfamily member of zinc transporters, which is involved in maintaining the intracellular homeostasis of zinc (47-49). Previous studies have demonstrated that SLC39A6 is overexpressed in breast (50,51), pancreatic and cervical cancer (52,53), as well as in esophageal squamous cell carcinoma (54,55). In liver cancer, a previous study indicated that miR-192 was a prognostic indicator, reducing liver cancer metastasis through the SLC39A6/SNAIL pathway (56). However, whether SLC39A6 expression is an independent prognostic factor for patients with liver cancer warrants further exploration.

In the present study, the mRNA expression of SLC39A family genes in liver cancer tissues and their association with OS of patients with liver cancer was analyzed. The results revealed that the expression levels of SLC39A1, SLC39A3, SLC39A4, SLC39A6, SLC39A7, SLC39A10 and SLC39A13 were significantly higher in the tumor compared with healthy tissues, while SLC39A5, SLC39A9, SLC39A11 and SLC39A14 were expressed at higher levels in healthy tissues. The results demonstrated that high expression of SLC39A1, SLC39A6, SLC39A10 and SLC39A12 was associated with unfavorable OS of patients with liver cancer. The expression of SLC39A members was associated with the prognosis of specific patients with liver cancer by subgroup analysis. In addition, Cox regression analysis confirmed that SLC39A6 was a key

gene influencing the survival of patients with liver cancer. All of these results indicated that SLC39A6 may be a biomarker for the early detection and prognosis of liver cancer.

As metastasis is the main cause of liver cancer-related mortality, the identification of new genes involved in this process is crucial in order to elucidate the molecular mechanism of liver cancer metastasis (57,58). The present study observed the upregulation of SLC39A6 expression in liver cancer tumor tissues and cell lines. GSEA also revealed that SLC39A6 was associated with liver cancer tumor proliferation and metastasis.

In recent years, studies have indicated that SLC39A6 has an important role in tumor progression, metastasis and invasion. With regard to breast cancer, the expression of SLC39A6 has been indicated to be significantly increased compared with that in normal breast tissue and the expression of the adhesion protein, E-cadherin, has been reported to be significantly decreased following overexpression of SLC39A6. It was thus revealed that SLC39A6 may promote epithelial-mesenchymal transition (EMT) in breast cancer (59). Furthermore, it was reported that SLC39A6 is activated by STAT3 in breast tumor cells, triggering the influx of zinc ions and promoting Snail to remain in the nucleus as an E-cadherin transcription inhibitor, thus promoting the migration process. Therefore, SLC39A6 has been proposed as a novel target for breast cancer (60). In addition, the expression of SLC39A6 has been indicated to be markedly higher in pancreatic tumors than in normal pancreatic tissues. Furthermore, the proliferative and migratory ability of pancreatic cancer cells has been indicated to decrease significantly following SLC39A6 knockdown (61). Another study demonstrated that SLC39A6 enhanced the invasive phenotype by inducing the EMT of pancreatic cancer cells (52). With regard to non-small cell lung cancer, knockdown of SLC39A6 has been reported to significantly inhibit the proliferation, migration and invasion of lung cancer cells and to induce cell cycle arrest in G1 phase (62). These results indicate that SLC39A6 promotes the development of multiple tumor types, which is consistent with the findings of the present study on liver cancer.

In the present study, in order to verify whether SLC39A6 promotes the proliferation, migration and invasion of liver cancer cells, the expression of SLC39A6 in HepG2 and Hep3B cells was knocked down. The results of a CCK-8 assay indicated that knockdown of SLC39A6 significantly decreased the viability and proliferation of HepG2 and Hep3B cells. Furthermore, in the Transwell assays, it was observed that knockdown of SLC39A6 significantly reduced the number of migrating and invading HepG2 and Hep3B cells. These results demonstrated that SLC39A6 significantly regulated the proliferation, migration and invasion of liver cancer cells, thus regulating the malignant progression of liver cancer. Of note, *in vivo*, it was demonstrated that the knockdown of SLC39A6 significantly inhibited the progression of liver cancer.

In conclusion, in the present study, SLC39A6 was identified as a key gene of the SLC39A family in liver cancer. The results demonstrated that SLC39A6 was upregulated in liver cancer, indicating that it may be used as a potential biomarker for the diagnosis of liver cancer. Furthermore, knockdown of SLC39A6 expression significantly reduced the proliferation, migration and invasion of HepG2 and Hep3B cells *in vitro*

and also significantly suppressed liver cancer growth *in vivo*. Collectively, the results of the present study demonstrated that SLC39A6 may have a crucial tumor-promoting role in liver cancer and may exhibit the potential for use as a therapeutic target for liver cancer.

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Availability of data and materials

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

Authors' contributions

ZW participated in the conception and design of the study. ZW and XW performed the experiments, data analysis and interpretation. ZW provided the resources. ZW and XW wrote the manuscript. ZW and XW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Human Research Ethics Committee of Nanchang University (Nanchang, China; approval no. 2021-027). All of the patients were fully informed of the study procedures orally and signed an informed consent form.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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