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Original Article

Low *SLC29A1* expression is associated with poor prognosis in patients with hepatocellular carcinoma

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Abstract: Overall survival of patients with hepatocellular carcinoma (HCC) remains poor, and the multidrug resistance of HCC cells contributes to the limited efficacy of anti-cancer drugs, and reduced time to recurrence. We systematically screened the expression of transporter genes in HCC samples and found that solute carrier family 29 member A1 (*SLC29A1*) expression was significantly elevated in human HCC cells compared with para-carcinoma cell samples. The results of tissues microarray showed that *SLC29A1* was an independent prognostic factor for overall survival and tumor recurrence, especially for patients with AFP ≤ 20 ng/ml, no microvascular invasion and early staging. In vivo and vitro analyses showed that down-regulation of SLC29A1 expression could enhance tumor cell proliferation, invasion and reduced drug sensitivity. Further microarray-based gene expression profile indicated that low *SLC29A1* expression may contribute to HCC progression by promoting the epithelial-mesenchymal transition through zinc finger E-box binding homeobox 2 and transforming growth factor beta receptor activation, modifying cell adhesion through up- or down-regulation of cell adhesion molecules, and activating the nuclear factor-kappaB pathway through tripartite motif-containing protein 9 inhibition. In conclusion, low *SLC29A1* expression correlated with high recurrence risk and poor outcomes for patients with HCC after surgery. *SLC29A1* might be a promising prognostic factor, a potential tumor suppressor, and a drug sensitizer for patients with HCC through its interaction with various signaling pathways involved in this disease.

Keywords: *SLC29A1*, hepatocellular carcinoma, drug resistance, epithelial-mesenchymal transition, cell adhesion, NF-κB

Introduction

Hepatocellular carcinoma (HCC) is one of the most commonly diagnosed liver cancers worldwide [1]. Despite improvements in monitoring programs and diagnostic tools, only 30-40% of patients with HCC are eligible for curative treatment [2, 3]. The multikinase inhibitor sorafenib was approved as a standard treatment for patients with advanced HCC, but the survival benefit remains modest [4]. Because of its heterogeneity and multiple etiologies, HCC is highly refractory to conventional systemic chemotherapy and treatment with the kinase-targeting agent sorafenib [5]. Improved understanding HCC is urgently needed.

Cellular uptake of anti-cancer drugs is an important first step in the mechanism of drug action. Membrane transporters are responsible for anti-cancer drug uptake and export processes, which effect anti-tumor agent efficacy. Studies have investigated the multidrug resistance mechanism in HCC [6, 7]. Two important proposed mechanisms of drug resistance, which act together or alone, are elevated drug efflux and decreased drug uptake in cancer cells. These processes are regulated by membrane transporters [8]. The two main transporter superfamilies are the solute carrier (SLC) transporters and the ATP-binding cassette transporters. The SLC transporter superfamily is important in endogenous compound homeostasis, xenobiotic disposition, and drug delivery [9]. The expression levels of drug transporters in HCC have been investigated [10-13]. However, the expression of the SLC trans-

Table 1. Correlation between SLC29A1 expression and clinical characteristics of patients with HCC

	Low SLC29A1 expression (n=204)	High SLC29A1 expression (n=189)	*P*-value*
Sex			
Female	26	24	1.000
Male	178	165	
Age, years			
≤ 50	94	103	0.107
> 50	110	86	
HBsAg			
Negative	37	33	0.896
Positive	167	156	
HCV			
Negative	202	182	0.094
Positive	2	7	
Cirrhosis			
No	38	34	0.897
Yes	166	155	
AFP, ng/mL			
≤ 20	89	52	0.001*
> 20	115	137	
Tumor encapsulation			
Complete	122	111	0.838
None	82	78	
Tumor size, cm			
≤ 5	111	120	0.081
> 5	93	69	
Tumor number			
Single	164	150	0.803
Multiple	40	39	
Microvascular invasion			
No	145	151	0.047*
Yes	59	38	
Tumor differentiation			
I-II	140	117	0.169
III-IV	64	72	
CLIP			
0	159	150	0.806
1	45	39	
ALT			
≤ 75 U/L	185	171	1.000
> 75 U/L	19	18	
Child			
A	192	182	0.354
B	12	7	
BCLC			
A	156	152	0.391
B	48	37	

Abbreviations: HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; AFP, α-fetoprotein; CLIP, Cancer of the Liver Italian Program; ALT, alanine transaminase; BCLC, Barcelona Clinic Liver Cancer. **P*-values < 0.05 were considered statistically significant. The Pearson chi-square test was used for between-group comparisons.

porter family in HCC has not been thoroughly researched, and the roles of the SLC gene family in HCC-associated drug resistance have not been well-elucidated.

Expression of SLC transporter-family genes was comprehensively investigated in HCC tissues and peritumor tissues. We demonstrate that low *SLC29A1 expression* correlated with high recurrence rates and poor outcomes for patients with HCC after surgery. Low *SLC29A1* expression mechanistically enhanced tumor cell proliferation, invasion and reduced drug sensitivity through changing cell adhesion status, induction of the epithelial-mesenchymal transition (EMT) process, and nuclear factor-kappaB (NF-κB) pathway activation.

Materials and methods

Clinical materials

A total of 480 patients with HCC from the Liver Cancer Institute of Fudan University in Shanghai, China were recruited for this study. A cohort of 75 HCC patients who underwent surgery from 2000 to 2002 were screened by qPCR array for aberrant transporter genes in HCC. Additional 12 fresh tumors with matched peritumoral tissues from HCC patients who underwent surgery in 2010 were chosen for qRT-PCR and western blot analyses. Paraffin-embedded specimens from another cohort of 393 patients with HCC who underwent curative resection were collected and used to construct tissue microarrays (TMAs). TMAs were used to test the *SLC29A1* expression in HCC and para-carcinoma tissues. The histopathological diagnoses were based on World Health Organization criteria [14]. Follow-up data were summarized at the end of May 2016; the median follow-up period was 65 months (range: 4 to 181 months). Patients with high risk of recurrence based on clinical features such as vascular invasion and microsatellite lesions, received prophylactic transcatheter arterial chemoembolization (TACE; doxorubicin, cisplatin, 5-fluorouracil, and iodized oil). Overall survival (OS) was defined as the interval between the date of surgery and death (or the last observation point taken). Time to recurrence (TTR) was defined as any diagnosed relapse (intrahepatic recurrence or extrahepatic metastasis). The follow-up procedures, postoperative treatment modalities, and surveillance followed previous-

ly-published uniform guidelines [15]. The clinicopathologic characteristics of patients are presented in **Table 1**. Ethical approval was obtained from the research ethics committee of Zhongshan Hospital. Each patient provided written informed consent.

Cell lines and animals

Human HCC MHCC97L and MHCC97H cell lines were established at the Liver Cancer Institute, Zhongshan Hospital, Fudan University. L02, Hep3B, and Huh-7 cell lines were obtained from the Shanghai Institute for Biological Science (China). Male, athymic, 8-week-old BALB/c nude mice (Chinese Academy of Science, Shanghai, China) were raised under specific-pathogen-free conditions. Animal care and experimental protocols were performed following the guidelines established by the Shanghai Medical Experimental Animal Care Commission.

qRT-PCR and western blot assays

The RT^2 Profiler PCR Array System (Qiagen, Valencia, CA, USA) was used to screen the expression levels of SLC transporters in HCC and para-carcinoma tissues. This array contained 46 SLC transporter gene primers, 5 housekeeping gene primers (for normalization), 1 genomic DNA primer (for detecting genomic DNA contamination), 3 reverse transcription elements, and 3 positive PCR controls.

Thirty randomly selected HCC samples were used to investigate messenger RNA (mRNA) and protein expression levels. Expression of mRNA and protein in tissues was determined using qRT-PCR and western blot assays, respectively, as previously described [14]. The primers used are listed in Supplementary Table 1. Three independent repeats were performed. The primary antibodies used were against *SLC29A1* (1:1000; Abcam, Cambridge, MA, USA), β-actin (1:3000; Abcam), E-cadherin, N-cadherin, and Vimentin (1:1000; Cell Signaling Technology, Danvers, MA, USA).

Immunohistochemistry

Tissue sections were treated with citrate buffer to retrieve antigen. After removing the endogenous peroxidase activity, the tissue sections were blocked with goat serum and subsequently incubated with the primary antibody, anti-*SLC29A1* (1:500; Abcam, Cambridge, MA, USA), at 4°C. The samples were then incubated with secondary antibody, washed with PBS, and visualized using diaminobenzidine and hematoxylin re-staining.

Patients were divided into two subgroups for analysis of the associations between *SLC29A1* expression and the OS and cumulative recurrence rates. A positive reaction for *SLC29A1* was scored in four grade categories according to staining intensity (i.e., 0, 1, 2, and 3). The percentages of *SLC29A1*-positive cells were scored as 0 (0%), 1 (1 to 33%), 2 (34 to 66%), or 3 (67 to 100%). In cases with discrepancies between duplicated cores, the higher score of the two tissues was recorded as the final score. The sum of the intensity and percentage scores was used as the final staining score. The staining pattern was ranked by two independent investigators blinded of the clinicopathological data as previously defined [15]. Variations in enumeration within a range of 5% were re-evaluated to reach consensus.

Transfection and clone selection

The lentiviral construct PLKO-shRNA-*SLC29A1* was generated with the target sequences sh1-GGAACTCTCTCAGTGCCATCT and sh2-GCCACTCTATCAAAGCCATCC. *SLC29A1* in stably transfected clones was validated using qRT-PCR and western blot assays.

Cell proliferation and flat plate clone formation assays

Cell proliferation was analyzed using the CCK8 proliferation method. Briefly, 1×10^4 cells/well were seeded in triplicate in 96-well plates and incubated at 37°C and a 5% CO_2 humidified atmosphere. After 24, 48, and 72 hours, the CCK8 assay was performed according to the manufacturer's protocol. All experiments were performed in triplicate.

Cells in the logarithmic growth phase were seeded onto a 2-cm cell culture plate at a density of 500 cells/mL. The cells were cultured at 37°C in a well-humidified 95% air and 5% CO_2 incubator for approximately 2 weeks. Colony formation was photographed

and counted at 50× magnification. The assays were performed in triplicate for each cell line combination.

Cell invasion

The Oris™ Pro 96-well Invasion Assay was used to examine cell invasion. MHCC97-H-Mock, MHCC97-H-*SLC29A1*-sh1/2, Huh-7-Mock, and Huh-7-*SLC29A1*-sh1/2 cells. The Oris™ Pro Collagen I Overlay solution was added and incubated in a humidified chamber (37°C, 5% CO_2) for 1 hour. Pre-invasion images of the Detection Zone were captured. Then, 100 µL complete medium (containing serum) was added on top of the overlay and incubated for 96 hours to permit invasion. Post-invasion images of the Detection Zone were taken at 24, 48, 72, and 96 hours.

The Oris™ Pro 96-well Invasion Assay results were evaluated using high-content screening/high-content imaging analysis.

Tumor models

Mice were manipulated and housed according to protocols approved by the Shanghai Medical Experimental Animal Care Commission. To evaluate *in vivo* tumor growth, 1×10^7 MHCC97-H-*SLC29A1*-sh2 and Huh-7-*SLC29A1*-sh2 cells were suspended in 100 µL serum-free Dulbecco's Modified Eagle Medium and injected via the subcutaneous route into the left flank of each mouse (six in each group, 8-week-old male BALB/c-nu/nu); the control cells (MHCC97-H-Mock and Huh-7-Mock) were injected into the right flank. The tumor sizes were measured three times per week as soon as the tumors were measurable. The tumor volumes were calculated using the formula: (length × width^2)/2. The mice were euthanized on day 22, and the tumors were weighed immediately after dissection.

Chemosensitivity assay

MHCC97-H-Mock, Huh-7-Mock, MHCC97-H-*SLC29A1*-sh2, and Huh-7-*SLC29A1*-sh2 cells were plated in 96-well plates and treated with 0, 5, 10, 20, or 40 mg/L 5-fluorouracil (5-FU), with 0, 10, 15, 20, or 25 mg/L cisplatin, or with 0, 5, 10, 15, or 20 µM sorafenib for 48 hours, respectively. HCC cell viability was evaluated using a Cell Counting Kit-8 assay. The optical density OD was measured at 450 nm, and the relative viability was evaluated by normalizing the OD values from the test samples to the OD values of the control samples.

Microarray-based gene expression profile

We performed a microarray assay to compare the profiles of differentially expressed genes between SLC29A1 shRNA-treated and Mock Huh-7/MHCC97-H cells and to investigate the function of SLC29A1 and the potential mechanism by which the SLC29A1 shRNA promoted tumor cell proliferation. The 44K oligonucleotide microarray as constructed by Outdo Biotech (Shanghai, China). The pathways were analyzed by Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) using the DAVID tool.

Statistical analysis

The Student's t test was used to evaluate the differences between *SLC29A1* expression in HCC and para-carcinoma tissues. The correlation between *SLC29A1* expression and clinical parameters was calculated using Pearson rank correlation coefficients. The Kaplan-Meier method and log-rank test were used to analyze survival curves according to *SLC29A1* expression and clinical characteristics. Subsequently, all potential predictive factors were included in a Cox multivariate regression survival analysis. All statistical analyses were performed using SPSS 19.0 software (SPSS, Chicago, IL). A *P*-value < 0.05 was considered to indicate a statistically significant result.

Results

The SLC-family gene expression profile in HCC

The mRNA expression levels of various SLC-family genes in a cohort of 75 frozen HCC specimens along with the corresponding para-carcinoma samples were analyzed by qPCR array to screen for aberrant expression of SLC-family genes in HCC. We identified 11 SLC-family genes that were down-regulated more than 2-fold in HCC samples, compared with the gene expression patterns in the para-carcinoma samples (Supplementary Table 2). Three genes (*SLC7A6*, *SLC29A1*, and *SLC29A2*) were up-regulated (**Figure 1A**). We divided the speci-

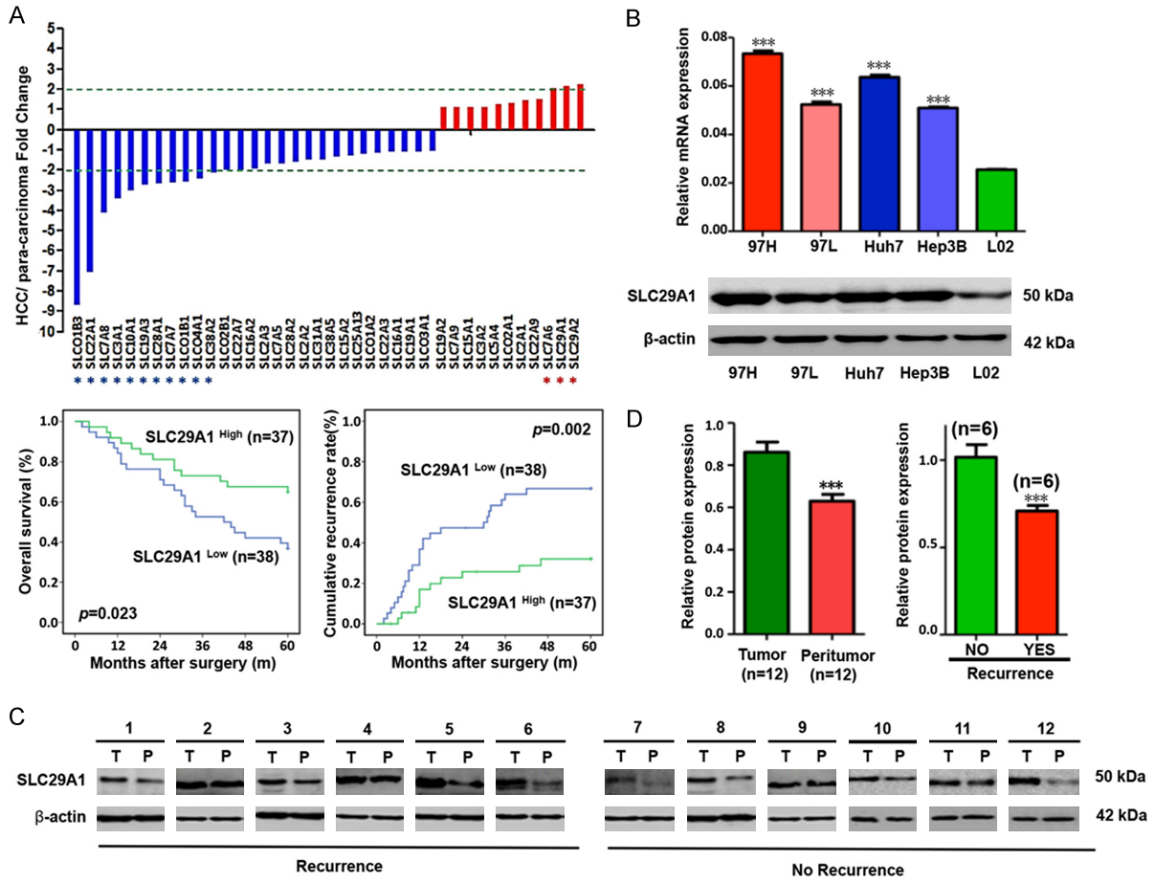

Figure 1. A. Solute carrier (SLC) family gene expression profile in hepatocellular carcinoma (HCC) samples. Eleven SLC genes (*SLCO1B3*, *SLC22A1*, *SLC7A8*, *SLC3A1*, *SLC10A1*, *SLC19A3*, *SLC28A1*, *SLC7A7*, *SLCO1B1*, *SLCO4A1,* and *SLC38A2*) were down-regulated more than two-fold in HCC samples. Three SLC genes (*SLC7A6, SLC29A1* and *SLC29A2*) were up-regulated. B. *SLC29A1* mRNA and protein levels in MHCC97H, Huh-7, MHCC97L, Hep3B, and L02 cells ($P < 0.05$). C. Western blot analysis of *SLC29A1* expression in a cohort of 12 patients with HCC. D. Patients with HCC recurrence had lower *SLC29A1* protein levels than those without recurrence.

mens into two groups according to the expression levels of the 14 identified genes and performed a survival analysis. *SLC29A1* was the only gene correlated with prognosis (Supplementary Figure 1). Low *SLC29A1* mRNA expression predicted poor survival (P=0.023) and high disease recurrence rates (P=0.002) after surgery. The qRT-PCR array also indicated that the *SLC29A1* mRNA expression level was significantly elevated in HCC, compared with para-carcinoma tissues ($P < 0.05$).

SLC29A1 expression in HCC cell lines and HCC tissues

Expression of SLC29A1 was confirmed in HCC cell lines and HCC tissues using qRT-PCR and western blot analyses. The *SLC29A1* mRNA and protein levels in MHCC97H and Huh-7 cells were higher than in MHCC97L and Hep-3B cells and significantly higher than in L02 cells ($P < 0.05$; **Figure 1B**). A cohort of 12 HCC samples with corresponding para-carcinoma tissues and qRT-PCR were used to validate the aberrant high *SLC29A1* transcription level. The functional *SLC29A1* protein expression in HCC tissues was clearly greater than that in para-carcinoma tissues (**Figure 1C**). Western blot analysis of the tumor tissues revealed that patients without tumor recurrence exhibited higher *SLC29A1* protein expression compared with those with recurrence (**Figure 1D**, ***$P < 0.001$).

Low SLC29A1 expression predicts poor prognosis in patients with HCC

We analyzed *SLC29A1* localization in the cytoplasm in TMAs of 393 HCC specimens using immunohistochemistry. High *SLC29A1* ex-

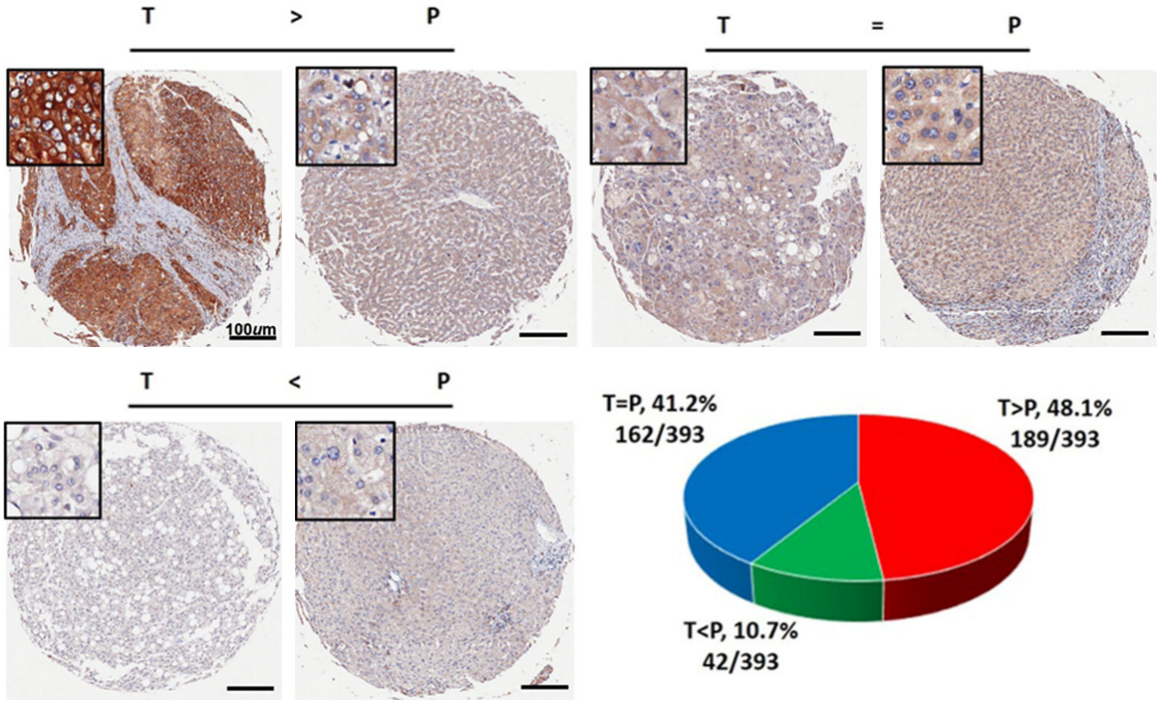

Figure 2. Immunohistochemistry results of 393 hepatocellular carcinoma specimens, by *SLC29A1* expression.

pression (score 3-6) was observed in 189 out of 393 HCC samples (48.1%), and low or negative *SLC29A1* expression (score 0-2) in 204 HCC samples (51.9%). All peritumoral tissues showed negative or low *SLC29A1* expression (score 0-2). There was a statistically significant difference in *SLC29A1* expression between tumor and peritumoral tissues ($P < 0.001$). When comparing *SLC29A1* expression in HCC samples and the corresponding para-carcinoma samples, higher *SLC29A1* expression in HCC samples than in para-carcinoma samples was defined as being in the *SLC29A1*^{high} group; equal or lower *SLC29A1* expression was defined as being in the *SLC29A1*^{low} group (**Figure 2**).

At the last follow-up, 28.2% (111/393) of the patients experienced recurrence and 63.6% (250/393) had died. The 1-, 3-, 5-, 10-, and 15-year OS rates were 90.1%, 68.2%, 53.3%, 39.9%, and 34.1%, respectively. The corresponding cumulative recurrence rates were 18.6%, 46.6%, 58.5%, 69.2%, and 74.1%. The univariate survival analysis revealed that liver cirrhosis, tumor size, tumor encapsulation, microvascular invasion, tumor number, and low *SLC29A1* expression were predictors of an unfavorable outcome for OS and tumor recurrence after surgery. The serum alpha-fetoprotein (AFP) level was only predictive of an unfavorable outcome for OS (**Table 2**). The results of further multivariate analysis indicated that *SLC29A1* was an independent prognostic factor for OS (HR=0.691, 95% confidence interval (CI) 0.535-0.894, P=0.005) and tumor recurrence (HR=0.677, 95% CI 0.534-0.860, P=0.001) (**Figure 3A**, **3B**). The median OS for *SLC29A1*^{low} patients was 50 months, compared with 78 months for the *SLC29A1*^{high} group, and the median TTR for *SLC29A1*^{low} patients was 38 months, compared with 64 months for the *SLC29A1*^{high} group. We further investigated the predictive value of *SLC29A1* within clinical subgroups of AFP levels ≤ 20 ng/ml, without microvascular invasion (MVI) and Barcelona Clinic Liver Cancer (BCLC) Staging 0-A. The prognostic significance of *SLC29A1* persisted in patients with HCC with normal AFP levels (P=0.041), without MVI (P=0.01) and BCLC 0-A (P=0.014) (**Figure 3C-E**). Forty-nine percent (145/296) of the no-MVI group patients expressed low levels of SLC29A1; these patients had a dismal prognosis. Most of the *SLC29A1*^{low} patients (59.7%) died of tumor recurrence within 5 years. In the AFP-normal group, 63.1% (89/141) expressed low levels of *SLC29A1*; the median TTR was 47 months and the 5-year recurrence rate was 57.8%. In the BCLC 0-A group patients, 50.6%

Table 2. Univariate and multivariate analyses of factors associated with overall survival and recurrence in 393 patients with hepatocellular carcinoma

Factors	OS				Cumulative recurrence			
	Univariate	Multivariate			Univariate	Multivariate		
	P-value	HR	95% CI	*P*-value	*P*-value	HR	95% CI	*P*-value
Sex (female vs. male)	0.205			NA	0.435			NA
Age, years (≤ 50 vs. > 50)	0.868			NA	0.782			NA
HBsAg (positive vs. negative)	0.216			NA	0.155			NA
HCVAb (positive vs. negative)	0.820			NA	0.922			NA
Liver cirrhosis (yes vs. no)	0.009*	1.513	1.061-2.159	0.022*	0.002*	1.587	1.139-2.211	0.006*
Serum AFP, ng/mL (≤ 20 vs. > 20)	0.011*	1.347	1.028-1.765	0.031*	0.061			NA
Serum ALT, U/L (≤ 75 vs. > 75)	0.855			NA	0.585			NA
Tumor size (diameter, cm) (> 5 vs. ≤ 5)	0.031*			NS	0.373			NA
Tumor encapsulation (absent vs. present)	0.017*			NS	0.074			NA
Microvascular invasion (yes vs. no)	< 0.001*	1.351	1.011-1.805	0.042*	0.001*	1.356	1.037-1.773	0.026*
Tumor number (multiple vs. single)	< 0.001*	1.785	1.322-2.412	< 0.001*	< 0.001*	1.831	1.381-2.427	< 0.001*
Tumor differentiation (III/IV vs. I/II)	0.316			NA	0.336			NA
SLC29A1 expression (high vs. low)	0.026*	0.691	0.535-0.894	0.005*	0.003*	0.677	0.534-0.860	0.001*

Abbreviations and Notes: OS, overall survival; NA, not adopted; NS, not significant; AFP, alpha-fetoprotein; ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; 95% CI, 95% confidence interval; HR, hazard ratio; Cox proportional hazards regression model. **P*-values < 0.05 were considered statistically significant.

(156/308) had low-level expression of SLC-29A1; the median TTR was 40 months and the 5-year recurrence rate was 57.4%.

Our previous studies found that TACE can improve the survival of patients with a high risk of residual tumor [16, 17]. Here, we did not find that adjuvant TACE after surgery improved the OS rates and TTR in the study population (Supplementary Figure 2). Patients who received adjuvant TACE after surgery had higher early recurrence (≤ 2 year) rates, compared with those did not (43% vs. 30.8%) (Supplementary Figure 2). The *SLC29A1*^{high} group patients who received postoperative adjuvant TACE had significantly higher early recurrence rates, compared with those did not (P=0.020, **Figure 3F**). In the *SLC29A1*^{low} group patients, the difference in recurrence rate between patients who did or did not receive adjuvant TACE after surgery was not statistically significant (43.1% vs. 37%) (Supplementary Figure 2).

We found that patients with low *SLC29A1* expression (score 0-2) were more likely to have MVI (P=0.047) and high serum AFP levels (P=0.001) (**Table 1**).

Down-regulation of SLC29A1 enhances tumor cells proliferation and invasion, induces EMT, and reduces drug sensitivity

We tested the hypothesis that *SLC29A1* is involved in HCC tumorigenesis and progression (**Figure 4A**). *SLC29A1* knockdown in MHCC97-H and Huh-7 cells promoted cell proliferation on day 3 (**Figure 4B**). The clonogenic ability of HCC cells was enhanced after *SLC-29A1* inhibition (**Figure 4C**). The *in vitro* invasion assay indicated that a greater proportion of the *SLC29A1* knockdown cells had invaded, compared with the control cells (Supplementary Figure 3).

EMT is a dominant characteristic of most cancers and has a crucial role in cancer metastasis and invasion. Therefore, we compared the expression of epithelial and mesenchymal markers between knockdown groups and Mock cells. MHCC97-H-SLC29A1-sh2 and Huh-7-SLC29A1-sh2 cells expressed lower levels of the epithelial gene E-cadherin than MHCC97-H-Mock and Huh-7-Mock cells (**Figure 4D**). Mesenchymal gene (Vimentin and N-cadherin) expression was significantly up-regulated in MHCC97-H-SLC29A1-sh2 and Huh-7-SLC29A1-sh2 cells, compared with MHCC97-H-Mock and Huh-7-Mock cells.

Using a successful subcutaneous xenograft tumor model constructed with HCC cells, we found that down-regulation of *SLC29A1* expression significantly enhanced tumor growth *in vivo* (**Figure 4E**). The weights and volumes of Mock cell-derived xenografts were significantly lower than those of tumor xenografts from MHCC97-H-*SLC29A1*-sh2 (MHCC97-H Mock: 0.275 ± 0.031 g vs. sh2: 0.525 ± 0.042 g and

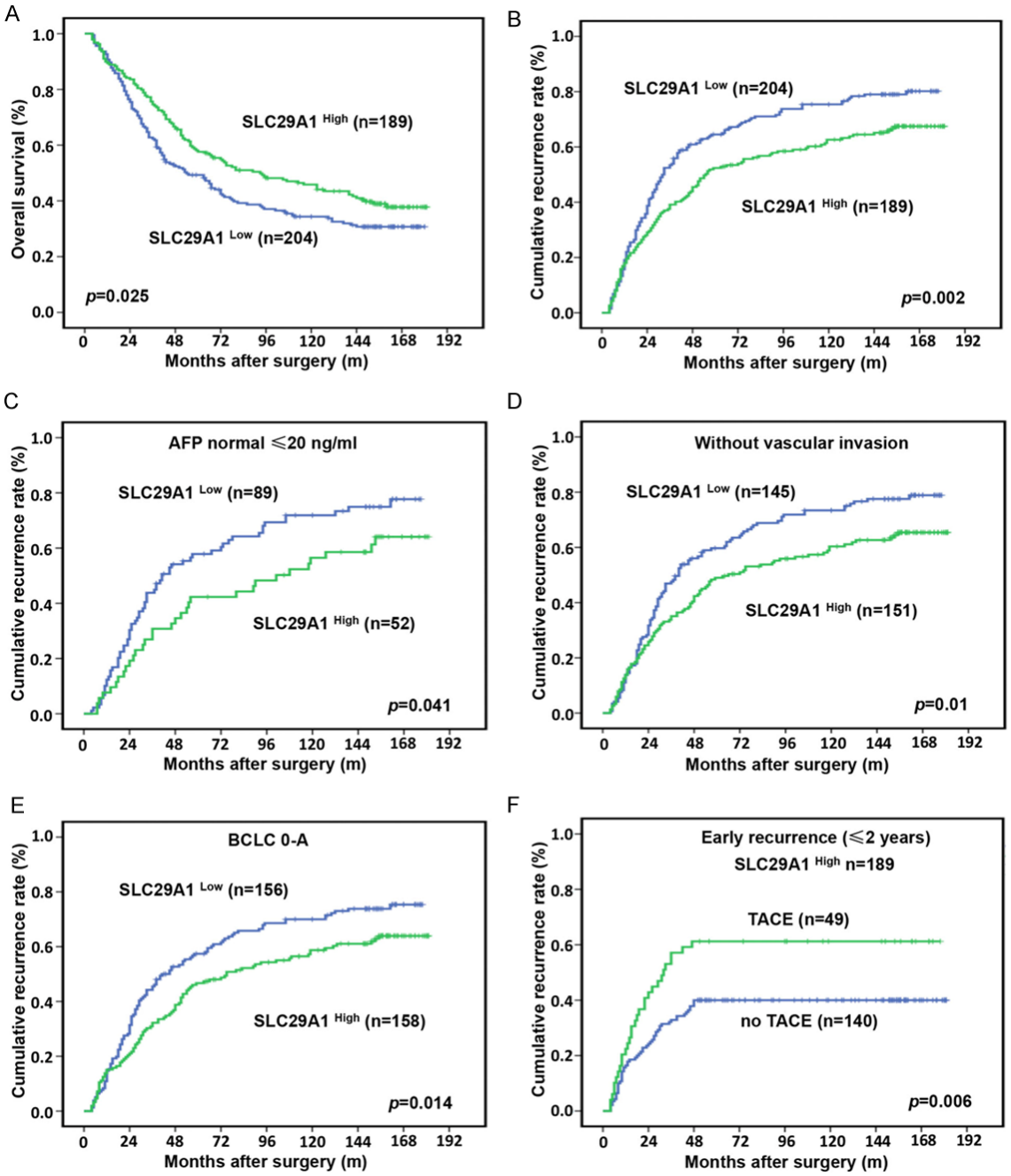

Figure 3. A and B. Kaplan-Meier analysis of overall survival (OS) and cumulative recurrence rates in 393 patients, by *SLC29A1* expression. Significantly greater OS and lower cumulative recurrence rates occurred in the *SLC29A1*^{high} group, compared with the *SLC29A1*^{low} group. C-E. The prognostic significance of *SLC29A1* in hepatocellular carcinoma (HCC) patients with normal alpha-fetoprotein (AFP) levels (P=0.041), without microvascular invasion (MVI; P=0.01), and Barcelona Clinic Liver Cancer (BCLC) Staging 0-A (P=0.014). F. Adjuvant transcatheter arterial chemoembolization (TACE) did not reduce the early recurrence rate in *SLC29A1*^{high} HCC patients (P=0.006).

387.72 ± 120.86 mm^3 vs. 627.29 ± 148.46 mm^3) and Huh-7-*SLC29A1*-sh2 hepatoma cells (Huh-7 Mock: 0.167 ± 0.180 g vs. sh2: 0.405 ± 0.023 g and 131.18 ± 30.26 mm^3 vs. 451.27 ± 100.14 mm^3) (**Figure 4F**).

The chemosensitivity assay was performed in Huh-7, MHCC97-H, Huh-7-*SLC29A1*-sh2, and MHCC97-H-*SLC29A1*-sh2 cells, which were treated with 0, 5, 10, 20, or 40 mg/L 5-FU or 0, 10, 15, 20, or 25 mg/L cisplatin or 0, 5, 10, 15,

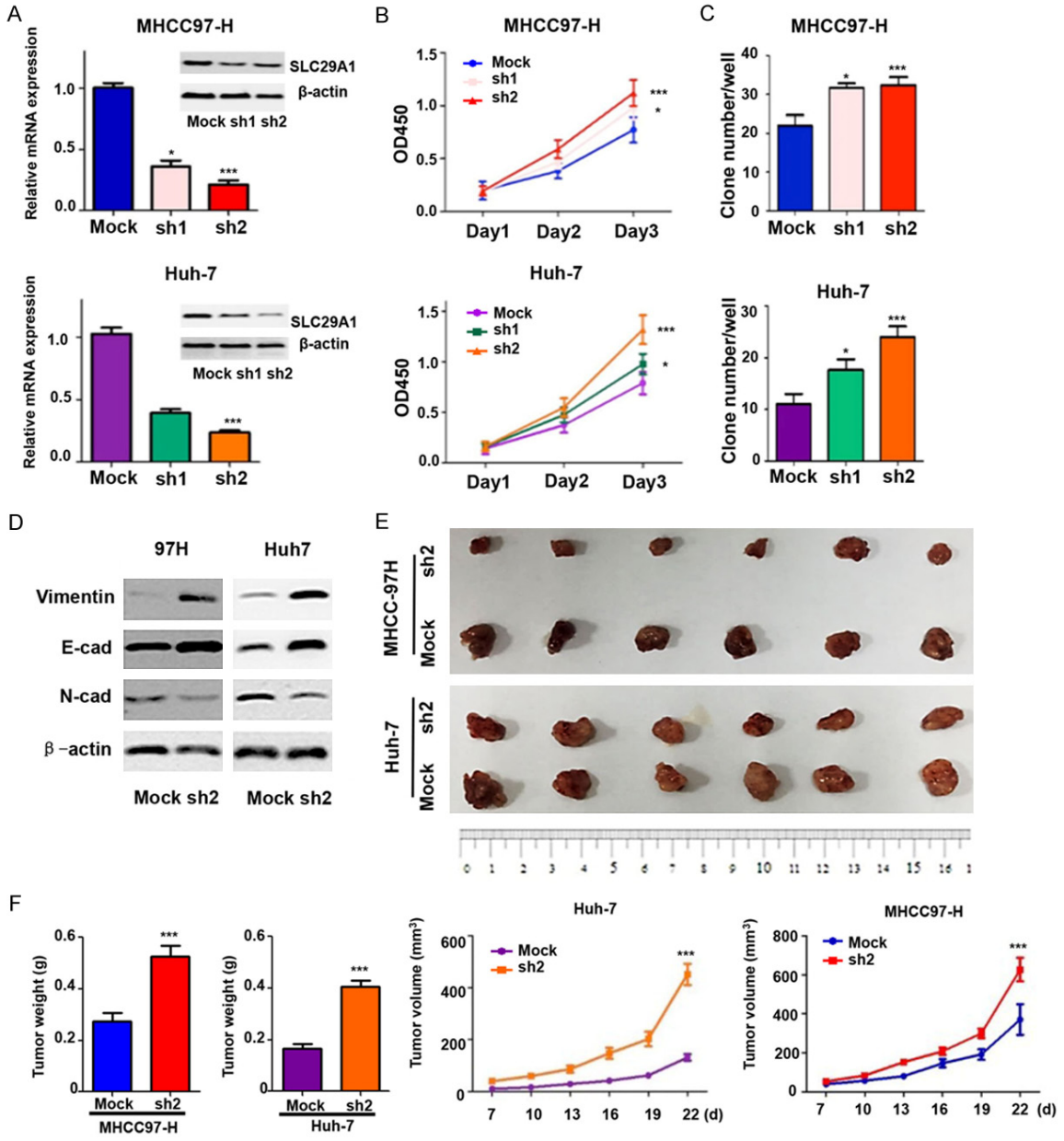

Figure 4. Inhibition of *SLC29A1* expression promotes hepatocellular carcinoma (HCC) cell growth *in vitro and in vivo*. Functional analysis, *in vitro*: A. *SLC29A1* expression in MHCC97-H and Huh-7 cells transfected with an shRNA. B. *SLC29A1* knockdown in MHCC97-H and Huh-7 cells promoted cell proliferation on day 3. C. The clonogenic ability of HCC cells was enhanced after *SLC29A1* inhibition. Functional analysis, *in vivo*: D. Differences in the expression of epithelial and mesenchymal markers were compared between cancer cells with high and low *SLC29A1* expression (MHCC97-H-Mock vs. MHCC97-H-*SLC29A1*-sh2 and Huh-7-Mock vs. Huh-7-*SLC29A1*-sh2 cells) using western blot assay. E. The morphological characteristics of tumors in the SLC29A1 shRNA and control groups. F. The differences in tumor weights and volumes between the SLC29A1 shRNA group and control group were statistically significant.

or 20 μM sorafenib for 48 hours; a CCK8 assay was used to evaluate cell viability (*, all $P < 0.05$). shRNA-mediated *SLC29A1* knockdown significantly decreased sensitivity of HCC cells to 5-FU, cisplatin, and sorafenib (**Figure 5**, ***, $P < 0.001$).

Gene expression profiling using a microarray analysis

The results of the microarray analysis indicated that 235 genes were differentially expressed between *SLC29A1* shRNA-treated Huh-7/MH-

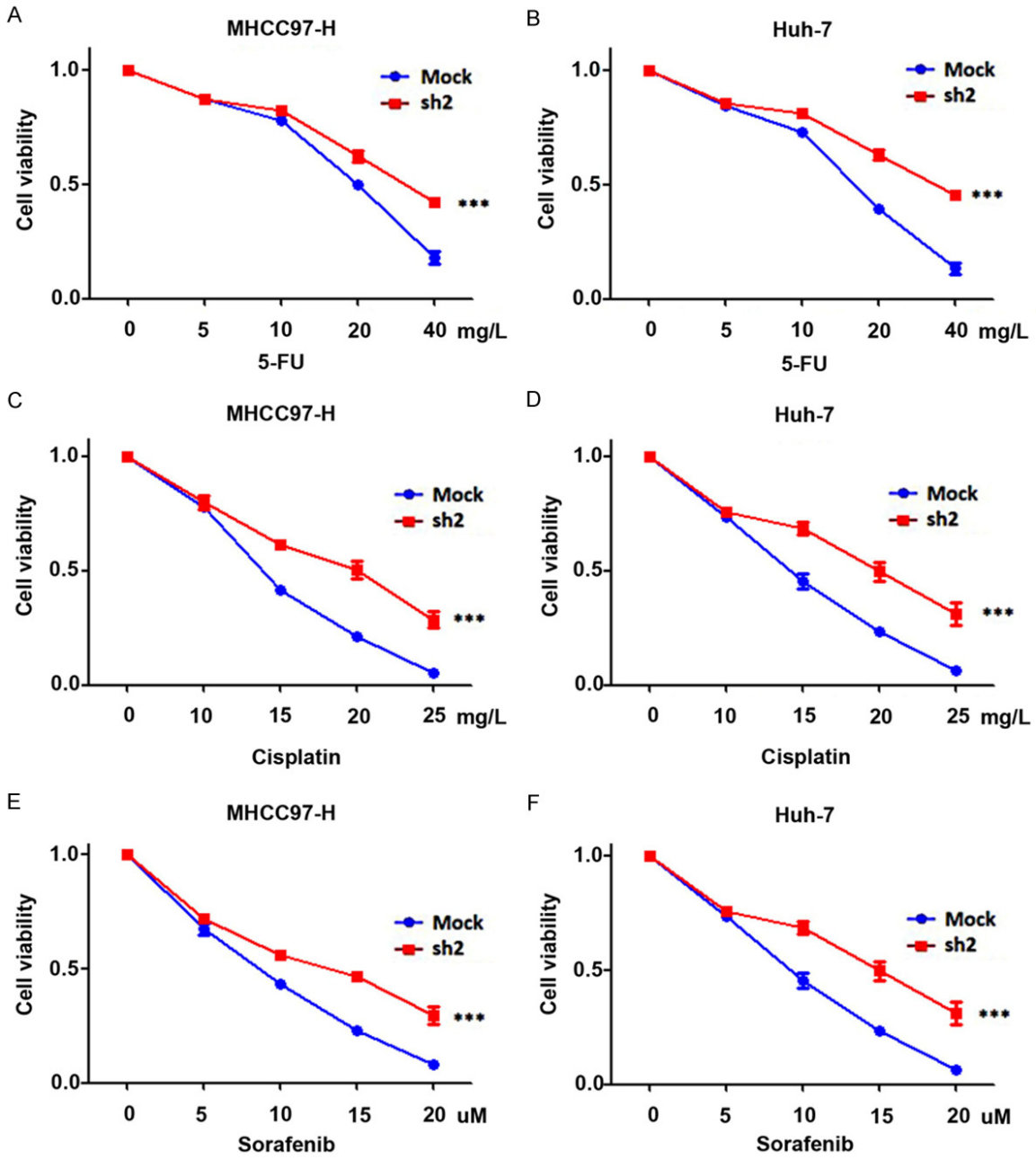

Figure 5. Inhibition of *SLC29A1* expression promotes drug resistance *in vitro*. ShRNA-mediated *SLC29A1* down-regulation inhibited the sensitivity of hepatocellular carcinoma cells to 5-FU (A and B), Cisplatin (C and D), and Sorafenib (E and F).

CC97-H cells and Mock cells. Of these genes, 112 were up-regulated (ratio > 2.0) and 123 were down-regulated (ratio < 0.5) (**Figure 6A**). The significant signaling pathways identified using the DAVID GO and KEGG databases are presented in **Figure 6B**. Many of these genes were related to growth factor a-c tivity (**Figure 6B**).

Six differentially expressed genes were further examined using qRT-PCR. Up-regulated and down-regulated genes were validated (**Figure 6C**). The possible functional relationship between *SLC29A1* and its target genes is presented in **Figure 6D**. *SLC29A1* might promote tumor cell proliferation, invasion, and drug resistance by regulating cell adhesion molecules

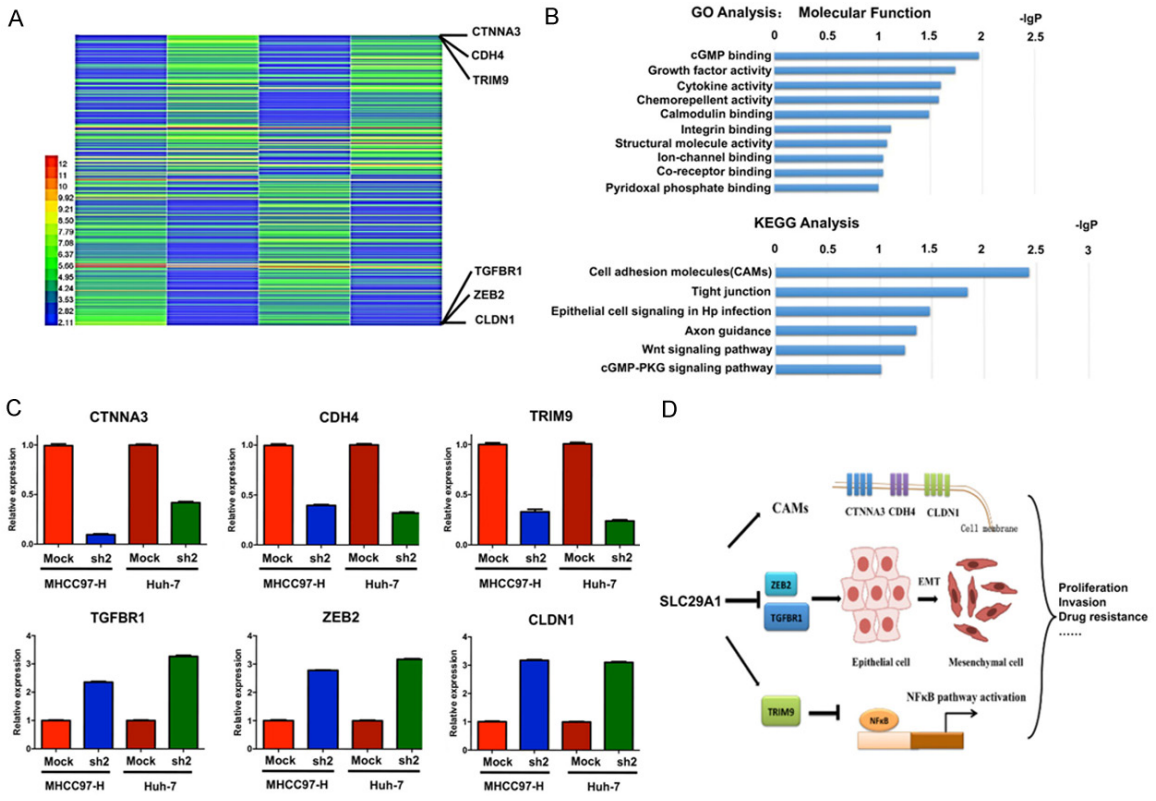

Figure 6. Microarray-based gene expression profiles after *SLC29A1* down-regulation. A. One hundred twelve genes were up-regulated (ratio > 2.0), and 123 genes were down-regulated (ratio < 0.5) between *SLC29A1* shRNA-treated and Mock Huh-7/MHCC97-H cells. B. GO and KEGG analysis results. C. *SLC29A1* expression was positively correlated with *CAM* and *TRIM* expression, thus increasing cell adhesion and inhibiting the NF-κB pathway, and negatively correlated with *ZEB2* and *TGFBR* expression, thus inhibiting the epithelial-mesenchymal transition (EMT). D. Schematic diagram of the mechanisms of *SLC29A1* involvement in hepatocellular carcinoma.

(*CLDN1*, *CTNNA3*, and *CDH4*), the EMT (*ZEB2* and *TGFBR1*), and the NF-κB pathway (*TRIM9* is a negative regulator).

Discussion

Aberrant expression of drug transporters is associated with HCC drug resistance, and SLC transporters have important roles in tumor drug resistance [11]. We investigated the expression of SLC-superfamily genes in HCC tissues and peritumoral tissues to explore aberrantly expressed genes. The *SLC29A1* mRNA was significantly elevated in HCC tissues. Low expression of SLC29A1 was also correlated with tumor recurrence after surgery and confirmed by western blot. TMA immunohistochemistry results from a larger series of human HCC specimens indicated that low *SLC29A1* expression was correlated with high recurrence rates and poor OS. Down-regulation of *SLC29A1* expression in HCC cells promoted tumor cell proliferation, invasion, EMT, and drug resistance, based on *in vitro* and *in vivo* studies.

SLC29A1 participates in cellular uptake of anti-cancer drugs and nucleosides [18]. *SLC29A1* enhances the cytotoxicity of some anticancer drugs and is a useful predictive marker of drug efficacy in cancer cells. Single nucleotide polymorphisms in *SLC29A1* contribute to the cytotoxic effects of AraC, an effective treatment for acute myeloid leukemia [19]. *SLC29A1* is positively associated with chemotherapy efficacy in Asian pancreatic cancer patients [20]. We found that *SLC29A1* knockdown enhanced drug resistance to 5-FU, cisplatin, and sorafenib *in vitro*. However, further studies of the mechanism by which *SLC29A1* increases anticancer drug efficacy are needed.

The anti-tumor effects of *SLC29A1* involve multiple signaling pathways. *CTNNA3*, *CDH4*, and *CLDN1* are cell adhesion molecules that are essential for the development and progression of cancer [21, 22]. *ZEB2* and *TGFBR1* regulate the EMT process [23, 24], and *TRIM9* inhibits the NF-κB pathway [25]. *SLC29A1* kn-

ockdown may result in decreased *CAM* and *TRIM9* function in HCC cells or increased *ZEB2* and *TGFBR1* function, which subsequently inhibits cell adhesion, and promotes the EMT and activation of the NF-κB pathway. These changes will increase tumor cell proliferation and invasion, and induce EMT and drug resistance. *SLC29A1*-induced changes in the expression of any one of these proteins has critical effects on carcinogenesis, progression, and drug resistance.

The prognostic significance of *SLC29A1* was present in patients with BCLC 0-A HCC: the 5-year tumor recurrence rates for *SLC29A1*^{high} and *SLC29A1*^{low} patients were 46.7% vs. 57.4%, respectively. AFP is the most widely used tumor marker; however, approximately 40% to 60% of patients with HCC have normal serum AFP levels [26]. Patients with normal AFP could be stratified according to *SLC29A1* expression into two groups with substantially different TTR (36.9% vs. 63.1% for *SLC29A1*^{high} and *SLC29A1*^{low}, respectively, $P < 0.05$). Our results indicated that *SLC29A1* might be a useful prognostic marker for HCC, especially in AFP-normal patients and those with low recurrence risk [27]. We also found that the risk of early recurrence for *SLC29A1*^{high} HCC patients with adjuvant TACE was high and there was not beneficial for TACE in *SLC29A1*^{high} HCC patients after surgery (**Figure 3F**). *SLC29A1* might be a useful index for choosing suitable adjuvant treatment for HCC patients after surgery.

To the best of our knowledge, our study is the first to reveal the correlation between *SLC29A1* expression and the prognosis of patients with HCC, and its role in drug resistance of HCC. Most patients with HCC in China are positive for the hepatitis B virus; 84.4% of our study population was hepatitis B virus-positive, which differs greatly from populations in the United States, Europe, and Japan [28]. The prognostic significance of *SLC29A1* should be validated in HCC patients from these countries. Prospective studies with large patient cohorts and additional studies into the mechanism of *SLC29A1* in HCC are needed.

In conclusion, *SLC29A1* has important roles in tumor cell proliferation and invasion, and induces EMT and drug resistance by interacting with various signaling pathways in HCC cells. Low expression of *SLC29A1* in tumor tissues was correlated with a high recurrence rate and poor outcomes for patients with HCC after surgery. *SLC29A1* might be a promising prognostic factor, a potential tumor suppressor, and a drug sensitizer for patients with HCC.

Acknowledgements

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Sequence of primers for *q*RT-PCR

Gene	Forward primer (5'-----3')	Reverse primer (5'-----3')
SLC29A1	5'-GGGTGTAGCCTCGGCATTT-3'	3'-CTCCACTGGCCTGCACAAG-5'
Actin	5'-CTGGAACGGTGAAGGTGACA-3'	3'-CGGCCACATTGTGAACTTTG-5'
CTNNA3	5'-TGGGCAATACTCTGTCCTGC-3'	3'-TGGTAACCTGGATTATGAGAGGC-5'
CDH4	5'-TGGCCATCCTCATCTGCATC-3'	3'-GTCGTAGTCCTGGTCCTCCT-5'
CLDN1	5'-CTGTCATTGGGGGTGCGATA-3'	3'-CTGACCAAATTCGTACCTGGC-5'
TRIM9	5'-TTTGCAGATTTCTGACGCCC-3'	3'-GATAGGGGTTGCTGGGACTG-5'
ZEB2	5'-CCTCTGTAGATGGTCCAGTGAA-3'	3'-GTCACTGCGCTGAAGGTACT-5'
TGFBR1	5'-TTGGTACCCAAGGAAAGCCA3'	3'-GAGAACTTCAGGGGCCATGT-5'

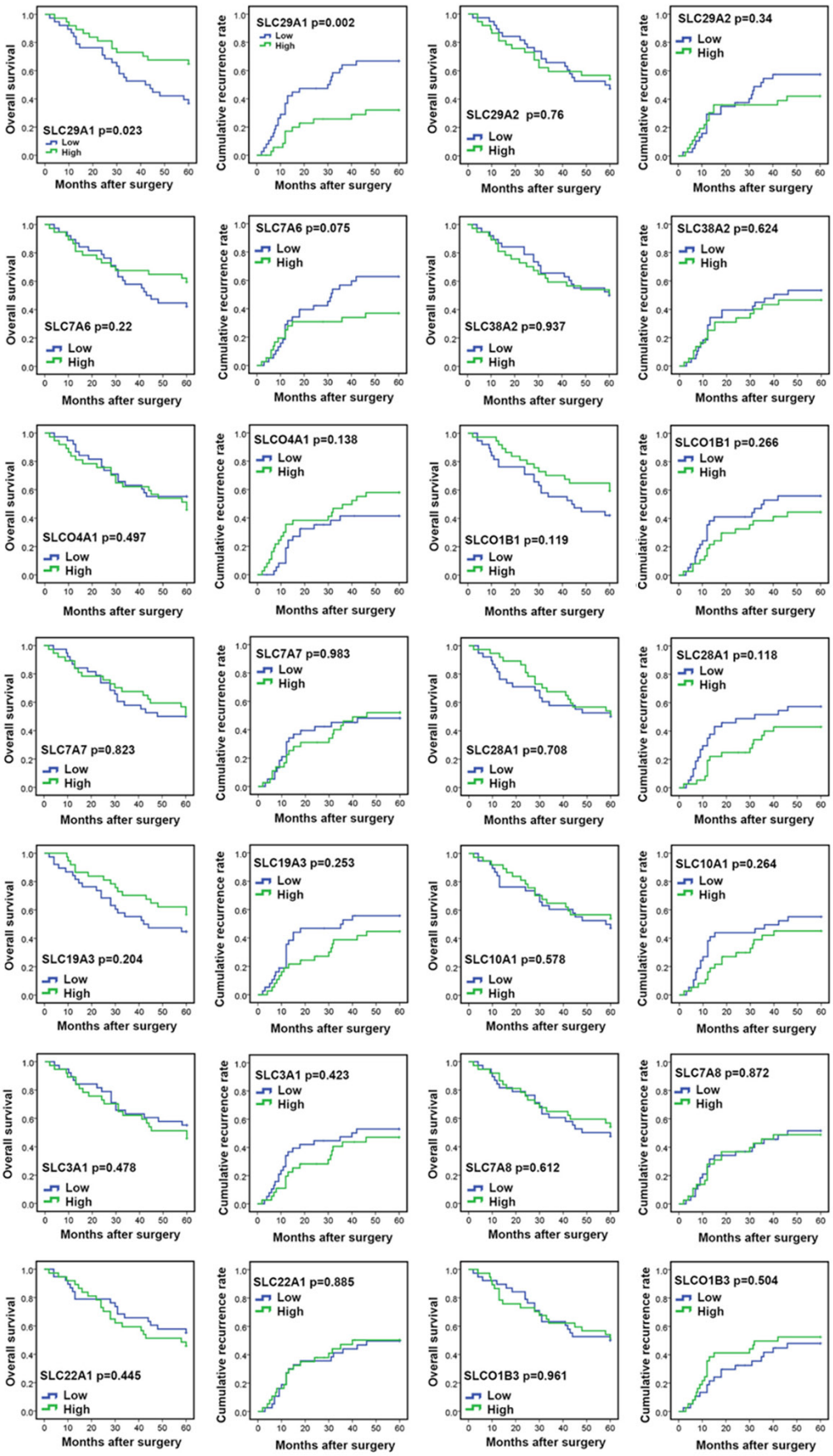
Abbreviations: *q*RT-PCR, quantitative real-time polymerase chain reaction.

Supplementary Table 2. The mRNA expressions of SLC-family genes in 75 patients with hepatocellular carcinoma

Symbol	Well	AVG ΔC_t (Ct(GOI) - Ave Ct (HKG))		$2^{-\Delta C_t}$		Fold Change	T-TEST	Fold Up- or Down-Regulation	Comments
		HCC	Para-carcinoma	HCC	Para-carcinoma	HCC/para-carcinoma	*p* value	HCC/para-carcinoma	
SLC10A1	C11	3.79	2.20	7.2E-02	2.2E-01	0.33	N/A	-3.02	OKAY
SLC10A2	C12	15.80	16.55	1.7E-05	1.0E-05	1.67	N/A	1.67	B
SLC15A1	D01	6.11	6.30	1.4E-02	1.3E-02	1.14	N/A	1.14	OKAY
SLC15A2	D02	10.06	9.67	9.4E-04	1.2E-03	0.76	N/A	-1.31	OKAY
SLC16A1	D03	4.96	4.82	3.2E-02	3.5E-02	0.91	N/A	-1.10	OKAY
SLC16A2	D04	5.99	5.02	1.6E-02	3.1E-02	0.51	N/A	-1.96	OKAY
SLC16A3	D05	15.70	16.56	1.9E-05	1.0E-05	1.81	N/A	1.81	B
SLC19A1	D06	9.93	9.82	1.0E-03	1.1E-03	0.93	N/A	-1.08	OKAY
SLC19A2	D07	7.03	7.16	7.7E-03	7.0E-03	1.10	N/A	1.10	OKAY
SLC19A3	D08	9.55	8.09	1.3E-03	3.7E-03	0.36	N/A	-2.75	OKAY
SLC22A1	D09	4.98	2.17	3.2E-02	2.2E-01	0.14	N/A	-7.04	OKAY
SLC22A2	D10	16.49	16.18	1.1E-05	1.4E-05	0.81	N/A	-1.24	B
SLC22A3	D11	7.97	7.80	4.0E-03	4.5E-03	0.89	N/A	-1.12	OKAY
SLC22A6	D12	14.13	14.46	5.6E-05	4.4E-05	1.26	N/A	1.26	B
SLC22A7	E01	3.91	2.93	6.7E-02	1.3E-01	0.51	N/A	-1.96	OKAY
SLC22A8	E02	13.18	15.97	1.1E-04	1.6E-05	6.94	N/A	6.94	B
SLC22A9	E03	4.14	4.72	5.7E-02	3.8E-02	1.49	N/A	1.49	OKAY
SLC25A13	E04	4.47	4.22	4.5E-02	5.4E-02	0.84	N/A	-1.19	OKAY
SLC28A1	E05	9.38	7.96	1.5E-03	4.0E-03	0.37	N/A	-2.67	OKAY
SLC28A2	E06	10.98	10.30	5.0E-04	7.9E-04	0.63	N/A	-1.59	OKAY
SLC28A3	E07	13.04	10.34	1.2E-04	7.7E-04	0.15	N/A	-6.49	A
SLC29A1	E08	3.71	4.81	7.7E-02	3.6E-02	2.15	N/A	2.15	OKAY
SLC29A2	E09	8.34	9.51	3.1E-03	1.4E-03	2.24	N/A	2.24	OKAY
SLC2A1	E10	11.25	11.78	4.1E-04	2.8E-04	1.44	N/A	1.44	OKAY
SLC2A2	E11	3.35	2.76	9.8E-02	1.5E-01	0.67	N/A	-1.50	OKAY
SLC2A3	E12	9.45	8.68	1.4E-03	2.4E-03	0.59	N/A	-1.70	OKAY
SLC31A1	F01	3.37	2.79	9.6E-02	1.4E-01	0.67	N/A	-1.49	OKAY
SLC38A2	F02	4.94	3.84	3.3E-02	7.0E-02	0.47	N/A	-2.15	OKAY
SLC38A5	F03	11.93	11.52	2.6E-04	3.4E-04	0.75	N/A	-1.34	OKAY
SLC3A1	F04	8.23	6.47	3.3E-03	1.1E-02	0.29	N/A	-3.40	OKAY
SLC3A2	F05	3.52	3.71	8.7E-02	7.6E-02	1.14	N/A	1.14	OKAY
SLC5A1	F06	13.41	9.44	9.2E-05	1.4E-03	0.06	N/A	-15.67	A
SLC5A4	F07	11.47	11.79	3.5E-04	2.8E-04	1.25	N/A	1.25	OKAY

SLC7A11	F08	12.89	15.31	1.3E-04	2.5E-05	5.36	N/A	5.36	B
SLC7A5	F09	9.10	8.36	1.8E-03	3.1E-03	0.60	N/A	-1.67	OKAY
SLC7A6	F10	8.85	9.90	2.2E-03	1.0E-03	2.07	N/A	2.07	OKAY
SLC7A7	F11	6.69	5.29	9.7E-03	2.6E-02	0.38	N/A	-2.65	OKAY
SLC7A8	F12	9.58	7.54	1.3E-03	5.4E-03	0.24	N/A	-4.12	OKAY
SLC7A9	G01	5.78	5.92	1.8E-02	1.6E-02	1.11	N/A	1.11	OKAY
SLCO1A2	G02	7.99	7.82	3.9E-03	4.4E-03	0.89	N/A	-1.13	OKAY
SLCO1B1	G03	5.00	3.63	3.1E-02	8.1E-02	0.39	N/A	-2.57	OKAY
SLCO1B3	G04	7.62	4.50	5.1E-03	4.4E-02	0.12	N/A	-8.69	OKAY
SLCO2A1	G05	9.44	9.82	1.4E-03	1.1E-03	1.30	N/A	1.30	OKAY
SLCO2B1	G06	4.99	4.00	3.1E-02	6.2E-02	0.50	N/A	-1.99	OKAY
SLCO3A1	G07	8.13	8.05	3.6E-03	3.8E-03	0.95	N/A	-1.06	OKAY
SLCO4A1	G08	10.50	9.22	6.9E-04	1.7E-03	0.41	N/A	-2.42	OKAY
TAP1	G09	4.34	4.75	4.9E-02	3.7E-02	1.33	N/A	1.33	OKAY
TAP2	G10	6.79	7.29	9.0E-03	6.4E-03	1.41	N/A	1.41	OKAY
VDAC1	G11	1.74	2.09	3.0E-01	2.4E-01	1.27	N/A	1.27	OKAY
VDAC2	G12	2.48	2.53	1.8E-01	1.7E-01	1.04	N/A	1.04	OKAY
ACTB	H01	-1.30	-0.92	2.5E+00	1.9E+00	1.30	N/A	1.30	OKAY
B2M	H02	-0.15	-1.08	1.1E+00	2.1E+00	0.53	N/A	-1.90	OKAY
GAPDH	H03	0.00	0.00	1.0E+00	1.0E+00	1.00	N/A	-1.00	OKAY
HPRT1	H04	6.73	6.65	9.4E-03	9.9E-03	0.95	N/A	-1.05	OKAY
RPLP0	H05	-1.60	-1.07	3.0E+00	2.1E+00	1.44	N/A	1.44	OKAY

Legend: Fold-Change (2^(- Delta Delta Ct)) is the normalized gene expression (2^(-Delta Ct)) in the Test Sample divided the normalized gene expression (2^(-Delta Ct)) in the Control Sample. Fold-Regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. Fold-change values less than one indicate a negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change. Fold-change and fold-regulation values greater than 2 are indicated in red; fold-change values less than 0.5 and fold-regulation values less than -2 are indicated in blue. *p*-values: The *p* values are calculated based on a Student's t-test of the replicate 2^(-Delta Ct) values for each gene in the control group and treatment groups, and *p* values less than 0.05 are indicated in red. Comments: A: This gene's average threshold cycle is relatively high (> 30) in either the control or the test sample, and is reasonably low in the other sample (< 30). These data mean that the gene's expression is relatively low in one sample and reasonably detected in the other sample suggesting that the actual fold-change value is at least as large as the calculated and reported fold-change result. This fold-change result may also have greater variations if *p* value > 0.05; therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene. B: This gene's average threshold cycle is relatively high (> 30), meaning that its relative expression level is low, in both control and test samples, and the *p*-value for the fold-change is either unavailable or relatively high (P > 0.05). This fold-change result may also have greater variations; therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene. C: This gene's average threshold cycle is either not determined or greater than the defined cut-off (deault 35), in both samples meaning that its expression was undetected, making this fold-change result erroneous and un-interpretable.


Overall survival
Cumulative recurrence rate
Months after surgery
Low
High
SLC29A1 p=0.023
SLC29A1 p=0.002
SLC29A2 p=0.76
SLC29A2 p=0.34
SLC7A6 p=0.22
SLC7A6 p=0.075
SLC38A2 p=0.937
SLC38A2 p=0.624
SLCO4A1 p=0.497
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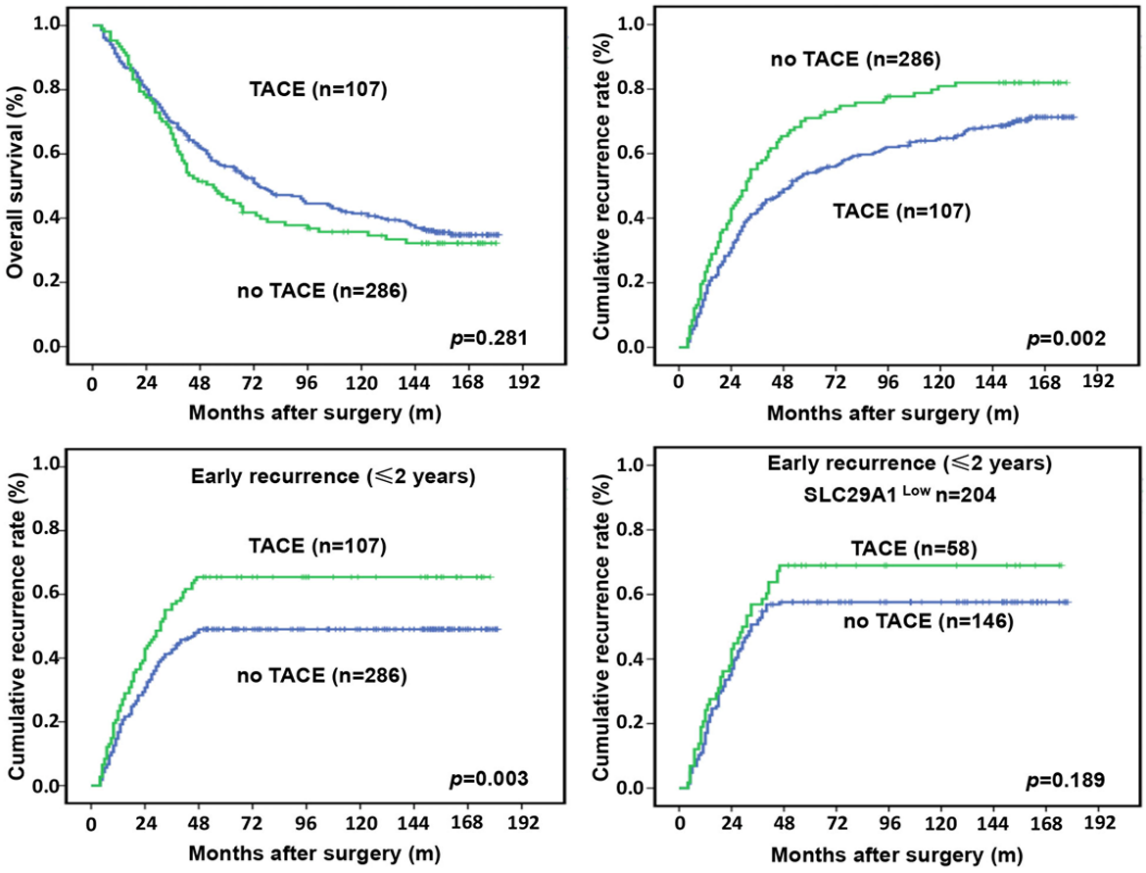
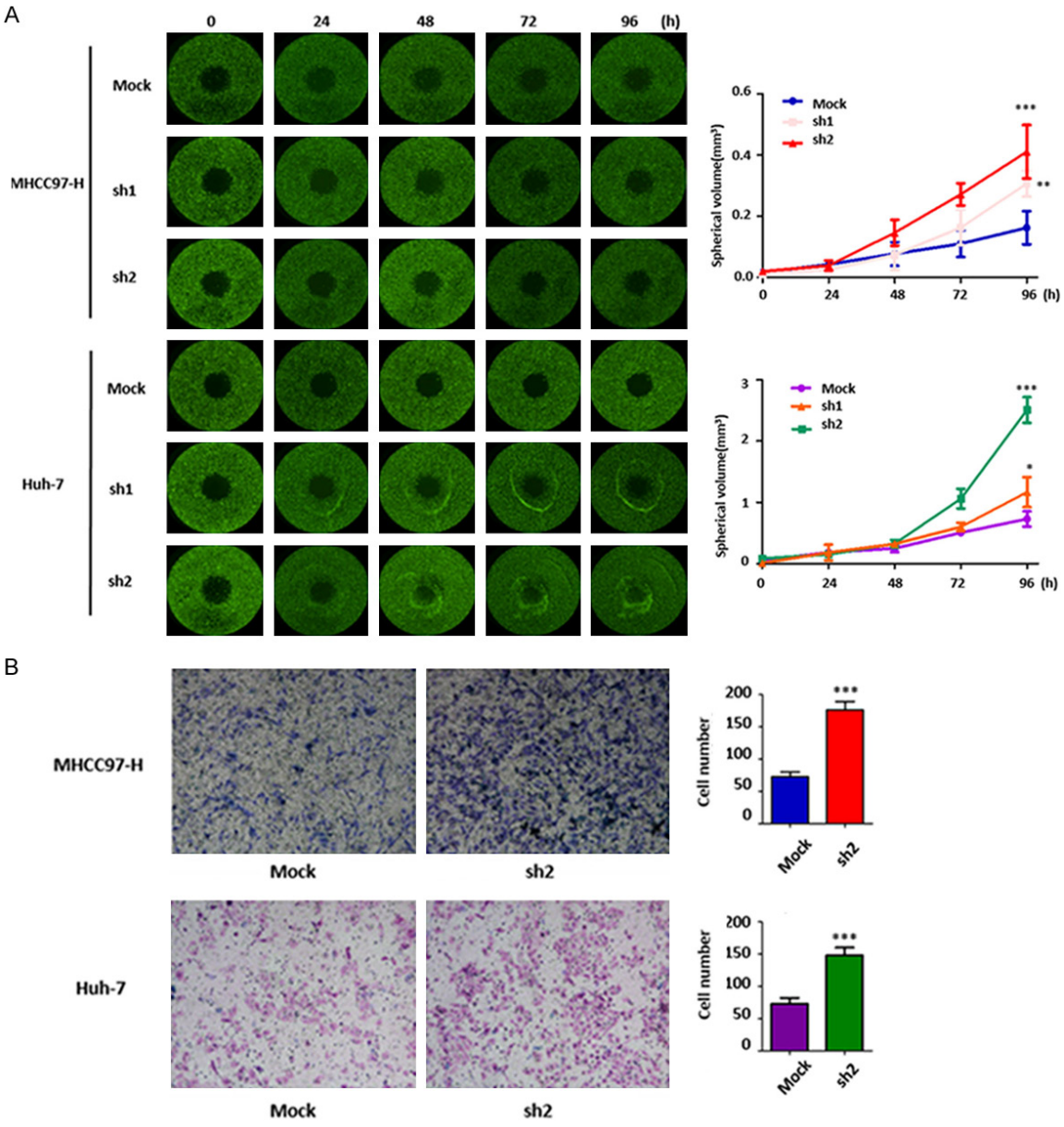
SLCO1B1 p=0.119
SLCO1B1 p=0.266
SLC7A7 p=0.823
SLC7A7 p=0.983
SLC28A1 p=0.708
SLC28A1 p=0.118
SLC19A3 p=0.204
SLC19A3 p=0.253
SLC10A1 p=0.578
SLC10A1 p=0.264
SLC3A1 p=0.478
SLC3A1 p=0.423
SLC7A8 p=0.612
SLC7A8 p=0.872
SLC22A1 p=0.445
SLC22A1 p=0.885
SLCO1B3 p=0.961
SLCO1B3 p=0.504

Supplementary Figure 1. Survival and recurrence analyses of 75 patients with hepatocellular carcinoma according to *SLCO1B3*, *SLC22A1*, *SLC7A8*, *SLC3A1*, *SLC10A1*, *SLC19A3*, *SLC28A1*, *SLC7A7*, *SLCO1B1*, *SLCO4A1*, and *SLC38A2*, *SLC7A6*, *SLC29A1*, and *SLC29A2* expression levels.



Supplementary Figure 2. Adjuvant transcatheter arterial chemoembolization (TACE) after surgery did not reduce the early recurrence (≤ 2 year) rate, compared with no adjuvant TACE treatment.



Supplementary Figure 3. A. Cancer cell migration was measured using an Aurora nucleic acid extraction system. B. The invasion of cancer cells was measured using transwell migration assay.