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Overexpression of Uric Acid Transporter SLC2A9 Inhibits Proliferation of Hepatocellular Carcinoma Cells

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Hepatocellular carcinoma (HCC) is the third leading cause of cancer-associated mortality worldwide. Although the mechanisms of HCC progression are not well understood, recent studies demonstrated the potential contribution of uric acid transporter SLC2A9 to tumor suppression. However, the roles and underlying mechanisms are still unknown. We aimed to study the roles and mechanisms of SLC2A9 in HCC. The present study showed that SLC2A9 expression was decreased in human HCC tissues and cell lines. In addition, overexpression of SLC2A9 inhibited HCC cell proliferation. SCL2A9 induced HCC cell apoptosis by inhibiting the expression of caspase 3. Our study also revealed that upregulation of SLC2A9 reduced intracellular reactive oxygen species (ROS) accumulation. Furthermore, SLC2A9 increased the mRNA and protein expression of tumor suppressor p53 in HCC cells. Probenecid inhibits SLC2A9-mediated uric acid transport, which promotes cell proliferation, inhibits cell apoptosis, induces intracellular ROS, and decreases the expression of p53 in HCC cells. Therefore, the present study demonstrated that SLC2A9 may be a novel tumor suppressor gene and a potential therapeutic target in HCC.

Key words: SLC2A9; HCC; Cell proliferation; Apoptosis; ROS; P53

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality and has growing trends worldwide[1,2]. Although treatments for HCC have improved, the majority of patients do not have the opportunity for surgery because of late diagnosis. The long-term prognosis for patients with HCC is not optimistic, with only a 30% 5-year survival rate[3,4]. The lack of early diagnosis with biomarkers and the shortage of effective therapeutic targets contribute to the unfavorable outcome. The carcinogenesis of HCC is complex and involves multiple genes which alter biological processes[5]. Therefore, the molecular mechanisms of HCC pathogenesis urgently need to be elucidated to find novel diagnostic biomarkers and therapeutic targets for HCC[6,7]. A recent study indicated that the expression and the roles of solute carrier family 2 member 9 (SLC2A9) in various tumors have been investigated. Furthermore, the aberrant expression and function of SLC2A9 occurred in the pathogenesis of HCC[8].

SLC2A9 is an important urate transportsome and a member of the facilitated glucose transporter family 21[9,10]. Uric acid (UA) transporter SLC2A9 has vital roles in the metabolism of cells by transporting different sugars like glucose and fructose[11,12]. A novel homozygous SLC2A9 mutation is related to renal-induced hypouricemia[13]. Moreover, SLC2A9 genetic variants impact the age of onset of Parkinson's disease in patients[14]. Nuclear receptor family member HNF4 promotes the transcriptional regulation of SLC2A9[15]. In various embryonic as well as kidney, liver, placenta, and pancreas tissues, the expression of SLC2A9 is normally detected[16]. SLC2A9 mRNA was significantly downregulated in the kidney, adrenal gland, prostate, and testis cancers compared with the corresponding normal tissues. Also, the downregulated SLC2A9 is associated with poor survival in gastric cancer patients[17]. Thus, these data imply that SLC2A9 might be a potential tumor suppressor. However, the exact role of SLC2A9 in HCC has not been fully explored.

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In the present study, the effects of SLC2A9 in cell proliferation, apoptosis, and reactive oxygen species (ROS) were investigated in HCC. The results revealed that SLC2A9 was decreased in HCC tissues and cell lines. Upregulation of SLC2A9 inhibited cell proliferation, induced HCC cell apoptosis, and reduced intracellular ROS accumulation. The above results suggested that SLC2A9 played key roles in the development and progression of HCC due to its tumor suppressor effect.

MATERIALS AND METHODS

Patient Tissues

HCC tissues and their adjacent normal tissues were collected from 10 clinical patients undergoing HCC resection. All these tissues were immediately stored in liquid nitrogen after surgery. The present study was approved by the medical review board, and all patients signed a written informed consent before the experiments.

Cell Culture

Human HCC cell lines QGY-7701, SMMC-7721, and HepG2 and the human hepatocyte cell line LO2 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, P.R. China). The QGY-7701, SMMC-7721, HepG2, and LO2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) and incubated at 37°C in a 5% CO_2 humidified incubator.

Plasmids and Adenoviruses

The human SLC2A9 plasmid was constructed using full-length SLC2A9 complementary DNA (cDNA) amplified with reverse transcriptase polymerase chain reaction (RT-PCR) from HepG2 cells and pcDNA3.1 vectors (Invitrogen). All cloned constructs were identified by direct DNA sequencing (Geneseeq, Nanjing, P.R. China). The untagged SLC2A9 in recombinant adenoviruses was produced using the AdEasy XL Adenoviral Vector System (Stratagene, San Diego, CA, USA) in line with the manufacturer's protocol.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNAs were isolated from clinical tissues and HCC cells using TRIzol reagent (Invitrogen) and then reversely transcribed into cDNA applying RevertAid First Stand cDNA Synthesis (Thermo Fisher Scientific Inc., Hudson, NH, USA). Four microliters of 5× Reaction Buffer, 1 μg of RNA, and RNase-free ddH_2O were used for reverse transcription. The thermocycler conditions were set at: 25°C for 5 min, 42°C for 60 min, 75°C for 5 min, and 4°C for 5 min. In addition, a relative expression of mRNA was determined by qPCR with a SYBR® Premix Ex Taq™ Tli RNaseH Plus PCR Kit (Takara Bio Inc., Otsu, Shiga, Japan) and an Applied Biosystems Prism® 7300 Sequence Detector (Thermo Fisher Scientific Inc.). GAPDH was regarded as the internal reference, and 2− Cq was used for mRNA relative expression determination[18].

Western Blotting

Cells from each group were detached with trypsin and washed with prechilled PBS twice. Total cellular protein was isolated using lysis buffer and incubated on ice for protein extraction. Protein concentration was determined by the BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, P.R. China). Equal amounts of protein samples (2 μg) were separated via 12% SDS-PAGE and then transferred to a PVDF membrane (Millipore Corporation, Billerica, MA, USA). In addition, the membrane was blocked with 10% skimmed milk in PBS containing 0.1% Tween 20 for 2 h. The incubation with primary and secondary antibodies (Abcam, Cambridge, MA, USA) was 1 h and 45 min, respectively, at room temperature. Detection was by peroxidase-conjugated secondary antibodies (Kirkegaard & Perry Labs, Gaithersburg, MD, USA). ImageJ software (NIH, Bethesda, MD, USA) was used for densitometry analysis.

MTT Assay

The cell proliferation rate was measured employing the MTT method. Briefly, HepG2 cells were plated into 96-well plates (about 5 10^3 cells/well) and incubated at 37°C after infection. At various time points (12, 24, or 48 h), the cultivated medium of cells was removed, the cells were washed twice with PBS, and then incubated with 20 μl of MTT (Sigma-Aldrich, St. Louis, MO, USA) reagent at 37°C. Then 150 μl of DMSO was added to dissolve formazan and terminate the reaction. The absorbance value (OD) was measured at 490 nm on a microplate reader.

Flow Cytometry Assay

HepG2 cells transfected with Adv-SLC2A9, Adv-NC, control, or probenecid were seeded into 12-well plates and incubated at 37°C with 5% CO_2. For the apoptosis assay, cells were stained with propidium iodide (10 μg/ml; Sigma-Aldrich) and annexin V-FITC (50 μg/ml; BD Biosciences, San Jose, CA, USA) for 15 min at room temperature in the dark. Subsequently, the cells were examined by flow cytometry (FACScan; BD Biosciences).

Measurement of Intracellular ROS

Expression levels of intracellular ROS were measured by the oxidative conversion of 2,7-dichlorofluorescein diacetate (DCFH-DA) to the highly fluorescent compound 2,7-dichlorofluorescein (DCF). Cells were

trypsinized and incubated with 10 μM DCFH-DA at 37°C for 1 h in the dark. A GLOMAX Multidetection System (Promega Corporation, Madison, WI, USA) with a blue filter (excitation: 490 nm; emission: 510–570 nm) was used for DCF fluorescence evaluation. All experiments were performed in triplicate.

Statistical Analysis

All quantitative data are displayed as mean±standard deviation (SD). Moreover, the analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA, USA). Statistical comparisons between each group were conducted with unpaired *t*-test or one-way analysis of variance followed by Bonferroni's multiple comparisons test. A value of $p<0.05$ indicates a statistically significant difference.

RESULTS

Downregulated SLC2A9 in HCC Tissues and Cell Lines

To investigate the expression of SLC2A9 in the development of HCC, 30 pairs of human HCC tissues and adjacent tissues were analyzed using the qRT-PCR method. The results showed that significant downregulation of SLC2A9 was observed in HCC tissues compared with adjacent normal tissues (Fig. 1A). Next, we analyzed the SLC2A9 levels in the HCC cell lines QGY-7701, SMMC-7721, and HepG2 and the human hepatocyte cell line LO2. qRT-PCR analysis indicated that SLC2A9 was more downregulated in the QGY-7701, SMMC-7721, and HepG2 cells compared with LO2 cells (Fig. 1B). Consistent with the findings in HCC tissues, the expression of SLC2A9 in HCC cells was lower than that in the LO2 cells. Together, these results revealed that SLC2A9 was downregulated in HCC.

SLC2A9 was dysregulated in HCC. Furthermore, the results have been suggested that SLC2A9 may affect the levels of UA in HCC. Then we analyzed the UA concentration using the Uric Acid Assay Kit (Nanjing Jiancheng Bioengineering Institute) in HCC tissues and paired adjacent matched noncancerous tissue homogenates. Interestingly, as shown in Figure 1C, UA concentration was markedly decreased in HCC tissues. Furthermore, we analyzed the intracellular and secreted UA in HCC cell lines QGY-7701, SMMC-7721, HepG2, and LO2. We found that the intracellular UA was significantly increased in the QGY-7701, SMMC-7721, and HepG2 cells compared to the LO2 cells (Fig. 1D), and the secreted levels of UA was significantly decreased in the QGY-7701, SMMC-7721, and HepG2 cells compared to the LO2 cells (Fig. 1E). These data indicated that SLC2A9 was

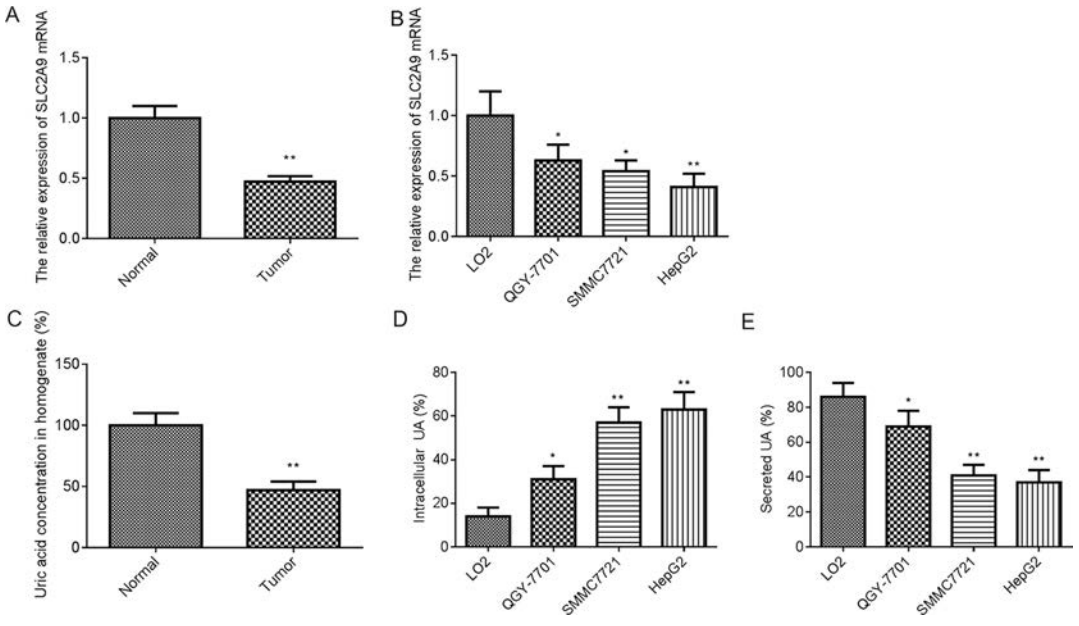

Figure 1. Expression of solute carrier family 2 member 9 (SLC2A9) in human hepatocellular carcinoma (HCC) tissues and cell lines. (A) The expression levels of SLC2A9 in 30 pairs of human HCC tumor and normal tissues were detected using quantitative real-time polymerase chain reaction (qRT-PCR). (B) The expression levels of SLC2A9 mRNA in the human HCC QGY-7701, SMMC-7721, and HepG2 cell lines and the human hepatocyte cell line LO2 were detected by qRT-PCR. (C) The uric acid concentration in HCC tissues and paired adjacent matched noncancerous tissue homogenates was analyzed. (D) The intracellular levels of uric acid in the HCC cell lines QGY-7701, SMMC-7721, and HepG2, and LO2 were analyzed. (E) Secreted levels of uric acid in HCC cell lines QGY-7701, SMMC-7721, and HepG2, and LO2 were analyzed. *$p<0.05$, **$p<0.01$.

critical in HCC urate homeostasis, so UA could be a new biomarker for HCC.

SLC2A9 Enhanced Cell Survival in HCC HepG2 Cells

In order to investigate the biological function of SLC2A9 in HCC cells, we assessed the mRNA level of SLC2A9 in the HCC cell lines and the LO2 cell line. Our results showed that SLC2A9 was lower in HepG2 than in the LO2, QGY-7701, and SMMC-7721 cell lines (Fig. 1B). In the present study, we used HepG2 cells with low expression of SLC2A9 as a model to explore the function of SLC2A9 on HCC cells. To evaluate whether SLC2A9 can act as a tumor suppressor, we measured the effect of SLC2A9 overexpression on cell proliferation. We transfected HepG2 cells with an Adv vector containing an SLC2A9 construct, or Adv-NC as negative control (Fig. 2A and B). Furthermore, probenecid is able to inhibit SLC2A9-mediated UA transport[19]. To evaluate the SLC2A9 UA transport activity in ROS reduction, we added the drug probenecid to HepG2 cells. As shown in Figure 2C, overexpression of SLC2A9 significantly inhibits cell proliferation in HepG2, and probenecid significantly promotes cell proliferation in HepG2.

SLC2A9 Induced Apoptosis in HCC HepG2 Cells

We also performed the flow cytometry assay to study the influence of SLC2A9 on cell apoptosis. As shown in Figure 3, the results demonstrated that cell apoptosis was observed in Adv-SCL2A9 transfection while the cell apoptosis decreased in the probenecid group in HepG2. Furthermore, the ratio of apoptotic cells was increased in Adv-SLC2A9 (44%) compared with the negative control groups (20%), and the ratio of apoptotic cells was reduced in the probenecid group (10%) compared with the negative control group (20%) (Fig. 3B). Caspase 3 is closely associated with apoptosis[20,21]. Therefore, Western blotting was also performed to detect the mRNA and protein expression of caspase 3. The results indicated that SLC2A9 increased the expression of caspase 3 mRNA and protein (Fig. 3C and D). Probenecid decreases the mRNA and protein expression of SLC2A9. These data indicate that SLC2A9 induced apoptosis in HCC HepG2 cells.

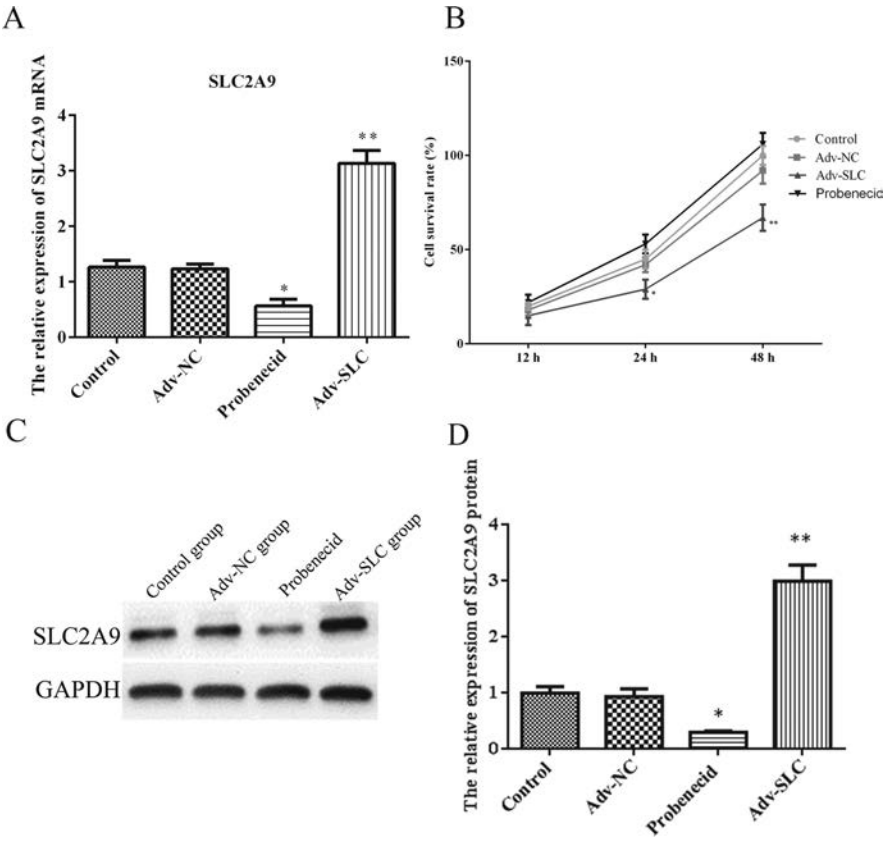

Figure 2. SLC2A9 suppresses proliferation of HepG2 cells. (A) qRT-PCR was used to verify the expression of SLC2A9 protein, and (C, D) Western blotting was used to determine the protein expression of SLC2A9 in HepG2 cells infected with Adv-SLC2A9, Adv-NC, control, or probenecid. (B) MTT assays were used to determine the cell survival of HepG2 cells infected with Adv-SLC2A9, Adv-NC, control, or probenecid. *$p < 0.05$, **$p < 0.01$.

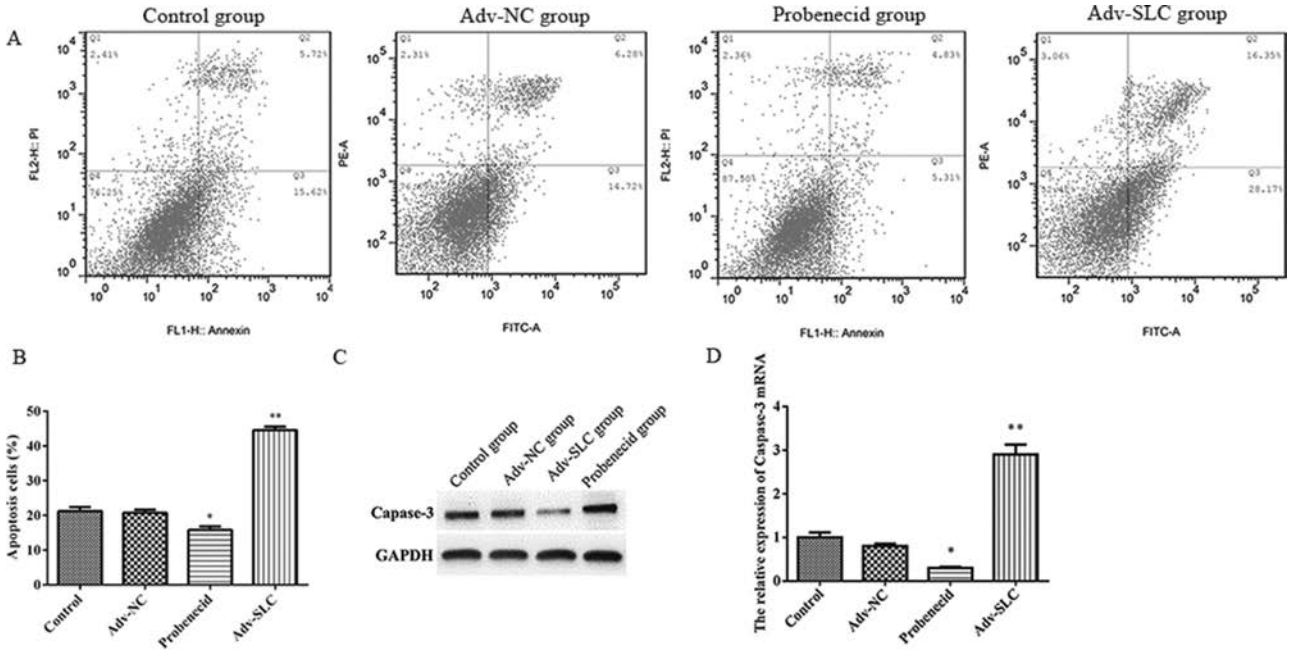

Figure 3. SLC2A9 induces cell apoptosis in HepG2 cells. (A) At 48 h after infection with Adv-SLC2A9, Adv-NC, control, or probenecid, HepG2 cells were collected for analysis of apoptosis. Flow cytometry was used to measure apoptosis. (B) Percent of cell apoptosis was calculated. (C) Western blotting was used to determine the protein expression of caspase 3, and (D) qRT-PCR was used to determine the mRNA expression of caspase 3 in HepG2 cells infected with Adv-SLC2A9, Adv-NC, control, or probenecid. *$p<0.05$, **$p<0.01$.

SLC2A9 Promoted the ROS Reduction in HCC

A recent study reported that SLC2A9 could facilitate UA traffic resulting in the ROS reduction[17]. SLC2A9 has not been reported to have an antioxidant function in HCC cells. Therefore, the effects of ectopic SLC2A9 expression on ROS production was performed. The results demonstrated that ectopic SLC2A9 successfully reduced ROS production in HepG2 cells (Fig. 4). Moreover, the increased probenecid significantly induced ROS in HepG2 (Fig. 4). These data suggest that SLC2A9 contributes to ROS reduction.

SLC2A9 Induced p53 mRNA and Protein Expression in HCC

The effects of SLC2A9 on p53 were also measured to investigate the apoptosis pathway. The results suggested that the expression of p53 mRNA and protein was increased by SLC2A9 and decreased by probenecid in HCC HepG2 cells (Fig. 5). Thus, SLC2A9 induces p53 mRNA and protein in HCC.

DISCUSSION

The development of HCC occurs in a multistep manner, and the mechanisms of HCC remain to be further explored. In the regions of Asia, HCC is mainly associated with hepatitis B virus (HBV) infection[9]. Previous studies have demonstrated that differential expression of genes in patients with HCC is correlated with cancer development and progression[22,23]. The investigation of novel targets for HCC requires the mechanisms underlying HCC carcinogenesis to be revealed.

In humans and nonhuman primates, the final product of purine degradation is UA. However, in mammals, the enzyme uricase converts UA to allantoin. Due to the oxidative properties and potential induction of inflammation by

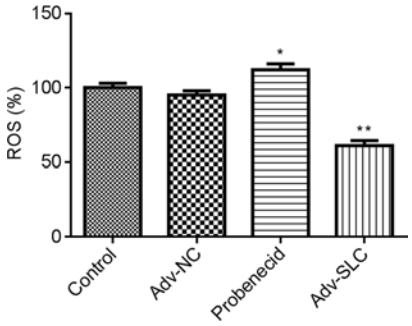

Figure 4. SLC2A9 reduces intracellular reactive oxygen species (ROS) levels in HepG2 cells. The levels of ROS in HepG2 cells infected with Adv-SLC2A9, Adv-NC, control, or probenecid. *$p<0.05$, **$p<0.01$.

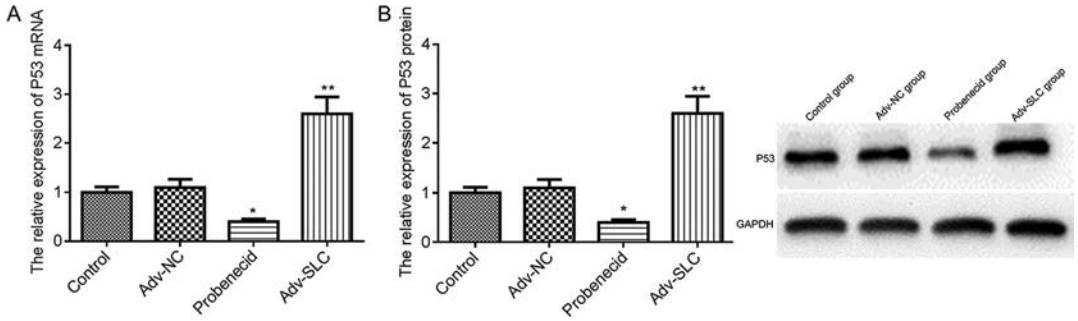

Figure 5. SLC2A9 promotes the mRNA and protein expression of p53 in HepG2 cells. (A) qRT-PCR was used to determine the mRNA expression of p53, and (B) Western blotting was used to determine the protein expression of p53 in HepG2 cells infected with Adv-SLC2A9, Adv-NC, control, or probenecid. *$p<0.05$, **$p<0.01$.

UA, this could directly cause injury of endothelial cells. Therefore, SLC2A9 has been recognized as the UA transporter with high capacity in humans[24,25]. SLC2A9 plays an essential role in maintaining blood UA levels[26]. The mutation of SLC2A9 could lead to renal hypouricemia due to the reduced UA reabsorption in renal proximal tubules[27].

SLC2A9 is widely expressed in various tissues including the liver, lung, heart, kidneys, and intestines[28,29]. The increased expression of SLC2A9 could inhibit the excretion of urate in the kidney[30]. Although SLC2A9 was known as the main factor in urate homeostasis, studies of the functions of SLC2A9 in cancers are limited over the past decades. A new study has reported that UA may function as an antioxidant due to the reduced ROS levels by SLC2A9 via increased UA traffic in various cell types[17]. The present study demonstrated that SLC2A9 was downregulated in HCC tissues and cell lines. Overexpression of SLC2A9 inhibited HCC cell proliferation. By contrast, increased probenecid concentrations significantly inhibited the SLC2A9 UA transport activity while promoting cell survival and inhibiting cell apoptosis.

Interestingly, we found that upregulation of SLC2A9 decreased levels of ROS, and probenecid significantly induced ROS in HepG2 cells. We hypothesized that upregulation of SLC2A9 could induce cell apoptosis due to the impact of ROS reduction. Furthermore, the previous studies indicated that the increased expression level of intracellular ROS could mediate the early and late steps of apoptosis involving mitochondrial dysfunction[31,32]. In our study, an increased SLC2A9 expression level contributes to the reduction of ROS in HCC. A new study suggested that the p53–SLC2A9 pathway could potentially prevent cancer development because of the antioxidant mechanism resulting in the prevention of ROS-associated damage[17]. p53 is an important tumor suppressor gene, and it is mutated in various cancers, including HCC[33,34]. We found that SLC2A9 decreased the mRNA and protein expression of p53 in HCC HepG2 cells.

In conclusion, the present study demonstrated that SLC2A9 was downregulated in HCC tissues and cell lines. SLC2A9 inhibited the proliferation of HepG2 cells and induced cell apoptosis. SLC2A9 contributed to the reduction of ROS in HepG2 cells. SLC2A9 increased the mRNA and protein expression of p53 in HepG2 cells. The findings of the present study provide a deeper perspective of the functional mechanisms of SLC2A9 in HCC.

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