

Knockdown by shRNA identifies SLC44A5 as a potential therapeutic target in hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) has been ranked the second leading cause of cancer-associated mortality in China and the third leading cause of cancer-associated mortality worldwide. A number of previous studies investigating SLC44A5 have revealed important biological insight and disease-specific functions. Therefore, the present study investigated the expression of SLC44A5 in HCC tissues and cell lines, and assessed the effect of SLC44A5 on the viability, cell cycle, apoptosis and invasion of HCC cell lines. The mRNA expression of SLC44A5 in 35 HCC tissues was significantly higher compared with that in 35 normal tissues. The protein expression of SLC44A5 was notably high in MHCC-97H and SMMC-7721 cells compared with that in four other HCC cell lines. Knockdown of SLC44A5 using short hairpin RNA inhibited cell viability and arrested the cells in G1 of the cell cycle by reducing the expression of cell cycle markers, proliferating cell nuclear antigen and cyclin-dependent kinase 2 in MHCC-97H and SMMC-7721 cells. Furthermore, SLC44A5 knockdown cells also exhibited cell apoptosis by reducing the expression levels of apoptosis markers, caspase-3 and caspase-9 in MHCC-97H and SMMC-7721 cells, and suppressed invasion. The present results suggested that SLC44A5 is involved in HCC carcinogenesis and progression in HCC, indicating that SLC44A5 may be a molecular target in cancer therapy.

Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-associated mortality and one of the most lethal malignancies worldwide (1). Early stage HCC with preserved liver function can be effectively treated by resection, liver transplantation or percutaneously, and with a 5 year survival rate (2). Generally, HCC progression can be defined by a decrease in differentiation, the loss of tissue-specific gene expression, acceleration of cell proliferation and, ultimately, invasion (3). Patients with HCC often exhibit tumor cell invasion and metastasis prior to conventional diagnosis (4). Therefore, it is vital to study the molecular basis of HCC and explore novel therapeutic agents.

Choline is an essential nutrient that is required to make the major membrane phospholipid, phosphatidylcholine (PC) (5). Choline has been suggested to serve multiple roles in cancer development. Choline metabolites can affect DNA methylation and lead to a disruption of DNA repair (6). Choline can also modify cell signaling that is mediated by intermediary phospholipid metabolites, and can support the synthesis of cell membranes and, therefore, cell proliferation (7). In this sense, the identification and characterization of choline transporters in cancer may offer a novel target for the design of antitumor strategies. Therefore, it is important to identify choline transporters in cancer cells.

The choline transport system has been categorized into three transporter families: Polyspecific organic cation transporters (OCTs/SLC22A1-2) with low affinity for choline, high-affinity choline transporter 1 (CHT1/SLC5A7) and intermediate-affinity choline transporter-like proteins (CTLs/SLC44A1-5) (8). Previously, the CTLs/SLC44A1-5 were shown to be present in various human tissues (9). The presence of SLC44A1 protein in the rat and human central nervous systems, where it is found in neuronal, glial and endothelial cells, suggests that malfunction of this transporter may have important implications in nervous system development and repair following injury, and in neurodegenerative diseases (10). SLC44A2 is expressed as two isoforms, SLC44A2-P1 and SLC44A2-P2, in the heart, colon, lung, kidney and liver, which suggests that tissue-specific differences may influence its function in each tissue (11). Moderate SLC44A3 expression

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is present in the kidney, ileum and colon, while a notably strong SLC44A4 expression can be detected in the intestine, stomach and kidney. Much fewer data are available regarding the expression of SLC44A5, which was markedly low in the brain and higher in the spinal cord, however, to a lesser extent than SLC44A1 (12). However, SLC44A3-5 remain to be characterized functionally.

The gene expression suggests that SLC44A5 is important in development and progression of HCC. The effect of SLC44A5 knockdown on viability, cell cycle, apoptosis and invasion of HCC cell lines were assessed and the possible mechanism was also explored. The present study provided original documentation for the upregulation of SLC44A5 in HCC and it may be an effective therapeutic target for this disease.

Materials and methods

Clinical HCC samples. Specimens of HCC and paired non-cancerous tissues were obtained from 35 patients with stage I-IV HCC, admitted to Zhongnan Hospital of Wuhan University (Hebei, China), were enrolled in the present study. Ethical approval for the present study was provided by the independent Ethics Committee of Zhongnan Hospital of Wuhan University (Hebei, China). Written informed consent was obtained from all patients involved in the present study. All research was performed in accordance with the Helsinki Declaration of 1975 (13). No patient had received radiotherapy or chemotherapy. The percentage of tumor cellularity in the patients with HCC's tissue section was at least 70%, as determined by pathological examination of histology slides in hospital patient's cohort. HCC and paired non-cancerous tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until the total RNA was extracted. Tumor samples were composed of at least 80% of viable-appearing tumor cells on histological assessment.

Cell cultures and transfection. The HCC cell lines, including SMMC-7721, BEL-7404, MHCC-97H, MHCC-97L, HepG2 and HuH7, were obtained from the Cell Bank of Academia Sinica (Shanghai, China) and grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/μl penicillin and 100 μg/μl streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified incubator containing 5% CO₂ in air at 37°C. Short hairpin (sh)RNA targeting position 1,946-1,968 (GUUGCAGUUACAGAUGAAG) of human SLC44A5 mRNA was cloned into a lentiviral vector (PLKO.1-EGFP). The cells were transfected with shRNA (40 nM) using the Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according the manufacturer's protocol. A non-specific scramble shRNA sequence was used as negative control and the selective silencing of SLC44A5 was confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The cells were analyzed 48 h after transfection.

RT-qPCR. The total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according

to the manufacturer's protocol. cDNA was synthesized from RNA using a MMLV RT reagent kit (Thermo Fisher Scientific, Inc.). The DyNamo Flash SYBR Green qPCR kit (Finnzymes Oy, Espoo, Finland) was used, according to the manufacturer's instructions. qPCR was performed to detect the mRNA expression levels of indicated genes. The primers sequences are list in Table I. Relative quantification of SLC44A5 expression levels was determined using the 2^{-ΔΔC_q} method.

Western blot analysis. HCC tissues and cell lines transfected with SLC44A5 shRNA or negative controls vector were lysed using radioimmunoprecipitation buffer, supplemented with protease inhibitor (Beyotime Institute of Biotechnology, Inc., Shanghai, China). The protein concentration was estimated using the bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.). Equal quantities of protein (30 μg) were subsequently separated on 12% SDS-PAGE gels, and were subsequently transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Following blocking, the membranes were immunoblotted overnight at 4°C with primary antibodies: polyclonal goat anti-SLC44A5 (1:1,000; cat. no. sc-68054; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), monoclonal rabbit anti-proliferating cell nuclear antigen (PCNA; 1:5,000; cat. no. ab92552; Abcam, Cambridge, MA, USA), monoclonal rabbit anti-cyclin-dependent kinase (CDK)1 (1:1,000; cat. no. ab32384; Abcam), monoclonal rabbit anti-caspase-3 (1:3,000; cat. no. ab32351; Abcam), polyclonal rabbit anti-caspase-9 (1:1,000; cat. no. ab2014; Abcam) or monoclonal rabbit anti-GAPDH (1:1,500; cat. no. 5174; Cell Signaling Technology, Inc., Danvers, MA, USA). After washing, the membranes were incubated with goat anti-rabbit (cat. no. A0208) or donkey anti-goat (cat. no. A0181) horse-radish peroxidase-conjugated secondary antibodies (1:1,000; Beyotime Institute of Biotechnology, Inc.) at 37°C for 1 h. The membranes were washed with Tris-buffered saline containing 20% Tween 20 (Amresco, LLC, Solon, OH, USA) Signals were detected using an enhanced chemiluminescence system (Pierce, Rockford, IL, USA).

Cell viability assay. Cell viability was assessed using the Cell counting kit (CCK)-8 (Dojindo, Kumamoto, Japan). Briefly, control, negative control vector and SLC44A5 shRNA-treated cells were seeded into 96-well plates at an initial density of 3x10³ cells/well for 72 h. At specified time points, 100 μl CCK-8 solution was added to each well of the plate and the plate was incubated for 1 h. Cell viability was determined by scanning with an iMark microplate absorbance reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm. All samples were assessed in triplicate for each group and the experiment was repeated at least twice.

Cell cycle analysis. A total of ~1x10⁴ cells were removed at specified time points, washed twice with phosphate-buffered saline (PBS) and fixed in cold ethanol for 30 min. The cells were subsequently incubated with propidium iodide (PI) and 0.5 μg/μl RNase A for 30 min. Thereafter, the cells were analyzed on a BD Accuri C6 flow cytometer (BD Biosciences, San Diego, CA, USA).

Table I. Primes sequences used in this study.

Primer	Sequence
SLC44A5	
Forward	5'-GACATCGGGATTGACTAAG-3'
Reverse	5'-ATAATGGCGACTCGGATTC-3'
PCNA	
Forward	5'-GCCTGACAAATGCTTGCTGAC-3'
Reverse	5'-TTGAGTGCCTCCAACACCTTC-3'
CDK1	
Forward	5'-ACCATACCCATTGACTAAC-3'
Reverse	5'-ATAAGCACATCCTGAAGAC-3'
Caspase-3	
Forward	5'-AACTGGACTGTGGCATTGAG-3'
Reverse	5'-AAACACATTAGGCACAATCC-3'
Caspase-9	
Forward	5'-GGAAGAGGGACAGATGAATG-3'
Reverse	5'-TTGTTTGGCACCCTCAG-3'
GAPDH	
Forward	5'-CACCCACTCCTCCACCTTTG-3'
Reverse	5'-CCACCACCCTGTTGCTGTAG-3'

PCNA, proliferating cell nuclear antigen; CDK, cyclin dependent kinase.

Apoptosis assays. Apoptosis was determined by flow cytometry using an Annexin-V fluorescein isothiocyanate (FITC)/PI double-staining, according to the manufacturer's protocol (BioVision, Mountain View, CA, USA). Briefly, at 48 h after transfection, the cells were collected and resuspended in 500 μ l binding buffer, containing 5 μ l annexin-V/FITC and 5 μ l PI, and subsequently incubated for 5 min in the dark at room temperature. Analysis was immediately performed using a flow cytometer.

In vitro invasion assay. The upper well of the Transwell (Corning, Corning, NY, USA) was coated with Matrigel (BD Biosciences) at 37°C in a 5% CO₂ incubator for 1 h. The indicated cells were serum starved for 24 h. Subsequently, 5x10⁴ cells in 500 μ l serum-free DMEM were seeded into the upper well of the Transwell chamber. Culture medium, supplemented with 10% FBS (750 μ l) was added into the lower well of the chamber. After 48 h incubation, the cells in the upper well were removed with a cotton swab and the cells that migrated into the lower well were washed with PBS, fixed in 3.7% para-formaldehyde and stained with 0.2% crystal violet. Images of the cells were captured and cell number was counted using an Olympus CX41RF microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis. Statistical analyses were performed using the GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The comparison of different groups was analyzed using two-tailed Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

SLC44A5 is upregulated in HCC tissues and cell lines. To study the biological role of SLC44A5 in HCC, RT-qPCR was performed to detect the expression levels of SLC44A5 in tissues from patients with HCC. A total of 35 HCC tissue samples and 35 normal tissue samples were collected from Zhongnan Hospital of Wuhan University. As shown in Fig. 1A, the expression of SLC44A5 was higher in the HCC tissues compared with the normal control tissue (P<0.001). The protein expression levels of SLC44A5 were next determined in the HCC cell lines, SMMC-7721, BEL-7404, MHCC-97H, MHCC-97L, HepG2 and HuH7, by western blotting. SLC44A5 was expressed at a higher level in MHCC-97H and SMMC-7721 cell lines compared with other four cell lines (Fig. 1B). Therefore, MHCC-97H and SMMC-7721 cell lines were selected for further investigation.

Knockdown of SLC44A5 represses HCC cell viability. To investigate the functions of SLC44A5 on HCC, shRNA was designed and transfected into MHCC-97H and SMMC-7721 cells. The mRNA and protein expression levels of SLC44A5 in response to specific shRNA were assessed. The mRNA and protein expression levels of SLC44A5 were remarkably reduced in MHCC-97H and SMMC-7721 cells transfected with shRNA (Fig. 2A-D). No apparent change was observed in the cells with the negative control vector. To determine the role of SLC44A5 on the viability of HCC cell lines, the viability of MHCC-97H and SMMC-7721 cells transfected SLC44A5 shRNA was assessed by CCK-8 assay. As shown in Fig. 2E and F, 37±0.6 and 38±0.1% inhibition of cell viability was observed 72 h after shRNA transfection in MHCC-97H and SMMC-7721 cells, respectively.

Knockdown of SLC44A5 induces HCC cell cycle arrest at G0/G1 phase. To further validate the inhibition of cell viability by SLC44A5 shRNA, the cell cycle was analyzed in MHCC-97H and SMMC-7721 cells (Fig. 3). Cell cycle analysis revealed that knockdown of SLC44A5 with shRNA notably increased the rate of G0/G1 phase cells and reduced the S-phase cell population in both cell lines. These results indicated that knockdown of SLC44A5 in HCC cells may inhibit cell viability by arresting cell cycle progression at G0/G1 phase.

Knockdown of SLC44A5 induces HCC cell apoptosis. The apoptotic function of SLC44A5 was then assessed in MHCC-97H and SMMC-7721 cells using an annexin V-FITC/PI staining assay. As shown in Fig. 4, flow cytometry analysis revealed that inhibition of SLC44A5 in MHCC-97H cells significantly induced cell apoptosis by 29% compared with the corresponding cells transfected with negative control vector. Increasing cell apoptosis was also observed in SMMC-7721 cells transfected with SLC44A5 shRNA.

Knockdown of SLC44A5 induces HCC cell invasion. To assess whether SLC44A5 affected the invasive ability of HCC cells, Matrigel-coated membrane chamber invasion assays were performed. As shown in Fig. 5, a marked reduction in the invasive ability was observed in the SLC44A5 knock-down MHCC-97H and SMMC-7721 cells compared with

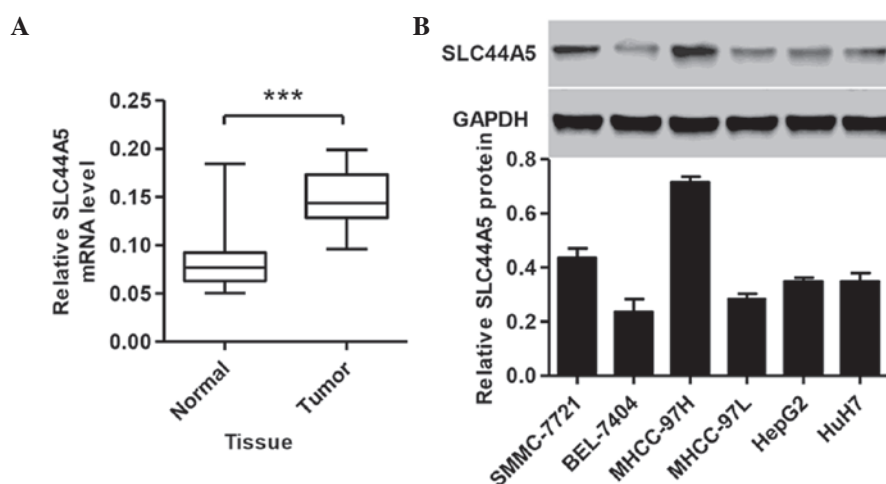


Figure 1. Expression of SLC44A5 in HCC tissues and cell lines. (A) The mRNA expression of SLC44A5 was significantly higher in the HCC tissues compared with that in the normal tissues. (B) The protein expression levels of SLC44A5 in MHCC-97H and SMMC-7721 cells were higher compared with the expression levels in four other HCC cell lines, and were therefore selected for further analysis. The data are presented as the mean \pm standard deviation of at least two independent studies each performed in triplicate (***) $P < 0.0001$, compared with the control). HCC, hepatocellular carcinoma.

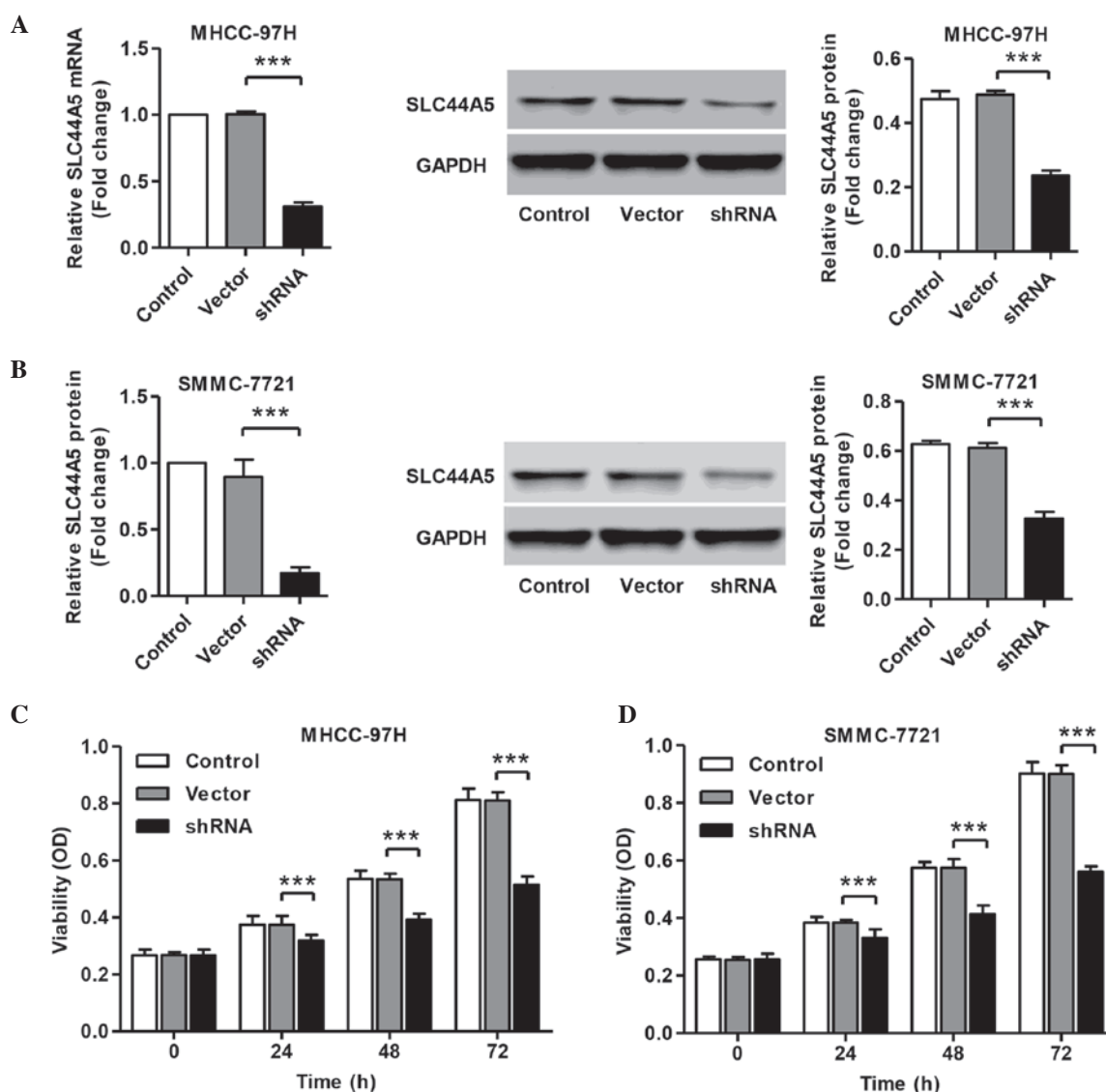


Figure 2. Knockdown of SLC44A5 inhibits HCC cell viability. Reverse transcription-quantitative polymerase chain reaction and western blotting revealed that the expression levels of SLC44A5 were significantly inhibited by SLC44A5 shRNA in (A) MHCC-97H and (B) SMMC-7721 cells. Cell counting kit-8 analysis identified significant inhibition of cell viability in (C) MHCC-97H and (D) SMMC-7721 cells. The data are presented as the mean \pm standard deviation of at least two independent studies each performed in triplicate (***) $P < 0.0001$). HCC, hepatocellular carcinoma; OD, optical density; sh, short hairpin.

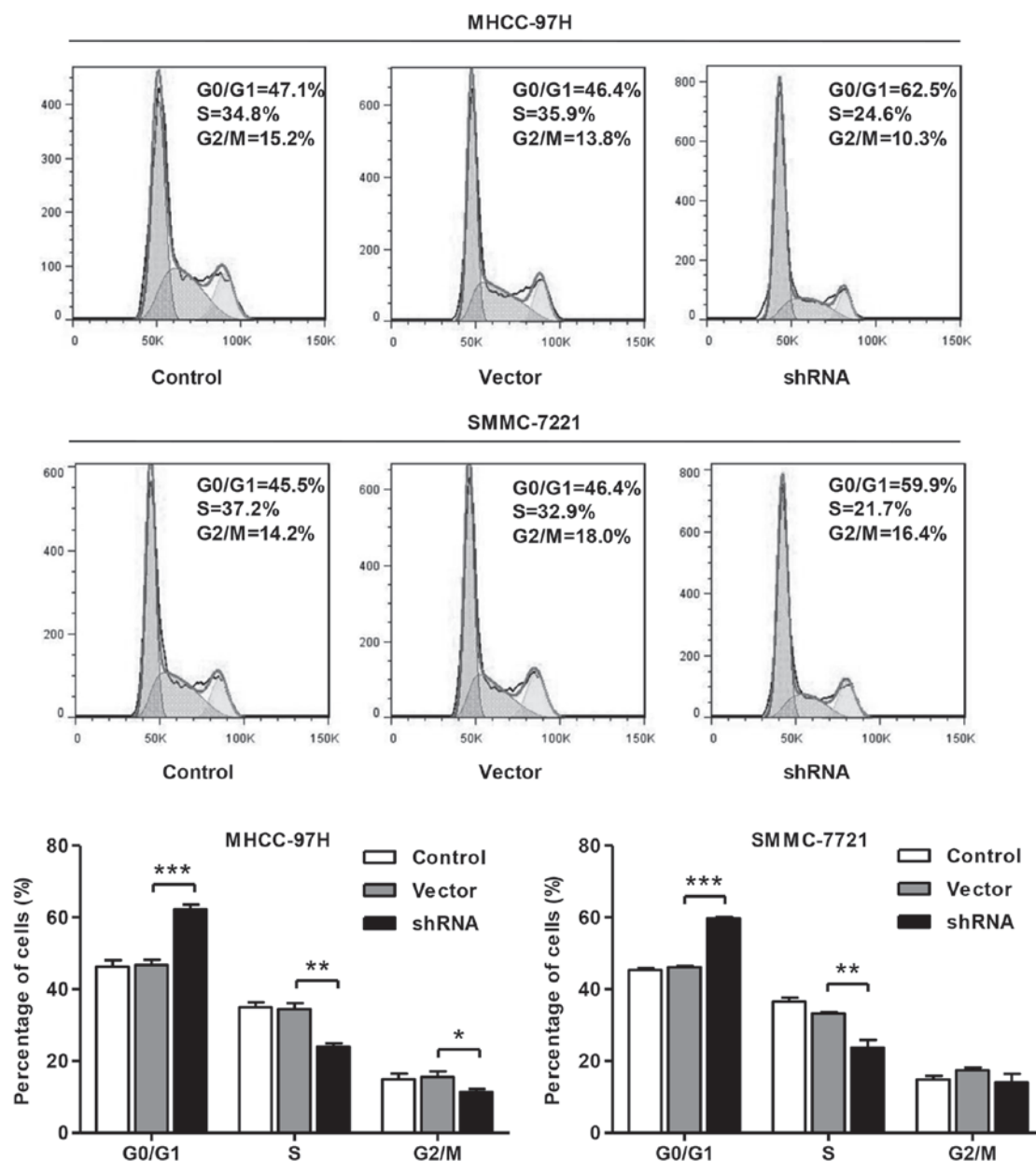


Figure 3. Knockdown of SLC44A5 arrests HCC in G0/G1 of the cell cycle. Flow cytometry analysis demonstrated a significant arrest in G0/G1 of the cell cycle of MHCC-97H and SMMC-7721 transfected with SLC44A5 shRNA. The data are presented as the mean \pm standard deviation of at least two independent studies each performed in triplicate (* $P < 0.01$, ** $P < 0.001$ and *** $P < 0.0001$). HCC, hepatocellular carcinoma; sh, short hairpin.

the negative control vector group. The number of invasive SLC44A5 shRNA MHCC-97H and SMMC-7721 cells was 47 ± 0.5 and $59 \pm 1.9\%$ of that of the negative control vector group, respectively.

Knockdown of SLC44A5 represses the expression of cell cycle and apoptosis markers. Having documented significantly induced cell cycle arrest and apoptosis of SLC44A5 knockdown in HCC cell lines, the present study wondered how SLC44A5 affects HCC cell cycle and apoptosis. To investigate this, the expression of cell cycle and apoptosis markers in MHCC-97H and SMMC-7721 cells were determined by RT-qPCR and western blotting. The results revealed that SLC44A5 knockdown resulted in a significant reduction in the mRNA and protein expression levels of PCNA, CDK1, caspase-3 and caspase-9, compared with the negative

control vector group in MHCC-97H and SMMC-7721 cells (Fig. 6A and B). The present data suggested that knockdown of SLC44A5 inhibits the expression of cell cycle and apoptosis-associated markers, which may contribute to the induction of G1 cell cycle arrest and apoptosis.

Discussion

HCC is one of the most highly malignant and lethal cancer types. The development and progression of HCC is a complicated process that involves the deregulation of multiple genes that are essential for cell biological processes (14,15). Previously, a distinct choline transporter called the SLC44A1-5 family was shown to be present in various human cancer cells (16). Using RT-qPCR, the mRNA expression profiles of SLC44A5 were measured in various cancer cell lines, including NCI-H69 (small

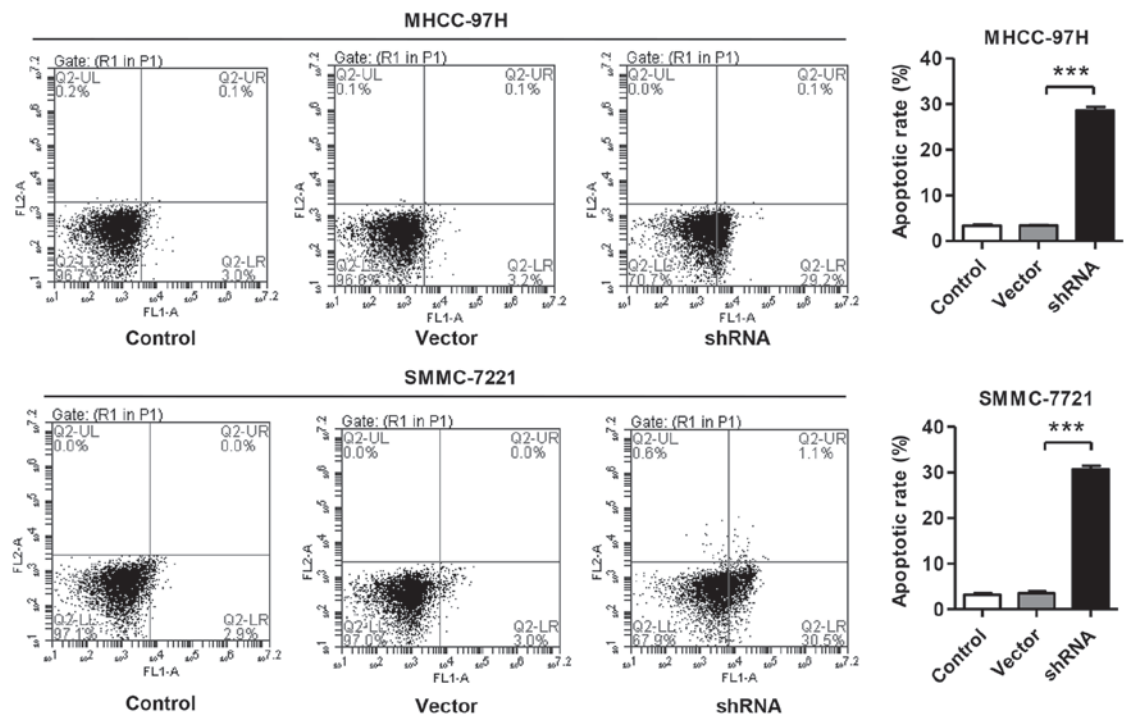


Figure 4. Knockdown of SLC44A5 induces HCC cell apoptosis. MHCC-97H and SMMC-7721 cells were stained with annexin V-fluorescein and apoptotic cells were analyzed by flow cytometry. The data are presented as the mean \pm standard deviation of at least two independent studies each performed in triplicate (**P<0.0001). HCC, hepatocellular carcinoma; sh, short hairpin.

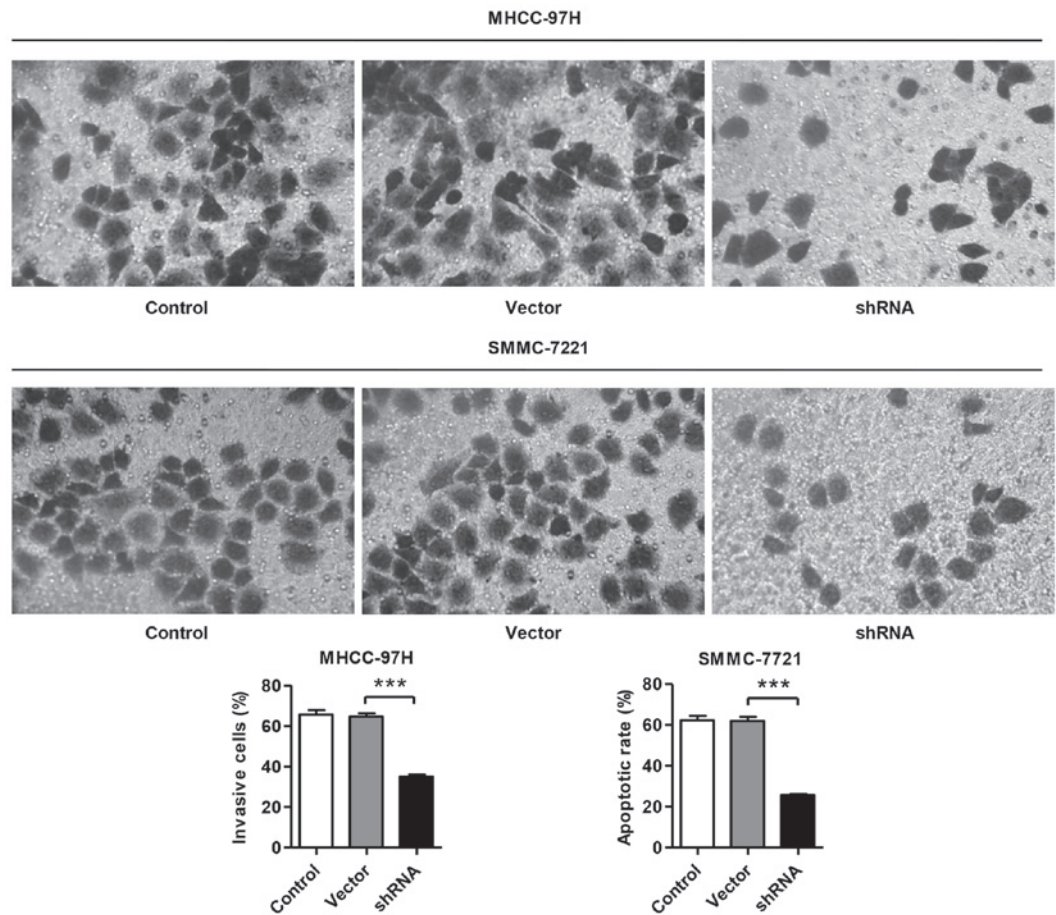


Figure 5. Knockdown of SLC44A5 induces HCC cell invasion. Invasion assays of MHCC-97H and SMMC-772 cells were performed using Transwell chamber coated with Matrigel. Cells that migrated from the upper well of a Transwell chamber into the lower well were stained, images were captured and the cells were counted. The data are presented as the mean \pm standard deviation of at least two independent studies each performed in triplicate (**P<0.0001). HCC, hepatocellular carcinoma; sh, short hairpin.

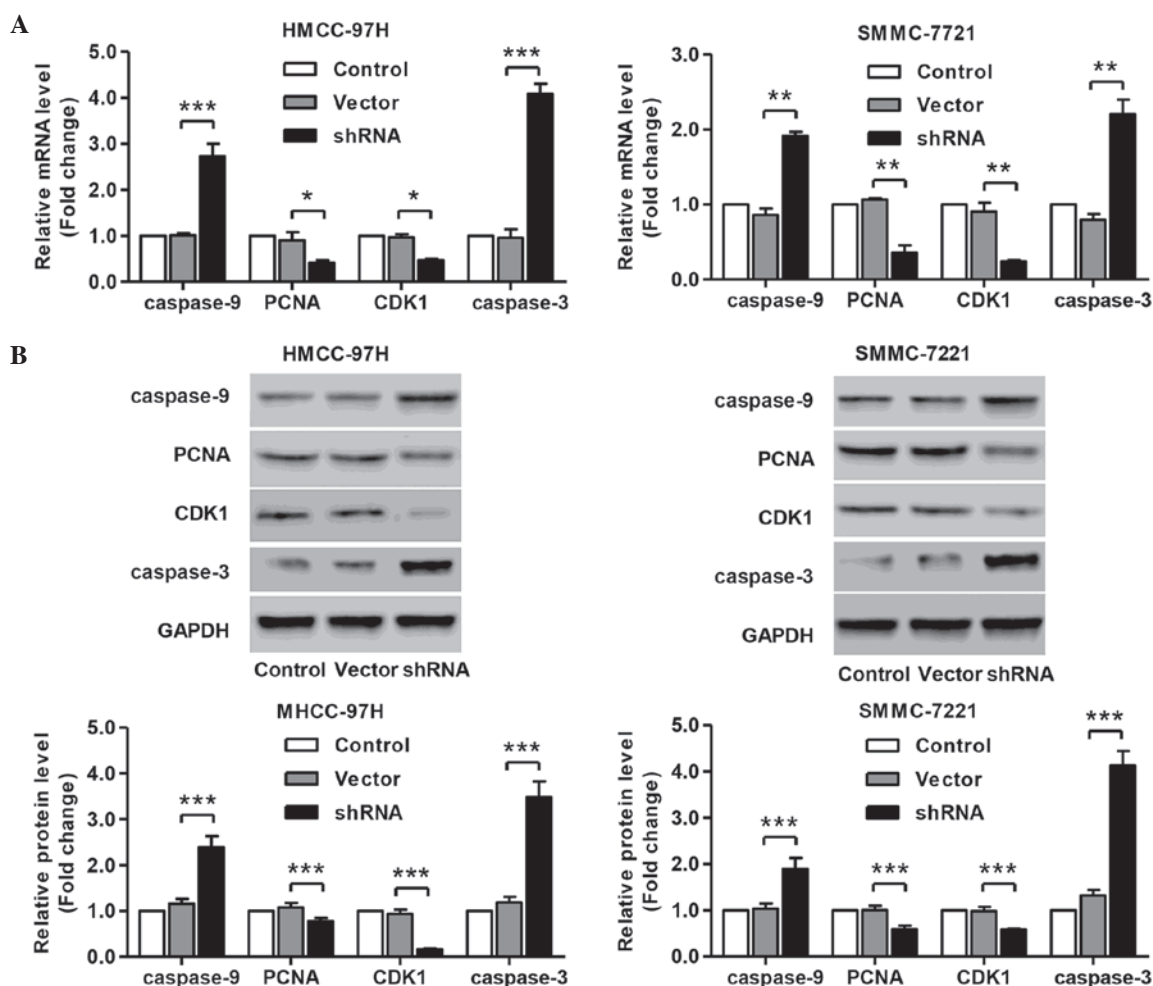


Figure 6. Cell cycle and apoptosis markers were assessed in SLC44A5 knockdown HCC cells. Equal quantities of mRNA and protein were analyzed by (A) reverse transcription-quantitative polymerase chain reaction and (B) western blotting with anti-PCNA, anti-CDK1, anti-caspase-3 and anti-caspase-9 antibodies. The data are presented as the mean \pm standard deviation of at least two independent studies each performed in triplicate (* P <0.01, ** P <0.001 and *** P <0.0001). HCC, hepatocellular carcinoma; PCNA, proliferating cell nuclear antigen; CDK, cyclin-dependent kinase; sh, short hairpin.

cell lung carcinoma), HT-29 (colon adenocarcinoma), Jurkat (acute T-cell leukemia), SH-SY5Y (dopaminergic, cholinergic, glutamatergic and adenosinergic neuroblastoma) and LA-N-2 (cholinergic neuroblastoma). Among these cell lines, SLC44A5 RNA was marginally expressed in HT-29 and Jurkat cells, however, it was notably expressed in NCI-H69, SH-SY5Y and LA-N-2 cells. Therefore, the expression of pattern of choline transporters differed according to the cancer cell type. Additionally, in the non-tumorigenic human mammary epithelial cell line, MCF-10A, the expression levels of SLC44A5 were very low compared with those in other cancer cells. The present study found that SLC44A5 mRNA levels were consistently upregulated in HCC clinical tissues compared with normal adjacent tissues; however, SLC44A5 protein levels varied in the six HCC cell lines, including SMMC-7721, BEL-7404, MHCC-97H, MHCC-97L, HepG2 and HuH7, suggesting that SLC44A5 expression differed according to the HCC cell type. Furthermore, it was shown that knockdown of SLC44A5 expression inhibited cell viability and invasion and promoted apoptosis in SMMC-7721 and MHCC-97H cells, indicating its role as an essential oncogene during HCC tumorigenesis.

Notably, the SLC44A1-5 family has been shown to be functionally important in the development of human lung,

prostate and colon carcinoma (17-20), however, the mechanisms underlying the effects of the SLC44A1-5 family remain to be elucidated. One of these proteins, SLC44A1, has previously been found to stimulate NCI-H69 cell growth and choline uptake, suggesting that SLC44A1 may function as a lung carcinogenic gene (17). The possible correlation between choline uptake and viability was also assessed (the effect of choline transporter inhibitors on the survival of various cancer cells). It has been reported that quinine, quinidine and desipramine inhibit choline uptake in various cell lines (20-22). These drugs can inhibit cell viability in various cancer cell lines, suggesting that cell viability may require an increased supply choline and induce cell death by obstructing the function of choline transporters. Such a potential oncogenic function of endogenous SLC44A5 in HCC had not previously shown *in vitro*, and the molecular mechanisms were also unknown. The present results revealed that knockdown of SLC44A5 effectively decreased the viability of SMMC-7721 and MHCC-97H cells, thus providing novel insights into the role of SLC44A5 in HCC development and progression.

Cell cycle regulation is frequently abnormal in most common malignancies, resulting in aberrant cell viability (23,24). Knockdown of SLC44A5 by shRNA significantly induced

G0/G1 cell cycle arrest in SMMC-7721 and MHCC-97H cells, which indicated that the inhibition of cell viability in HCC cells is due to the arrest of cell cycle progression. Cell cycle is mediated, directly or indirectly, by misregulation of cyclin-dependent kinases (CDKs) (25). In the present study, mRNA and protein expression levels of the cell cycle markers, CDK1 and PCNA, were notably reduced in SMMC-7721 and MHCC-97H cells treated with SLC44A5 shRNA, which was consistent with the results of induction of arrest G0/G1 cell cycle in SMMC-7721 and MHCC-97H cells treated with SLC44A5 shRNA, indicating an association between SLC44A5 function and the regulation of DNA replication and cell cycle progression in HCC cells.

G1-phase arrest of cell cycle progression provides an opportunity for cells to either undergo repairing or follow the apoptosis process. The effects of SLC44A5 knockdown on the induction of apoptosis were subsequently determined in SMMC-7721 and MHCC-97H cells. The flow cytometry data indicated that knockdown of SLC44A5 resulted in significant induction of apoptosis via increasing the mRNA and protein expression levels of the cell apoptosis markers, caspase-3 and caspase-9, which was consistent with previous studies showing that a gradual reduction in choline supplementation initially causes apoptosis in rat hepatocytes (8). In addition, knockdown of SLC44A5 also inhibited the cell invasion of SMMC-7721 and MHCC-97H cells. Due to its antiapoptosis and anti-invasion functions in HCC, SLC44A5 may be a potential therapeutic target worth further investigation.

Although choline has already been reported to be associated with HCC carcinogenesis, the present study revealed a critical role for choline transporter, SLC44A5, as a promoter of cell viability and invasion, and an inhibitor of apoptosis in HCC cells. Notably, the present study indicated the important role of SLC44A5 as a tumor promoter in HCC through the inhibition of choline uptake, suggesting that SLC44A5 may be a potential target for HCC therapy.

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References

- Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. *CA Cancer J Clin* 61: 69-90, 2011.
- Lin ZZ, Shau WY, Hsu C, Shao YY, Yeh YC, Kuo RN, Hsu CH, Yang JC, Cheng AL and Lai MS: Radiofrequency ablation is superior to ethanol injection in early-stage hepatocellular carcinoma irrespective of tumor size. *PloS One* 8: e80276, 2013.
- Lazarevich NL, Cheremnova OA, Varga EV, Ovchinnikov DA, Kudrjavitseva EI, Morozova OV, Fleishman DI, Engelhardt NV and Duncan S: Progression of HCC in mice is associated with a downregulation in the expression of hepatocyte nuclear factors. *Hepatology* 39: 1038-1047, 2004.
- Hashiguchi M, Ueno S, Sakoda M, Iino S, Hiwatashi K, Minami K, Ando K, Mataka Y, Maemura K, Shinchi H, *et al*: Clinical implication of ZEB-1 and E-cadherin expression in hepatocellular carcinoma (HCC). *BMC Cancer* 13: 572, 2013.
- Zeisel SH: Choline deficiency. *J Nutr Biochem* 1: 332-349, 1990.
- Anderson OS, Sant KE and Dolinoy DC: Nutrition and epigenetics: An interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. *J Nutr Biochem* 23: 853-859, 2012.
- Iorio E, Mezzanzanica D, Alberti P, Spadaro F, Ramoni C, D'Ascenzo S, Millimaggi D, Pavan A, Dolo V, Canevari S and Podo F: Alterations of choline phospholipid metabolism in ovarian tumor progression. *Cancer Res* 65: 9369-9376, 2005.
- Michel V, Yuan Z, Ramsdubir S and Bakovic M: Choline transport for phospholipid synthesis. *Exp Biol Med* (Maywood) 231: 490-504, 2006.
- O'Regan S, Traffort E, Ruat M, Cha N, Compaore D and Meunier FM: An electric lobe suppressor for a yeast choline transporter mutation belongs to a new family of transporter-like proteins. *Proc Natl Acad Sci USA* 97: 1835-1840, 2000.
- Machová E, O'Regan S, Newcombe J, Meunier FM, Prentice J, Dove R, Lisá V and Dolezal V: Detection of choline transporter-like 1 protein CTL1 in neuroblastoma x glioma cells and in the CNS, and its role in choline uptake. *J Neurochem* 110: 1297-1309, 2009.
- Kommareddi PK, Nair TS, Thang LV, Galano MM, Babu E, Ganapathy V, Kanazawa T, McHugh JB and Carey TE: Isoforms, expression, glycosylation and tissue distribution of CTL2/SLC44A2. *Protein J* 29: 417-426, 2010.
- Traffort E, Ruat M, O'Regan S and Meunier FM: Molecular characterization of the family of choline transporter-like proteins and their splice variants. *J Neurochem* 92: 1116-1125, 2005.
- Shephard DA: The 1975 Declaration of Helsinki and consent. *Can Med Assoc J* 115: 1191-1192, 1976.
- Kunter I, Erdal E, Nart D, Yilmaz F, Karademir S, Sagol O and Atabey N: Active form of AKT controls cell proliferation and response to apoptosis in hepatocellular carcinoma. *Oncol Rep* 31: 573-580, 2014.
- Hong X, Song R, Song H, Zheng T, Wang J, Liang Y, Qi S, Lu Z, Song X, Jiang H, *et al*: PTEN antagonises Tcl1/hnRNP-mediated G6PD pre-mRNA splicing which contributes to hepatocarcinogenesis. *Gut* 63: 1635-1647, 2014.
- Inazu M: Choline transporter-like proteins CTLs/SLC44 family as a novel molecular target for cancer therapy. *Biopharm Drug Dispos* 35: 431-449, 2014.
- Inazu M, Yamada T, Kubota N and Yamanaka T: Functional expression of choline transporter-like protein 1 (CTL1) in small cell lung carcinoma cells: A target molecule for lung cancer therapy. *Pharmacol Res* 76: 119-131, 2013.
- Wang T, Li J, Chen F, Zhao Y, He X, Wan D and Gu J: Choline transporters in human lung adenocarcinoma: Expression and functional implications. *Acta Bioch Bioph Sin* (Shanghai) 39: 668-674, 2007.
- Awwad HM, Geisel J and Obeid R: The role of choline in prostate cancer. *Clin Biochem* 45: 1548-1553, 2012.
- Kouji H, Inazu M, Yamada T, Tajima H, Aoki T and Matsumiya T: Molecular and functional characterization of choline transporter in human colon carcinoma HT-29 cells. *Arch Biochem Biophys* 483: 90-98, 2009.
- Inazu M, Takeda H and Matsumiya T: Molecular and functional characterization of an Na⁺-independent choline transporter in rat astrocytes. *J Neurochem* 94: 1427-1437, 2005.
- Yabuki M, Inazu M, Yamada T, Tajima H and Matsumiya T: Molecular and functional characterization of choline transporter in rat renal tubule epithelial NRK-52E cells. *Arch Biochem Biophys* 485: 88-96, 2009.
- Wang S, Bian C, Yang Z, Bo Y, Li J, Zeng L, Zhou H and Zhao RC: miR-145 inhibits breast cancer cell growth through RTKN. *Int J Oncol* 34: 1461-1466, 2009.
- Sevli S, Uzumcu A, Solak M, Ittmann M and Ozen M: The function of microRNAs, small but potent molecules, in human prostate cancer. *Prostate Cancer Prostatic Dis* 13: 208-217, 2010.
- Malumbres M and Barbacid M: Mammalian cyclin-dependent kinases. *Trends Biochem Sci* 30: 630-641, 2005.