Abstract. Hepatocellular carcinoma (HCC) is a malignant tumor that has become a global health issue. The aim of the present study was to examine the role of transmembrane protein 9 (TMEM9) in cell progression, such as cell growth, cell cycle, cell metastasis of hepatoma cells, and to discuss the TMEM9 gene-encoding protein as a potential therapy target of hepatoma. RT-qPCR was performed to examine TMEM9 expression in tumor tissues and adjacent tissues of patients with liver cancer. siRNAs were used to interfere TMEM9 in HepG2 and 7721 cells. A CCK-8 assay was performed to evaluate cell growth at 24, 48 and 72 h. Cell cycle and apoptosis were analyzed using flow cytometry. Transwell assays were used to determine cell invasion, migration and adhesion. The results showed that TMEM9 was expressed abnormally in liver cancers. TMEM9 expression increased significantly in the 34 examined patients. TMEM9 knock-down inhibited proliferation in the HepG2 and 7721 cells. The flow cytometric analysis revealed that TMEM9 knock-down by RNA interference resulted in G1 arrest and induced apoptosis. Cell invasion, migration and adhesion ability were also decreased. Western blotting indicated expression of the cell cycle-related proteins CDK1, EIF3H, RPL10L, S100A10, CCNB1 and CCNB2 was significantly decreased. In conclusion, TMEM9 plays an important role in the cell growth of hepatoma cells.

Introduction

The incidence of cancer is on the increase and one of the main causes of global mortality (1). Hepatocellular carcinoma (HCC) is a primary malignant tumor and the leading cause of cancer among cirrhotic patients, making it a global health issue (2,3). HCC usually develops in the context of inflammation and organ injury (4). The pathogenic factors are varied. Hepatitis B and C viruses, and autoimmune hepatitis cause progressive liver disease and are major risk factors for the development of HCC (5-9). Due to the lack of effective therapies, such as standard chemotherapeutic agents, and the challenges experienced in early diagnosis, HCC patients have a poor prognosis (10). Therefore, new therapeutic targets may be identified from investigations into the molecular mechanism involved in liver cancer (11).

The membrane protein is a unique structure of protein that plays an important role in cell contact, signal transduction and enzyme activity. It has various functions and becomes the ideal drug target. CD151, as a 4 transmembrane protein gene, is associated with the invasion and metastasis of HCC (12). The transmembrane protein (TMEM) family sequence functions remain unknown only individual protein function has been reported. TMEM9 was characterized as a novel human transmembrane protein, belonging to a new protein family (13). The gene is localized to chromosome 1q41. To the best of our knowledge, the role of TMEM9 in HCC studies remains to be investigated.

In the present study, we investigated the function of the TMEM9 gene in HCC. The results suggest that this gene is closely associated with liver cancer. Thus, this may be a candidate gene for further study of molecular or therapeutic targets.

Materials and methods

Patients and tissue samples. Between 2008 and 2013, 70 HCC patients presenting to the Zhongnan Hospital of Wuhan University (Hubei, China) were enrolled in the present study. All the patients had complete clinical and pathological follow-up data. Adjacent normal hepatocellular
tissues were also collected as negative controls. These normal hepatocellular tissues were resected within at least 5 cm of the tumor margin when the patients underwent definitive surgery. Clinical fresh tissue samples were detected by qPCR. Approval for the study was provided by the independent Ethics Committee of the Zhongnan Hospital of Wuhan University. Informed and written consent was obtained from all the patients or their advisers according to the ethics committee guidelines.

Cell culture and transfection conditions. Human 97H, 97L, HepG2, 7721, 7404 and HuH7 HCC cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% double antibiotics (penicillin/streptomycin) and maintained in a 37˚C incubator with a 5% CO2 humidified atmosphere. Transfections were performed using the Lipofectamine™ 2000 reagent according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). After 48 h of transfection, the cells were used for cell proliferation assays, cell cycle analysis, and apoptosis, Matrigel invasion, migration and adhesion assays. Silencer negative control siRNA was used as a negative control.

RT-qPCR analysis. Cellular RNA was isolated using the TRIzol kit (Invitrogen Life Technologies). SYBR-Green RT-qPCR was performed to detect the mRNA expression. GADPH was used to normalize the RNA inputs. The primers used were: TMEM9 sense, 5'-GGGCACATTTACAACCG-3' and antisense, 5'-ATCACGAAGGCCATGTAG-3'; GADPH sense, 5'-CACCCACTCCTCCACCTTTG-3' and antisense, 5'-CCACCCACCTGTGCGTTAG-3'.

Cell proliferation assay. Viability of cells 72 h after transfection was assessed using the Cell Counting Kit-8 (CCK-8) (Qihai, Shanghai, China). Briefly, cells were seeded at a density of 3x10^4 in each 96-well plate and cultured for 0, 24, 48 and 72 h, respectively. CCK-8 reagent (100 µl/well) was added to each well and incubated for 1 h at 37˚C. The optical density (OD) values were determined at 450 nm using a microplate reader. Three different experiments were performed for each experimental condition.

Flow cytometry. The cell cycle was assessed by flow cytometric analysis at different time points using a propidium iodide (PI) cell cycle detection kit (Beyotime, Shanghai, China). The cells were collected, treated and stained with PI according to the manufacturer's instructions. The cell cycle was detected using a flow cytometer (BD Biosciences, Heidelberg, Germany).

Apoptotic cells were visualized using an Annexin V-FITC/PI kit (BD Biosciences, San Jose, CA, USA). The apoptosis of HCC-transfected cells were determined by flow cytometric (FCM) analysis using a FACSCalibur.

Cell invasion and migration. After transfection, the cells were detached and washed twice in PBS. Then, 1x10^5 cells/ml were seeded in the upper chamber of a Transwell insert (8-µm pore size) coated (invasion) or not coated (migration) with 80 µl Matrigel (BD Biosciences). The lower chamber was filled with 0.75 ml of DMEM. After a 48-h (invasion) or 24-h (migration) incubation period, the non-migrated cells in the upper chamber were scraped away, and adherent cells were stained with formaldehyde solution. Any cells on the underside were counted and photographed under x200 microscope fields.

Cell adhesion. To determine the adhesion cells, 12-well plates were used. Cell suspension (1x10^5 cells/ml) was added to the well and incubated for 1 h at 37˚C. Adherent cells were fixed with 4% methanol and stained with crystal violet for 20 min. The number of adherent cells were photographed and counted from three random selected x200 fields of microscope.

Channel protein expression detection. To detect the role of TMEM9 in liver cancer cells, we selected the proteins CCNB1, CCNB2, CDK1, PRL10A, S100A10 and EIF3H to detect the protein expression using western blotting. Protein lysates were prepared. Equal amounts of samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then blocked with 5% low-fat milk for 1 h or overnight at 4˚C; incubated with CCNB1, CCNB2, CDK1, PRL10A, S100A10 and EIF3H with primary antibodies for 2 h, followed by secondary antibodies for 1 h at room temperature; and analyzed. GADPH protein levels were determined as a loading control.

Statistical analysis. Statistical significances were determined using the GraphPad Prism v5.0 software (GraphPad Software, La Jolla, CA, USA). Kaplan-Meier analysis was used to determine that the overall survival time between low and high expression of HCC. Data are presented as the mean ± SD of at least three independent replicates. Differences were considered significant when P<0.05 or P<0.01.

Results

TMEM9 is highly expressed in HCC with poor patient survival. To investigate the expression of TMEM9, we used RT-qPCR to investigate in the HCC tissues of 30 patients. The results showed a higher level of TMEM9 expression (Fig. 1A). Then we investigated the correlation between TMEM9 expression and prognosis of the patients with HCC. As shown in Fig. 1B, Kaplan-Meier analysis showed that the overall survival time of lower-TMEM9-expressing patients was notably higher than that of higher-TMEM9-expressing patients. The expression levels of TMEM9 in the six HCC cells were also evaluated by qPCR and western blotting (Fig. 1C and D). The HepG2 and 7721 cell lines had a higher TMEM9 mRNA and protein expression.

Knockdown of TMEM9 inhibits cell proliferation and induces apoptosis. To assess the potential effects of RNAi silencing TMEM9 on proliferation, CCK-8 analysis was performed 72 h after transfection. The proliferative ability of HepG2 and 7721 cells was significantly inhibited at 24, 48 and 72 h (Fig. 2).

Moreover, we investigated the effects of TMEM9 on cell cycle and apoptosis in HCC. The flow cytometric analysis...
revealed that the population of G0/G1 phase was significantly increased but that of S and G2/M phase was decreased in HepG2 and 7721 cells, when compared with the negative control (NC) (P<0.01) (Fig. 3).

In addition, we assessed the apoptotic function of TMEM9 in HepG2 and 7721 cells using the Annexin V-FITC/PI staining assay. As shown in Fig. 4, the results showed that knockdown of TMEM9 in HCC cells markedly induced the cell apoptotic rate compared with NC (P<0.01).

Knockdown of TMEM9 decreases metastasis of HCC cells. Cell metastasis plays an important role in cancer progression. We determined whether TMEM9 regulated metastasis of HCC cells. Cell invasion, migration and adhesion assays were then used to detect the metastatic capacity. As shown in Fig. 5, the cell invasion ability was reduced when compared with NC (P<0.01). The migration and adhesion cells were also decreased (P<0.01) (Figs. 6 and 7).

Knockdown of TMEM9 decreases protein expression in HCC. Signaling pathways are often activated in tumor cells. We assessed the protein expression using western blotting. The results showed that the protein expression of CDK1, EIF3H, RPL10L, S100A10, CCNB1 and CCNB2 was decreased compared to the control group (P<0.01) (Fig. 8).

Discussion
HCC is one of the most common types of cancer worldwide. Of an estimated 700,000 cancer-associated mortalities that
arising in 2008, 50% occurred in China (2,14). The 5-year survival rate remains at <40% following surgery (15). HCC cause serious damage to human health; thus, investigation of its development mechanism and identification of effective measures of prevention, diagnosis and treatment of HCC is crucial. In the present study, we investigated the biological function of TMEM9 in HCC cells (16). The clinical data show that TMEM9 was highly expressed in HCC patients. Moreover, TMEM9 expression was associated with the patient survival rate. The in vitro experiments showed that knockdown of TMEM9 in HCC HepG2 and 7721 cancer cells inhibited cell growth and metastasis, and promoted cell apoptosis. Thus, TMEM9 serves as a potential target for the treatment of HCC.

In order to elucidate the possible mechanism involved, we identified the related protein expression. CDK1 is a highly conserved protein and a key player in the cell cycle regulation (17). Eukaryotic translation initiation factors (EIFs) are involved in the protein translation initiation process, and EIF2,
EIF3, EIF4 and EIF5 have been previously investigated (18). EIF3H is an important subunit of the EIF3 family. The EIF3H expression level is closely associated with a variety of tumors, and is overexpressed in numerous malignant tumors (19,20).

Figure 5. TMEM9 knockdown decreased invasion capacity of HCC cells in vivo. (A and B) Images of HepG2 and 7721 invasion cells were captured in 48 h after seeding. (C and D) Invasion assays showed that invasion cells were decreased than those in the negative group. **P<0.01, compared with the negative control. TMEM9, transmembrane protein 9; HCC, hepatocellular carcinoma.

Figure 6. Knockdown of TMEM9 decreases cell migration in HCC cells. (A and B) The images of HepG2 and 7721 migrated cells were captured in 48 h after seeding. (C and D) Migrations of HCC cells were assayed using the 24-Transwell system. Data are one of three similar experiments. **P<0.01, when compared with the negative controls. TMEM9, transmembrane protein 9; HCC, hepatocellular carcinoma.
Ribosomal protein L 10-like (RPL10L), a protein-coding gene, has a structural constituent of ribosome. Furthermore, S100A10, CCNB1 and CCNB2 are involved in mitosis and the regulation of cell cycle progression (21-23). Our results show that the cell cycle-related protein expressions were significantly decreased when compared with NC.

In summary, to the best of our knowledge, the present study provides evidence for the first time that TMEM9 is crucial in the cell proliferation, apoptosis and metastasis of HCC cells. Additionally, TMEM9 regulates these biological processes by regulating cell cycle-related proteins. As TMEM9 expression level is associated with the patient survival rate, inhibition
of TMEM9 in tumor tissues provides a therapeutic strategy. However, additional investigations should be conducted to validate its therapeutic function in the future.

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