During the process of long-term carcinogenesis, cells accumulate many mutations. Deregulated genes expression causes profound changes in cell proliferation, which is one of the hallmarks of HCC. A comprehensive understanding of these changes will contribute to the molecular mechanism of HCC progression. Through clinical sample analysis, we found that TMEM220 is downregulated in tumor and lower levels of TMEM220 is associated with poor prognosis in HCC patients. Through overexpressing TMEM220 in HCC cell lines, we found that the proliferation of cancer cells was significantly slowed down and metastasis was significantly reduced. For further study of its molecular mechanism, we performed a reverse-phase protein array (RPPA). The results suggest that phenotypic changes caused by TMEM220 in HCC cells might be associated with FOXO and PI3K-Akt pathways. Mechanism studies showed that overexpression of TMEM220 could regulate β-catenin and FOXO3 transcriptional activity by altering their subcellular localization, affecting the expression of downstream gene p21 and SNAIL, and ultimately reducing the progression of HCC. Altogether, our study proposes a working model in which upregulation of TMEM220 expression alters the genes expression involved in cell proliferation, thereby inhibiting HCC progression, which suggests that TMEM220 might serve as a clinical biomarker.

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INTRODUCTION

At present, hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world, and its mortality rate is very high in Asia, Africa, and Southern Europe [1]. However, due to the advanced stage at the time of diagnosis, most HCC patients have lost the opportunity of surgical treatment. Therefore, the early diagnosis of HCC patients needs to be improved.

A transmembrane protein (TMEM) is a type of protein that spans the entire width of the lipid bilayer and is permanently anchored. Differential expression of TMEMs has been reported in colorectal cancer [2], lung adenocarcinoma [3], and thyroid cancer [4]. Many of them, such as TMEM176A [5], TMEM97 [6], and TMEM88 [4], act as tumor suppressor genes and were downregulated in cancer tissues. Specifically, TMEM7 and TMEM97 were reported to be involved in HCC development [7, 8].

Increasingly, a growing number of work suggests that TMEM proteins play an important role in tumor development through Akt and Wnt/β-catenin pathways [9–11]. Nuclear accumulation of β-catenin has been found in 17–40% of HCCs [12–14], and its downstream gene SNAIL has been implicated in the process of epithelial-mesenchymal transition (EMT) and promote metastasis [15–17]. It is reported that TMEM88 interacts with DVL1 and regulates cancer progression through the Wnt/β-catenin pathway [18]. Many TMEM proteins on the plasma membrane have been found to act as ion channels (potassium, chloride, sodium, calcium), which can regulate intracellular ion concentration and thus affect the activation of Akt pathways [10, 19]. However, the underlying molecular mechanisms by which these pathways are activated or inhibited in HCC have not been well defined yet.

Transmembrane protein 220 (TMEM220) is a family member of TMEMs that is poorly described. The subcellular localization, physiological function, and its role in the cancer of TMEM220 remain blank. Recent studies reported the expression of LncRNAs CTC-297N7.9, which is associated with HCC patient survival, positively correlated with TMEM220 expression, and TMEM220 gene expression is downregulated in gastric cancer [20, 21]. These studies suggest that TMEM220 might be involved in tumorigenesis and development, however, the role of TMEM220 in HCC is still unknown. Here, the aim of this study was to explore the subcellular localization, the clinical significance of HCC, and the molecular role of TMEM220 during HCC progression.

METHODS

Patients and samples

One hundred and twenty paired tumors and adjacent noncancerous liver samples were collected from May 2015 to October 2019 in The First Affiliated Hospital of Xi'an Jiaotong University, Xiangya Hospital of Central South University, and Shanghai Tenth People's Hospital of Tongji University. Detailed clinical-pathological parameters were listed in Table S1. The average age was 55.6 years, and ages range from 39 to 75. A total of 74 males and 46 females were included. There were no patients who...
received chemotherapy or radiotherapy before surgical excision. Informed consent was obtained from each patient, and the study protocol, which conformed to the ethical guidelines of the 1975 Declaration of Helsinki, was approved by the Institute Research Ethics Committee at The First Affiliated Hospital, Xiangya Hospital, and Shanghai Tenth People’s Hospital.

Data sources

Different tissue expression of TEMM220 was obtained from the National Center of Biotechnology Information (NCBI) gene database (https://www.ncbi.nlm.nih.gov). TEMM220 expression trend frequency plot among different cancer types was obtained from The HIVE Lab (https://hive.biochemistry.gwu.edu/biopress). TEMM220 copy number in normal liver and HCC samples (Guichard Liver: normal liver n = 86; HCC n = 99; TCGA LIHC: normal liver n = 59; HCC n = 97) were obtained from Oncomine (https://www.oncomine.org). Data of mRNA expression and overall survival (normal liver n = 50; liver cancer n = 374) were obtained from The Cancer Genome Atlas Program (TCGA) and The Cancer Genomics Portal (http://cbioportal.org).

Cells and mice

Cell lines used (SMMC7721, MHCC97H, and HCCLM3) were obtained from ATCC and CAS Cell Bank (http://www.cellbank.org.cn/) and routinely cultured in DMEM medium and 10% FBS and 1% penicillin-streptomycin. The cells were seeded in 500 μl media and HCC samples (Guichard Liver: normal liver n = 86; HCC n = 99; TCGA LIHC: normal liver n = 59; HCC n = 97) were obtained from Oncomine.

RNA isolation, reverse transcription PCR (RT-PCR), and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was carried out using oligo-dT primers. 18s RNA was used as an internal control. Real-time PCR was performed in an Applied Biosystems 7500 system using Power SYBR Green PCR Master Mix (Applied Biosystems).

Western blot

The cells were lysed in RIPA buffer in presence of a protease inhibitor cocktail (Roche). Whole-cell lysates were prepared and subjected to 12% SDS-PAGE and transferred to NC membrane (Bio-Rad). The membranes were incubated overnight at 4 °C with the specific antibodies (1:1000 dilution). Membranes were washed and incubated for 1 h at room temperature with a secondary antibody conjugated with peroxidase. Membrane-bound immune complexes were detected using Super ECL detection reagent on Amersham Imager 600 (GE Healthcare).

Immunohistochemistry staining

Formalin-fixed, paraffin-embedded tissue sections cut at 4 μm were used to perform immunohistochemistry for TEMM220. Tissue sections were subjected to antigen retrieval with Tris-EDTA buffers (Agilent-DAKO) kit at 120 °C for 1 h then underwent antigen retrieval with Tris-EDTA buffers (Agilent-DAKO) kit at 120 °C for 1 h. The sections were treated with 3% H2O2 for 10 min to quench endogenous peroxidase activity and blocked with 10% normal goat serum. The sections were incubated overnight at 4 °C in primary antibodies. Fluorescent Alexa-Fluor-488 or -555-labeled secondary antibodies (Life Technologies, Carlsbad, CA, USA) were used for confocal microscopy detection. NIS-Elements (Nikon) were applied to reconstructs and renders 3D images from multiple Z-axis planes.

Promoter luciferase assay

Cells were transfected with reporter constructs containing the promoter together with an expression vector or empty vector using Lipofectamine 3000 (Thermo Fisher Scientific). After 48 h, the luciferase activities of whole-cell lysates were measured using the dual-luciferase reporter assay system (Promega).

ChIP

ChIP assay was carried out using Chromatin Immunoprecipitation Kit (Sigma-Aldrich) according to the manufacturer’s protocol. Briefly, cells were fixed in 1% formaldehyde at room temperature for 10 min and then washed in the lysis buffer. Chromatin solutions were prepared with enzymatic shearing, and immunoprecipitated overnight at 4 °C using 2 μg of specific antibodies or control IgG. Input and immunoprecipitated chromatin were incubated for 15 min at 95 °C to reverse cross-link. After 1 h of proteinase K digestion at 37 °C, Proteinase K Stop Solution was added to abolish proteinase K activity. ChIP DNA was then analyzed by qPCR.

Cell cycle assay

Cells were fixed with 70% ethanol overnight at 4 °C, washed with PBS, and resuspended in 500 μl PBS with 100 μg ml−1 RNase, and incubated for 30 min at 37 °C. Next, 2.5 μl propidium iodide solution (10 mg ml−1) was added and the cell cycle was analyzed by flow cytometry.

Cell viability and colony formation assays

Cell viability was analyzed using the Cell Counting Kit-8 (CCK-8) (Dojindo). Cells at a density of 5 × 103 per well were seeded into 96-well plates and cultured in 100 μl of DMEM containing 10% FBS for 5 days. In total, 10 μl of CCK-8 solution was added to each plate, and the cells were incubated for 3 h at 37 °C. The absorbance value (OD) of each well was measured at 450 nm. Cells were plated in 6-well culture plates. After incubation for 12 days at 37 °C, the cells were washed twice with PBS and stained with 0.1% crystal violet solution. The number of colonies was counted under a microscope.

Migration and invasion assays

Cell motility was assessed by cell invasion and migration assays using Transwell chambers with or without Matrigel (BD, Biosciences). Cells in medium without FBS were seeded on Transwell chambers with or without Matrigel and incubated at 37 °C for 15 h. Medium containing 2% FBS was put in the lower chamber. The invasive cells attached to the lower surface of the membrane insert were fixed, stained using Giemsa, and quantified.

Wound healing assay

Cells were seeded in 12-well plates and incubated until >80% confluence. A straight wound was created by scratching with a 200-μl pipette tip. Floating cells were removed by washing with serum-free medium twice. The cells were then cultured in a serum-free medium and allowed to migrate into the wound area. Images of the migrated cells were acquired with an inverted microscope.

H&E staining

Tissues were immediately fixed in 10% formalin at room temperature for 24 h. The slices were embedded in paraﬃn, sectioned, and mounted on glass microscope slides. The sections were stained with hematoxylin and eosin and examined using light microscopy.

Xenograft and metastasis assays

HCCLM3 or MHCC97H (1.0 × 107 cells/mouse) TEMM220 overexpression and control vector cell lines were subcutaneously injected into the nude mice. The tumor was measured every other day after injection and tumor volume was calculated using the formula [(small diameter)² × (large diameter)] × 0.5. Mouse survival was analyzed using survival analysis. To evaluate the metastasis potential of the cells to the lung and liver, briefly, HCCLM3 or MHCC97H (2.0 × 105 cells/mouse) cells were suspended in PBS with calcium and magnesium and then inoculated into the nude mice by intraperitoneal or tail vein injection. The mice were monitored every day and sacrificed 4–8 weeks later, and the metastatic tumor colonies in the lung and liver were measured.
RESULTS

TMEM220 is downregulated in human HCC and associated with poor clinical outcomes

First, we noticed the presence of high expression levels of TMEM220 in the healthy human liver as compared to the other tissues or organs in the body (Fig. S1a). Moreover, we performed database mining in multiple publicly available databases (Fig. 1a, BioXpress; Fig. 1b, The Cancer Genome Atlas Liver Hepatocellular Carcinoma, TCGA-LIHC). TMEM220 expression was significantly decreased in tumors compared with normal liver tissue (Fig. 1a, b). And TMEM220 gene copy number was also decreased (Fig. 1c, Guichard Liver; Fig. 1d, TCGA-LIHC) and methylation of the TMEM220 gene was increased in HCC tissues (Fig. 1c, d and Fig. S1b). On this basis, we analyzed the expression of TMEM220 in 120 pairs of HCC clinical samples collected from May 2015 to October 2019 (Table S1). Consistent with the findings above, the overall expression level of TMEM220 in the tumor was much lower than adjacent normal livers (ANLs) (Fig. 1e), and specifically, 91.7% (110/120) of them showed underexpression compared with its matched ANL (Fig. 1f, g). Moreover, the immunohistochemistry of clinical liver cancer samples showed that the expression of TMEM220 locates on the cell membrane of the ANL tissue, which is significantly lower in the matched tumor tissue (Fig. S1c). Most importantly, by grouping the samples with TMEM220 expression level, we found that the overall survival curve of the high expression group was significantly better than that of the low expression group through a 5-year follow-up (Fig. 1h and Fig. S1d). In addition, TMEM220 mRNA levels were much lower in SMMC7721, MHCC97H, and HCCML3 HCC cell lines compared with normal hepatic cell lines (Fig. S1e), therefore we choose these 3 cell lines for subsequent research. These data suggest that the expression of TMEM220 is significantly decreased in HCC tissues, and its low expression is closely related to poor prognosis in HCC patients.

Overexpression of TMEM220 blocks HCC cell growth and decreases metastasis

Although it is known that TMEM220 is a TMEM, the subcellular localization of TMEM220, whether located on the plasma membrane or the organelle membrane, is yet to be reported. Using immunofluorescence, we clearly identified that TMEM220 mainly localized on the plasma membrane in HCC cells (Figs. 2a and S2).

To study the effect of TMEM220 on HCC cells, we generated HCC cell lines that overexpress TMEM220. First, cell cycle analysis by flow cytometry showed that the percentage of TMEM220 overexpressed cells at the G1 stage was significantly higher than the control cells (Fig. 2b). Subsequent cell proliferation assay and colony formation assay indicated that the growth of TMEM220 overexpression cells was slowed down markedly (Fig. 2c, d). Moreover, a tumor xenograft study in nude mice showed that the tumor growth of overexpression cells was blocked (Figs. 2e and S3), and accordingly, these mice survived longer than those of the control group (Fig. 2f). Taken together, data here suggested that overexpression of TMEM220 inhibited the growth of HCC cells both in vitro and in vivo.

In addition to growth, metastasis is another important indicator of HCC malignancy. The migration assay, invasion assay, and wound healing assay results showed that the migratory and invasive capacity of TMEM220 overexpression HCC cells were decreased in vitro (Fig. 3a, b). At the same time, in vivo metastasis assay showed that the liver metastasis (Fig. 3c) and lung metastasis (Fig. 3d) of TMEM220 overexpressed cells were significantly reduced. These data suggested that overexpression of TMEM220 suppressed metastasis in HCC cells.

Signaling downstream of TMEM220 in HCC

To further explore the role of TMEM220 in HCC, we performed RPPA in TMEM220 overexpression of SMMC7721 cell lines and control cell lines (Table S2). RPPA represents an antibody-based functional proteomic analysis that can be used for both tumor tissue and cultured cells. It characterizes the basal protein expression and modification levels. Based on the RPPA results, we found 131 altered proteins (P < 0.05; more than 1.5-fold change), 58 of which were upregulated or phosphorylation levels were increased, while 73 were downregulated or phosphorylation levels were decreased (Fig. S4a). Some of the altered proteins (Fig. 4a) were verified by Western blot in MHCC97H and HCCML3 (Fig. 4b). Furthermore, the protein–protein interaction (PPI) network is established to investigate the signal network affected by TMEM220 (Figs. 4c and S4b, c). The proteins that were upregulated or had increased phosphorylation levels in TMEM220 overexpression cells were significantly enriched in the FOXO signaling pathway (Fig. 4d upper panel), whereas the proteins that were downregulated or had decreased phosphorylation levels were highly enriched in the PI3K-Akt signaling pathway (Fig. 4d lower panel). CDKN1A (p21), PTEN and FOXO3 (FOXO3-pS318/321 inactive form decreased) were involved in the activated PPI subset (Fig. S4b), while PCNA, AKT, MAPK, and β-catenin (β-catenin pT41/S45 inactive form increased) were present in the inactivated subset (Fig. S4c). Data reported here indicated that TMEM220 might be involved in HCC progression through PI3K-Akt and FOXO3 pathways.

TMEM220 affect downstream gene expression by altering β-catenin and FOXO3 subcellular localization

The RPPA result of over-expressed TMEM220 suggested that TMEM220 might function via regulating two hub transcription factors, β-catenin, and FOXO3. RPPA results also implied that TMEM220 overexpression also can inhibit AKT and GSK3β phosphorylation. Therefore we proposed that TMEM220 could regulate the transcription activity of β-catenin and FOXO3.

To address this, we first examined the effect of TMEM220 on the subcellular localization of β-catenin. When transfected with TMEM220, β-catenin was translocated from the nucleus to the cytoplasm; and if the cells were treated with BIO (GSK3β inhibitor), the rate of nuclear-cytoplasmic shuttling of β-catenin could be blocked efficiently (Fig. 5a–c). Meanwhile, TOPFLASH/ FOPFLASH reporter assay showed that the transcription activity of β-catenin induced by Wnt3a or β-catenin was gradually suppressed by TMEM220 overexpression (Fig. S5a, b). β-catenin S33Y, which is insensitive to GSK-3β, abrogated the inhibition caused by TMEM220 transfection (Fig. S5c). Moreover, TMEM220 overexpression could inhibit SNAIL expression, the downstream gene of β-catenin, in cell culture (Fig. 5d) and Xenograft tumors (Fig. S5d). Through the analysis of the expression level in HCC samples, we found a negative correlation between the expression of TMEM220 and SNAIL (Fig. S5e). As a β-catenin downstream gene, SNAIL is not only involved in the regulation of cancer cell proliferation but also related to epithelial-to-mesenchymal transition (EMT) in HCC. Based on this, we also detected the expression of vimentin and E-cadherin, EMT prototypical markers. As shown, vimentin expression decreased while E-cadherin increased in TMEM220 overexpression cells (Fig. S5f), which indicates that EMT was inhibited in overexpression cells. Collectively, these results suggested that TMEM220 might negatively regulate β-catenin through AKT-GSK3β cascade, affecting the expression of downstream gene SNAIL.
Fig. 1  TMEM220 is downregulated in human HCC and associated with poor clinical outcomes. a Expression trend frequencies for TMEM220 in different cancer types from BioXpress (https://hive.biochemistry.gwu.edu/tools/bioxpress). b The Cancer Genome Atlas (TCGA) Liver Hepatocellular Carcinoma (LIHC) data indicating TMEM220 expression in Normal liver (n = 50) vs. Liver cancer (n = 374). c, d TMEM220 gene copy numbers in normal liver and HCC in the Guichard Liver microarray and TCGA LIHC (Oncomine database). e TMEM220 mRNA expression was determined for 120 pairs of matched ANL and tumor tissues in collected HCC patients by PCR. f Comparison of TMEM220 expression trend in paired ANL and tumor tissues in (e). g Representative TMEM220 expression levels in paired A (ANL) and T (Tumor) tissues in collected HCC patients by western blot. h Kaplan–Meier survival curves in relation to the expression levels of TMEM220 expression in collected HCC patients.
On the other hand, we found that overexpression of TMEM220 also changed the subcellular localization of FOXO3. However, FOXO3 is transferred from the cytoplasm to the nucleus (Figs. 5e, f and S5g, h). Consistently, the phosphorylation level of FOXO3, its inactivated form, was decreased with TMEM220 overexpression (Fig. 4b). Correspondingly, TMEM220 overexpression increased FOXO3 binding on the p21 (FOXO3 target gene) promoter and activated its expression (Fig. 5g, h).
Fig. 3  TMEM220 overexpression suppresses HCC metastasis in vivo and in vitro. a, b Migration and invasion assays (a) and Wound healing assay (b) of TMEM220 overexpression cells compared with control cells using Transwell membrane without or with Matrigel respectively. Scale bar = 100 μm. c, d Representative image and H&E staining and tumor nodules numbers of liver (c) or lung (d) metastasis in nude mice metastasis model by intrasplenic or tail vein injection for indicated cell lines (n = 8). *P < 0.05, **P < 0.01 (two-tailed t-test).
same trend can be observed in the xenograft tumor model (Fig. S5i). Meanwhile, the expression of p21 and TMEM220 showed a positive correlation in the clinical HCC samples collected (Fig. 5j).

Consistent with critical cyclin-dependent kinase (Cdk) inhibitory roles for p21, p21 arrests the cell cycle by blocking the G1 phase (Fig. 2b). Collectively, our data implicated that TMEM220 could promote FOXO3 nuclear accumulation and increase p21 to inhibit HCC cell growth.

**DISCUSSION**

In this study, by database mining and clinical sample analysis, we found that TMEM220 showed significantly low expression in HCC and was associated with a poor prognosis. Subsequent studies showed that TMEM220 overexpression could block growth and metastasis in vitro and in vivo. To further study the molecular mechanisms underlying the effects of TMEM220 in HCC, we performed RPPA to analyze its associated signaling networks in...
HCC cell lines. Results indicated that TMEM220 may influence downstream gene expression by regulating FOXO3 and β-catenin subcellular location. Consistently, it is well known that FOXO3 acts as a tumor suppressor in cancer progression by promoting p21 transcription, and β-catenin promotes metastasis in HCC [23, 24]. Collectively, results reported in this study promoting us to propose a working model of the role of TMEM220 in HCC progression (Fig. S5k). Overexpression of TMEM220 leads to β-catenin inhibition and FOXO3 activation and ultimately inhibits cell proliferation and metastasis in HCC cell lines.

As we mentioned above, many TMEM proteins located on the plasma membrane, such as TMEM97 and TMEM98 [25, 26], have...
been found to act as ion channels (potassium, chloride, sodium, calcium), which can regulate intracellular ion concentration and thus affect the activation of Akt pathways. In our study, RPPA results show that TMEM220 overexpression could upregulate PTEN expression and inhibit Akt phosphorylation. As a consequence, the decrease of Akt phosphorylation allows GSK to be released, thus inhibiting β-catenin function. Meanwhile, AKT-mediated FOXO3 phosphorylation has been blocked, thereby promoting the expression of its target gene such as p21. Moreover, RPPA data have shown that the phosphorylation of c-MET (mesenchymal-epithelial transition factor/scatter factor receptor) (Y1234/1235) could be inhibited by TMEM220 overexpression in HCC (Fig. 4a, b). Binding with its ligand HGF/SCF (ligand hepatocyte growth factor/scatter factor), c-MET could trigger various of downstream signaling pathways including PI3K/AKT, JAK/STAT, Ras/MAPK, and GSK3/β-catenin [27–29]. The question arises as to how TMEM220 inhibits the activation of c-MET. Given that an increased level of intracellular calcium could negatively regulate c-MET [30], one candidate mechanism is that TMEM220, like TMEM176A/B which also has multiple transmembrane helical domains as TMEM220, might act as a cation channel to control the cytosolic calcium level, thereby suppressing the c-MET activation [31], which will be further confirmed by patch-clamp technology.

By mechanism study, we found that overexpression of TMEM220 in HCC cell lines could downregulate β-catenin pathway and upregulate FOXO3 pathway respectively by changing their subcellular localization, thus affecting the expression of target genes. Recent reports indicate that FOXO3a, as a negative regulator of the canonical Wnt/β-catenin pathway in cervical carcinoma and prostate cancer [32, 33]. This suggests that overexpression of TMEM220 might have a crosstalk effect on FOXO3 pathway promotion and β-catenin pathway inhibition, which synergistically enhances the effect of TMEM220 as a tumor suppressor.

CONCLUSION

Altogether, our study has shown that TMEM220 is decreased in tumors and associated with a poor prognosis in HCC. Based on our findings, we proposed a working model in which upregulation of TMEM220 expression alters the genes expression involved in cell proliferation, thereby inhibiting HCC progression. Taken together, data reported here suggest that TMEM220 might participate in the HCC progression through FOXO3 and Wnt/β-catenin pathways.

AVAILABILITY OF DATA AND MATERIAL

The datasets generated and/or analyzed during the current study are involved in this published article (and its supplementary information files) or available on published databases (TCGA).

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AUTHOR CONTRIBUTIONS

Conception and design: X.Y.L. and H.Q.Z.; Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.L., L.G., G.B.T., B.H., J.W., L.L.H., M.Y.L., Y.X.B.; Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.L., G.L., J.W., B.H., J.P.W.; Technical, or material support (reporting or organizing data, constructing databases): S.S.D.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Each patient consented to the approved protocol which was confirmed by the Institute Research Ethics Committee at The First Affiliated Hospital, Xiangya Hospital, and Shanghai Tenth People’s Hospital.

CONSENT FOR PUBLICATION

All authors have agreed to publish this manuscript.

ADDITIONAL INFORMATION

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