Expression of TMEM16A/ANO1, a Ca\(^{2+}\)-activated Cl\(^{-}\) channel, is dramatically decreased in hepatocellular carcinoma tissue

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Abstract: The role for TMEM16A/ANO1, a Ca\(^{2+}\)-activated Cl\(^{-}\) channel, in hepatocellular carcinogenesis has not been well characterized. In this study, we illustrate that TMEM16A expression in human hepatocellular carcinoma (HCC) tissue was dramatically lower than adjacent non-tumor and normal liver tissue, using real time PCR and western blotting. To investigate the mechanism for reduced TMEM16A expression in HCC tissue, we treated HCC-derived SMMC-7721 cells with a methyltransferase inhibitor to induce demethylation of the TMEM16A promoter. We found that the inhibitor enhanced TMEM16A expression in the cancer cells. Bisulfite sequencing PCR revealed that methylation of the TMEM16A promoter was ~three-fold higher in HCC tissue than adjacent non-tumor liver tissue. Thus, we suggest that the hypermethylation of the TMEM16A promoter is a possible mechanism for reduced TMEM16A expression in HCC tissue. Lastly, we found that overexpression of TMEM16A in the SMMC-7721 cells inhibited cellular proliferation and enhanced apoptosis. Taken together, these findings illustrate that hypermethylation of TMEM16A gene may cause reduction of its expression in HCC tissue, and we argue that TMEM16A possibly plays a proliferation-inhibiting role in liver cancer cells.

Keywords: Hepatocellular carcinoma, TMEM16A, ANO1, Ca\(^{2+}\)-activated Cl\(^{-}\) channel, proliferation, methylation, apoptosis

Introduction

Although our understanding of hepatocellular carcinogenesis is progressing, many of the mechanisms regarding the hepatocellular cancers remain obscure [1, 2]. The reasons for high recurrence and mortality rates in hepatocellular carcinoma (HCC) are unclear. HCC is one of the five most common malignancies worldwide [3] and accounts for more than 90% of primary liver cancers [4]. Most HCCs are associated with chronic hepatitis B virus infection [5]. Due to the considerable prevalence of viral hepatitis in China, hepatitis virus-induced HCC has become a serious health issue facing the Chinese people [6]. A better understanding of HCC mechanisms is necessary in order to develop more efficacious treatments. Several putative tumor suppression genes (TSGs) in HCC have been found to be inactivated by DNA hypermethylation of upstream promoter regions [7]. Therefore, one possible mechanism for HCC may involve promoter hypermethylation of specific TSGs [8-10].

Ca\(^{2+}\) signaling has been demonstrated to be important in liver carcinogenesis [11]. In line with this perspective, TMEM16A or ANO1, a Ca\(^{2+}\)-activated Cl\(^{-}\) channel, has recently attracted attention because of its effect on carcinogenesis and its potential tumor-promoting role in some types of cancers [12]. In most cancer tissues, it seems that TMEM16A is overexpressed and enhances cancer proliferation, metastasis and invasion through its channel functions. Because of these findings, we sought
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to assess if TMEM16A plays a tumor-promoting role in HCC.

To our surprise, however, we found that TMEM16A expression was dramatically reduced in HCC tissues and an HCC-derived cell line, SMMC-7721. We hypothesized that TMEM16A may function as a proliferation-inhibiting factor in HCC, where decreased expression of TMEM16A may promote cellular proliferation and cause HCC. To test this hypothesis, we performed TMEM16A overexpression and flow cytometry experiments with SMMC-7721 cells. Our results raise a challenging question against the potential proliferation-promoting role of TMEM16A in liver cancer cells.

Materials and methods

Tissue specimens of patients
Forty pairs of HCC tissue, adjacent non-tumor liver tissue (ANLT) and normal liver tissue were randomly collected from patients who underwent liver resection at the Department of Hepatobiliary Surgery, Affiliated Hospital of Qingdao University. The study and all procedures performed involving human participants were approved by the Ethics Committee of Affiliated Hospital of Qingdao University. Informed consent, written or oral, had been obtained from the participants. The tissue samples were directly placed into RNAlater solution (Takara, Otsu, Japan) after surgical resection and stored at -80°C until extraction of RNAs or proteins was performed.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)
Total RNA from tissue and cells were extracted with RNAiso Plus (Takara, Japan), verified by agarose gel electrophoresis for integrity, reverse transcribed with PrimeScript™ RT reagent Kit with genomic DNA Eraser (Takara) and amplified quantitatively by qRT-PCR using SYBR-Green Premix (Takara), according to the instructions provided by the manufacturer. The expression of TMEM16A in tissues was normalized to the expression of a house-keeping gene, GAPDH. The data were analyzed by the ΔCt method. The ΔCt values for experiments or controls were obtained from the normalization of TMEM16A mRNA Ct (cycle threshold) values by GAPDH Ct values (ΔCt = Ct\text{GAPDH} - \text{TMEM16A}). ΔΔCt = ΔCt\text{Experiment} - ΔCt\text{Control}. The fold change of TM-EM16A mRNA expression between the experiment and control groups is equal to 2^\text{ΔΔCt}.

The primers used for qRT-PCR were the following: for TMEM16A, 5′-ATTCTCAATCTTCTTCCATCA-3′ (forward) and 5′-TGATAACTCCAGGAAAGATTGCA-3′ (reverse) for GAPDH, 5′-ACACCACCTCTCCACCTTTT-3′ (forward) and 5′-TTCTTTGAGGGCATGT-3′ (reverse).

Western blot
Cells or tissue were lysed in 50-100 ul RIPA Lysis Buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS, supplemented with sodium orthovanadate, sodium fluoride, EDTA and leupeptin, Beyotime, China) and Protease Inhibitor Cocktail (Roche, USA) with 1 mM PMSF (Beyotime) added freshly. The lysate was denatured by heat and centrifuged at 12,000× g for 20 min at 4°C. The protein concentration in the supernatant was measured using a bicinchoninic acid protein assay kit (Beyotime). The proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to 0.45 μm PVDF membranes (Millipore, USA). The membranes were blocked with 5% nonfat milk and incubated with anti-TMEM16A polyclonal antibody (Sigma, USA) at a ratio of 1/2000 overnight at 4°C. Anti-β-actin antibody (1/20000, Sigma) was used as an internal loading control. Following incubation with horseradish peroxidase-conjugated secondary antibody (1/10000, Sigma) for 1 hour at room temperature, the membranes were visualized with the enhanced chemiluminescence kit (Millipore).

Cell culture and treatment of 5-aza-2′-deoxycytidine
In this study, one HCC-derived cell line, SMMC-7721 (the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) was cultured in DMEM medium containing 10% fetal bovine serum (Hyclone, USA) in a humidified atmosphere of 5% CO₂ at 37°C. One day after being seeded into 60 mm dishes, the cells were treated with 5-aza-2′-deoxycytidine (5-AC, Sigma) at 2-30 µM in DMEM containing 10% FBS from 24 to 72 hours. The medium containing 5-AC and 10% FBS was removed and replaced everyday throughout the culture peri-
DNA isolation, bisulfite sequencing PCR and methylation analysis

Cells and tissues were stored at -80°C until they were used. DNA extraction and purification were performed with Wizard® genomic DNA Purification Kit (Promega, USA), according to the instructions of the manufacturer. Bisulfite sequencing PCR was performed as follows. The DNA samples (500 μg each) were treated with bisulfite using the EZ DNA Methylation Gold™ Kit (ZymoResearch, USA). Bisulfite-converted genomic DNA was amplified by PCR using Pfu DNA polymerase. Specific primers for the PCR were designed according to the location of the TMEM16A gene CpG sites. The primer sequences are as follows: Forward: CCTCTCGCGAATATTAGAAATTCCGTTTC; Reverse: CGAGTACTTCTCGTTGACCCTCATCG.

The PCR products were inserted into the pUC18-T vector using a TA cloning Kit (Invitrogen, Carlsbad, USA), and 10 clones were selected for DNA sequencing performed by Sangon Biotech Company (Shanghai, China) that also analyzed the methylation percentage of CpG sites.

Cell transfection

To overexpress TMEM16A, the expression plasmid pEGFP-TMEM16A or pcDNA3.1-TMEM16A, the empty pEGFP or pcDNA3.1 plasmid (Invitrogen) as a control was transiently transfected into SMMC-7721 cells using the Lipofectamine 3000 transfection reagent (Invitrogen) with DMEM. Transfection was performed according to the protocol of the manufacturer. Briefly, cells were transfected at around 70-80% confluence. Four hours after transfection, 10% FBS was added to the DMEM transfection medium.

Cell proliferation assay

SMMC-7721 cell survival or proliferation was evaluated using an MTT [(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide]
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colorimetric assay kit (Solarbio, Beijing, China). After transfection, cells were seeded into 96-well culture plates at a density of 2 × 10^4 cells per well and cultured for 0, 24 and 48 hours, respectively. Then, the cells were incubated with 100 μl medium containing 10 μl MTT (5 mg/mL in PBS) for 4 hours. MTT was converted into purple-colored formazen in living cells which were then solubilized with DMSO (dimethylsulfoxide). The light absorbance of the solution was measured at 490 nm wavelength using a microplate reader (Bio-Rad Model 680, USA).

Flow cytometry

SMMC-7721 cells were seeded at a density of 1 × 10^6 into 60 mm plates and cultured until reaching 80-90% confluence. Then, the cells were transfected with TMEM16A expressing plasmid or empty vector pcDNA3.1 as a control. For cell labeling with Annexin V conjugated with fluorescein isothiocyanate (Annexin V-FITC) and Propidium Iodide (PI), an Annexin V & PI kit (Jiamay Biotech, China) was used. After 24 hours, cells were washed with PBS and binding buffer, and then harvested with binding buffer according to the instructions provided by the manufacturer. The cell suspension was incubated in 300 μl binding buffer containing 5 μl Annexin V-FITC and 5 μl PI for 15 min at room temperature in the dark, and analyzed by flow cytometry (BD, Franklin, USA). The data were analyzed using Flowjo 7.6 software (Stanford University, USA).

Statistical methods

Statistical analysis was conducted using the SPSS17.0 statistical software (SPSS, Chicago, USA). Data are presented as mean ± SD. Statistical significance between groups was determined using an unpaired Student’s t test except for cell proliferation assays where a paired Student’s t test was used. Each experiment was repeated at least three times. A P-value < 0.05 was considered to indicate a statistically significant difference.

Results

TMEM16A expression was drastically reduced in HCC tissue

In order to quantify TMEM16A expression in HCC tissue, we first performed western blots and compared the difference of TMEM16A protein expression between HCC tissue, ANLT, and normal liver tissue. Surprisingly, we found that the HCC tissue expressed much lower levels of TMEM16A protein (Figure 1A and 1B). This finding is in contrast to a recent publication which showed that liver cancer tissue from HCC patients expressed higher levels of TMEM16A protein [13]. In order to confirm our western blot data, we performed qRT-PCR to investigate whether the TMEM16A mRNA expression was consistent with TMEM16A protein expression. As expected, TMEM16A mRNA levels were also much lower in HCC tissue than in normal tissue by 18.3 fold, strongly supporting our western blot result (Figure 1C).

Hypermethylation of the TMEM16A promoter may suppress TMEM16A gene transcription in HCC

Our results showed that the levels of TMEM16A expression in HCC tissue were extremely low (Figure 1). It is well known that breast cancer...
tissue or breast cancer cell lines express high levels of TMEM16A [12, 14]. Hence, we compared the TMEM16A expression levels between MDA-MB-231, a breast cancer cell line and SMMC-7721, a HCC-derived cell line. In Figure 2A, as expected, we noticed that the HCC cell line indeed expressed much lower levels of TMEM16A than the breast cancer cell line.

Hypermethylation of the CpG sites (regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide) has been demonstrated to inhibit TMEM16A gene transcription in squamous cell carcinoma of the head and neck [15]. Additionally, liver carcinogenesis has been shown to be associated with hypermethylation of several TSGs [16]. Therefore, we suspected that the low expression of TMEM16A in HCC or SMMC-7721 cells was probably due to hypermethylation of the TMEM16A gene as well. To demonstrate this, we first examined the effect of TMEM16A gene methylation on the TMEM16A expression in SMMC-7721 cells. The TMEM16A promoter contains about 100 CpG sites distributed before and after the gene initiation site (Figure 4A). We applied 5-aza-2’-deoxycytidine (5-AC), a methyltransferase inhibitor which lowers the level of DNA methylation, to SMMC-7721 cells and assessed whether 5-AC induced TMEM16A transcription. As expected, 5-AC enhanced TMEM16A protein expression dose- and time-dependently (Figure 2B and 2C). 5-AC treatment increased TMEM16A protein expression by roughly one-fold (Figure 3A and 3B) while TMEM16A mRNA levels increased roughly five-fold (Figure 3C). The protein bands were identified as specific for TMEM16A by a siRNA approach (not shown).

To estimate the changes to TMEM16A promoter methylation, genomic DNA was extracted from SMMC-7721 cells treated or untreated with 5-AC, as well as from HCC and ANLT tissue, and bisulfite sequencing PCR was performed on the sample DNA. Specifically, PCR products from TMEM16A promoter DNA leading up to the translation start site were analyzed for methylation levels (Figure 4A). The results showed that DNA methylation level in SMMC-7721 cells was reduced ~ 20% by 5-AC treatment. Methylation levels of the TMEM16A promoter were 20% in HCC tissue compared to 7% in ANLT (Figure 4B and 4C), indicating that the low expression of TMEM16A in the HCC tissue may involve hypermethylation of the TMEM16A promoter. This result reflects the inverse relationship between TMEM16A expression and its promoter methylation levels in HCC tissue. We hypothesize that hypermethylation of the TMEM16A promoter participates in the suppression of TMEM16A gene transcription in HCC.
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To explore whether TMEM16A expression levels affect hepatocellular biology, TMEM16A was overexpressed in SMMC-7721 cells. A plasmid expressing TMEM16A fused with green fluorescent protein (GFP) was transiently transfected into the cells. At 24, 48 and 72 hours after transfection, we observed abnormal morphology in many transfected cells. The cells were smaller in volume, and exhibited cellular fragmentation (Figure 5A). There were a significantly greater percentage of abnormal cells in the TMEM16A vs. control group (Figure 5B). These cellular morphologies led us to hypothesize that apoptosis may play a role in our observations. Therefore, we used an MTT assay to examine changes to cellular proliferation after TMEM16A overexpression in SMMC-7721 cells. As shown in Figure 5C, TMEM16A overexpression significantly reduced light absorbance, implying that cellular proliferation was inhibited by TMEM16A. We suggest that TMEM16A overexpression accomplishes this via apoptotic induction.

In the following experiments, SMMC-7721 cells transfected with TMEM16A-expressing plasmids, control plasmids or transfection reagents alone were examined with flow cytometry (Figure 6A-C). The transfected cells were double-labeled with Annexin V-FITC and PI, markers of “early” and “late” apoptotic cells, respectively. In the present study, “early” apoptotic cells are considered apoptotic while “late” apoptotic cells necrotic. Overexpression of TMEM16A caused the induction of “early” apoptosis, with three-fold more cells in this stage than in control cells (Figure 6D). The number of necrotic cells, however, was not different between the two groups. Transfection reagents alone did not induce significant apoptosis (Figure 6C). This result indicates that the increase in abnormal cells in Figure 5B was likely due to an increase in cells entering apoptosis.

Discussion

Generally, TMEM16A expression is thought to be a cancer-promoting factor because in many
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Types of cancer tissue, TMEM16A is overexpressed and TMEM16A promotes cellular proliferation when overexpressed in many cancer-derived cell lines [12]. Moreover, TMEM16A also is associated with invasion, metastasis and apoptosis of cancer cells through its chloride channel function [12, 18, 19]. However, in this study, we report for the first time that TMEM16A may be a cancer-inhibiting factor in liver carcinogenesis.

Expression of TMEM16A in cancer tissues and its effect on proliferation

TMEM16A has been studied in many types of cancers including lung, breast gland, gastrointestinal track, prostate, ovary, thyroid, liver, pancreas, skin, nasopharynx and thyroid, among others [12, 13, 15, 20-22]. In these cancer tissue and their corresponding cell lines, TMEM16A expression has been shown to be higher than normal. However, some exceptions where TMEM16A expression is reduced in cancer cell lines do exist. For example, in a cancer cell line, HEK293, TMEM16A expression is extremely low, and artificial overexpression does not promote proliferation [12, 23]. Another interesting phenomenon was that when TMEM16A was knocked out of SCCHN cancer cells, these cells did not decrease capability of proliferation but became more invasive and metastasized, indicating that TMEM16A protein was capable of preventing certain type of cancer cells from migrating [15]. In vascular smooth muscle cells, TMEM16A is a negative regulator of cell proliferation [17].

Figure 5. TMEM16A overexpression in SMMC-7721 cells depressed cellular proliferation. Cells were transiently transfected with GFP or TMEM16A-GFP expressing plasmids. (A) Abnormal cell morphologies. Cells with green fluorescence were observed under a fluorescent microscope. Bars indicate 20 μm. (B) Statistical analysis of abnormal cells. The percentage of abnormal cells was obtained by dividing the number of abnormal cells with the number of total cells in one microscope field and multiplying by 100. Bars with small squares indicate GFP; large squares TMEM16A-GFP. (C) MTT assay of cell proliferation. See Methods for details. ***P < 0.001, n = 3-5 for (B and C).
Our results are in contrast to a recent report which stated that TMEM16A was overexpressed in HCC tissue [13]. In our experiments, we initially used a TMEM16A antibody from the Abcam to detect TMEM16A expression in liver tissue and noticed that the antibody detected a higher expression level of TMEM16A in HCC tissue than ANLT and normal liver tissue. But, surprisingly, this result was contradictory to that obtained with qRT-PCR which showed that the TMEM16A mRNA level was much lower in the HCC tissue. Therefore, we examined expression using an antibody from the Sigma. This antibody produced western blot results for TMEM16A protein which were consistent with our qRT-PCR results. We therefore conclude that HCC tissue, in fact, express low levels of TMEM16A. Further, our observation is consistent with lower TMEM16A/ANO1 expression in most of HCC specimens presented in the Cancer Genome Atlas database.

We, therefore, hypothesized that high proliferation of HCC may be associated with the low expression of TMEM16A and selected the SMMC-7721 cell line to test this hypothesis. As expected, overexpression of TMEM16A in the cells inhibited cellular proliferation. Although the native expression of TMEM16A protein in SMMC-7721 cells was low (Figure 2A), we think that a valuable follow up experiment would be a knockdown of TMEM16A in SMMC-7721 cells to further examine the relationship between TMEM16A and proliferation. Further, repeating
these results in other cell lines is also a logical next step.

Regulation of TMEM16A expression via gene methylation

Studies have suggested that the methylation of some TSGs in liver cancers can be used as biomarkers for diagnosis and prognosis. Therefore, the clinical implications for gene methylation studies in HCC is self-evident [24]. Methylation of the TMEM16A promoter affects expression of the protein product [15, 25]. Our results indicate that the low levels of TMEM16A expression in HCC tissue was, at least in part, due to hypermethylation of the TMEM16A promoter. A demethylating agent, 5-AC has been demonstrated to be capable of demethylating many genes in HCC [26]. We treated the SMMC-7721 cells with 5-AC and found that the TMEM16A gene promoter methylation was reduced and TMEM16A protein expression was significantly increased. The result was consistent with observations from HCC tissue where TMEM16A promoter methylation levels were higher than normal and TMEM16A protein levels were dramatically lower. Therefore, we believe that low levels of TMEM16A in HCC are closely associated with the hypermethylation of the promoter. In the viewpoint, 5-AC may be effective in HCC treatment. In fact, 5-AC has been attempted in HCC patients and does have a therapeutic effect [26]. We hypothesize that the therapeutic effect likely involves increased TMEM16A expression. This point is supported by our result that TMEM16A inhibits cell proliferation via apoptosis in an HCC-derived cell line.

We plan on performing follow up experiments to test other aspects of this epigenetic repression of TMEM16A expression in HCC. For example, we plan on assessing the contribution of histone modifications to these effects through pharmacological manipulation of histone deacetylases [26].

TMEM16A likely prevents carcinogenesis via apoptosis

Our study has benefited from a TMEM16F (AN06) study which suggests that the protein may be an apoptosis-promoting factor [19, 27]. Since TMEM16F and TMEM16A are from the same gene family, the results from TMEM16F became a valuable clue for our study. Our results show that TMEM16A may also be a factor in promoting cellular apoptosis in hepatocytes, which may be a general mechanism by which the liver controls cellular proliferation. Once apoptosis is inhibited due to suppression of TMEM16A expression, the proliferation of hepatocytes or cancer cells could accelerate.

It has been well known that apoptosis is one important mechanism by which human body avoids cellular carcinogenesis [28]. In this study, the abnormal cellular morphologies caused by TMEM16A overexpression in SMMC-7721 cells led us to consider the role for apoptosis in HCC. Overexpression of exogenous TMEM16A caused significant apoptosis-like changes to the cells. Further examination by flow cytometry confirmed that the changes were apoptotic in nature. We therefore argue that TMEM16A is capable of containing proliferation of SMMC-7721 cells via apoptosis. In the future, the argument will be further confirmed with various hepatic cell lines. We think that a clinical strategy for treating HCC may involve increasing TMEM16A expression, e.g. via 5-AC, to enhance apoptosis [29].

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Disclosure of interest of conflict

None.

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