Molecular Immunology 80 (2016) 58–67

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Transmembrane protein 88 attenuates liver fibrosis by promoting apoptosis and reversion of activated hepatic stellate cells

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ARTICLE INFO

Article history:
Received 13 July 2016
Received in revised form 14 October 2016
Accepted 1 November 2016

Keywords:
Liver fibrosis
Transmembrane protein 88 (Tmem88)
Hepatic stellate cells (HSCs)
DNA metyltransferases (Dnmts)
Wnt/β-catenin

ABSTRACT

Transmembrane protein 88 (Tmem88) is a crucial inhibitor for Wnt/β-catenin pathway in the development of myocardial cells. Due to the important role of β-catenin in the activation and proliferation of hepatic stellate cells (HSCs), it is necessary to investigate the function of Tmem88 in HSCs. In this study, we found that Tmem88 expression was decreased in the human liver fibrotic tissues, primary HSCs from fibrotic mice and activated HSC-T6 cells. Functionally, Tmem88 could inhibit HSCs activation and proliferation by blocking Wnt/β-catenin pathway, and promoted the apoptosis of activated HSCs by initiating Bcl-2/Bax/Caspase3 pathway. Moreover, the level of DNA metyltransferase 3a (Dnmt3a) was upregulated in activated HSCs, and siRNA-mediated Dnmt3a silencing led to Tmem88 restoration. These results indicated that Tmem88 played an important role in HSCs activation, proliferation and apoptosis, and Tmem88 expression might be modulated by Dnmt3a.

1. Introduction

Liver fibrosis is the wound-healing respond to multiple factors that lead to liver injury (virus infection, alcohol abuse and high-fat diet) (Bataller and Brenner, 2005a; Moreira, 2007). Hepatic stellate cells (HSCs) are widely recognized as the main cell responsible for hepatic fibrogenesis (Reeves and Friedman, 2002; Sato et al., 2003). In the usual, HSCs are quiescent and function as vitamin A storage (Friedman, 2008). However, quiescent HSCs will be converted into activated HSCs by transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor (Henderson and Iredale, 2007; Lee and Friedman, 2011). Activated HSCs will up-regulate the expression of alpha-smooth muscle actin(α-SMA), and remodel the liver architecture by synthesizing types I collagen (Col1α1) and other extracellular matrix (ECM) (Brown et al., 2006; Rippe and Brenner, 2004). Therefore, uncovering the potential mechanisms of HSCs activation and exploring the possible strategies that inhibit HSCs activation will make a difference for the therapy of liver fibrosis.

Transmembrane protein 88 (Tmem88) is a two-transmembrane protein with the inhibitory effect on Wnt/β-catenin pathway (Lee et al., 2010). Emerging evidence suggests that Tmem88 has two isoforms: CRA-a (17 kDa), which interacts with Wnt/β-catenin pathway, and CRA-b (25 kDa), which lacks the motif combined with Dvl proteins and therefore dose not regulate Wnt/β-catenin pathway (Palpant et al., 2013). β-catenin is an important pro-fibrotic factor (Morrisey, 2003; Tan et al., 2014) and plays a crucial role in HSCs activation and proliferation (Ge et al., 2014; Jiang et al., 2006). Based on the observation mentioned above, we assumed that Tmem88 (17 kDa) could inhibit HSCs activation and proliferation by blocking Wnt/β-catenin pathway. To verify this assumption, Tmem88 expression was examined in the human liver fibrotic tissues, primary HSCs from fibrotic mice and activated HSC-T6 cells, and the function of Tmem88 in activated HSCs was assessed by modulating the level of Tmem88 expression. Furthermore, since the hypermethylated DNA of Tmem88 was found in primary HSCs from fibrotic mice, the relation between Tmem88 and DNA methylation was investigated.

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http://dx.doi.org/10.1016/j.molimm.2016.11.002
0161-5890/

2. Materials and methods

2.1. Specimen collection and mice treatment

Human liver fibrotic tissues were collected from several patients (n = 10, male = 6) undergoing partial liver resection in the First Hospital of Anhui Medical University. According to the Liver Cancer Study Group of Japan, the degree of fibrosis was classified as normal liver (Control) and mild to moderate fibrosis (Fibrosis). The causes of liver fibrosis included HBV infection (70%), alcohol abuse (20%) and HCV infection (10%). Informed consent was signed by all patients, and all experimental processes were in accordance with the Helsinki Declaration.

Normal male C57BL/6 mice (about 20 g) were purchased from the Animal Center of Anhui Medical University. The mice model of liver fibrosis was generated as previously described (Chen et al., 2016). Briefly, the CCL4 (20% in olive oil) was injected subcutaneously in mice back for 4 weeks (twice a week, 0.01 ml/g). XAV-939 (Selleck Chemicals, USA) was injected intraperitoneally at a dose of 5 mg/kg/day. All animal procedures complied with the guidelines which were approved by the University Animal Care and Use Committee.

2.2. Primary HSCs isolation

Mice were chosen randomly from the fibrosis group and control group (n = 6/group). Then, the mice were anesthetized with 10% chloral hydrate (0.15 ml/mouse) and fixed in experimental board. After the blood was flushed out, enzymes mixture, including protease E, collagenase IV and DNAase (Sigma, GER), was perfused to digest liver completely. Cells suspension was prepared via mincemeat, filtration and centrifuged in the cell-Nycodenz mixture (Sigma, GER) with density of 1.040–1.060 g/ml. To create discontinuous gradient, the cell-Nycodenz mixture was covered with Hank's fluid (Gibco, USA). Finally, HSCs were gathered by extracting the white cell layer from the gradient interface.

2.3. Cell culture

HSC-T6 cells (Fumeng Gene, China), an immortalized rat HSCs, were cultured in DMEM (Keygen Bio, China) supplemented with 5%(v/v) heat-inactivated FBS (Every Green, China). HSC-T6 cells were incubated at 37 °C with 5% CO_2 and propagated every two days. HSC-T6 cells were activated by 10 ng/ml TGF-β1 for 24 h and treated by 10uM XAV-939 and 2uM 5-azadC (Sigma, USA) for 24 h.

2.4. Total RNA isolation and quantitative real-time PCR

After splitting by TRIZOL reagents (Invitrogen, USA), total RNA was extracted from mice primary HSCs (n = 3/group) and HSC-T6 cells. RNA quantification was determined by Nanodrop 2000 (Thermo Scientific, USA). The mRNA levels of α-SMA, Tmem88, Col1α1, β-actin and GAPDH were determined. The primer sequences (Sangon Biotech, China) were listed in Table 1. The mRNA level of other genes was normalized by β-actin/GAPDH. All samples were performed in triplicate and repeated at least three times.

2.5. Transfection with Tmem88 plasmid

HSC-T6 cells were seeded in 6 wells plate for 12 h and transfected with GV141-Tmem88 and GV141-Control (Shanghai Gene Corporation, China) in opti-MEM culture medium (Gibco, USA) by using LipofectamineTM2000 (Invitrogen, USA). The opti-MEM was replaced by DMEM after 6 h, and 10 ng/ml TGF-β1 (Peprotech, USA) was added for 24 h.

Table 1
Primers used in RT-qPCR.

Gene	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)
Mouse		
Colla1	TGTAAACTCCCTCCACCCCA	TCGTCTGTTTCCAGGGTTGG
α-SMA	CGGGCTTTGCTGGTGATG	CCCTCGATGGATGGGAAA
Tmem88	GCCCTGTTGGTCACTGGATT	GAACCTGGTGAGACTGGGATTT
GAPDH	GGACCTCATGGCCTACATGG	TAGGGCC TCTCTTGCTCAGT
Rat		
Colla1	GATCCTGCCGATGTCGCTAT	TGTAGGCTACGCTGTTCTTGCA
α-SMA	CGAAGCGCAGAGCAAGAGA	CATGTCGTCCCAGTTGGTGAT
Tmem88	ACTCCCAGTTTCTGCGTTCT	AATCCAATGACCAACAGGGT
β-actin	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC

2.6. RNA interference analysis

The sequences of siRNA were as follows: Tmem88-siRNA (5′-CCAGAAUUUACAGGACUGATT-3′, 5′-UCAGUCCUGUAAAUUCUGGTT-3′); DNA metyltransferase 1 (Dnmt1)-siRNA (5′-CCCAGAGUAUGCACCAAUATT-3′, 5′-UAUUGGUGCAUACUCUGGGTT-3′); DNA metyltransferase 3a(Dnmt3a)-siRNA (5′- GCGUCACACAGAAGCAUAUTT-3′, 5′-AUAUGCUUCUGUGUGACGCTT-3′); DNA metyltransferase 3b (Dnmt3b)-siRNA (5′- AGAUGACAGGUGCCCAGAGUU-3′, 5′- CUCUGGGCACCUGUCAUGUUU-3′); scrambled-siRNA (5′-UUCUCCGAACGUGUCACGUTT-3′, 5′-ACGUGACACGUUCGGAGAATT-3′). All siRNA were purchased from the Shanghai GenePharma Corporation. HSC-T6 cells were seeded in 6 wells plate for 12 h and transfected with siRNA in opti-MEM culture medium. The opti-MEM was replaced after 6 h by DMEM, and 10 ng/ml TGF-β1 was added for 24 h.

2.7. Cell proliferation assays

HSC-T6 cells were trypsinized and the number of cells was adjusted to 4×10^4/ml. Then, 100ul cells suspension was added in each of 96 wells plate and the edge wells were filled with sterile PBS. After attachment, HSC-T6 cells were transfected with GV141-Tmem88 or GV141-Control for 6 h in opti-MEM and incubated for 24 h in DMEM or DMEM with 10 ng/ml TGF-β1. Then, 20ul MTT (5 mg/ml, Sigma, USA) was added for 4 h and supernatant was replaced by 150 μl DMSO (Sigma, USA). The value of absorbance (A) was examined at the wavelength of 490 nm. Cell viability = the A value of transfection wells/the A value of control wells * 100%. All experiments were performed in triplicate and repeated at least three times.

2.8. Flow cytometer analysis

Cell cycle analysis: HSC-T6 cells were trypsinized and collected in 15 ml centrifuge tubes. The cells suspension was centrifuged (1000*g*, 3–5 min), mixed sufficiently and fixed in cold ethanol (70%, 1 ml) at 4 °C for 12–24 h. After being centrifuged and resuspended, the HSC-T6 cells were added with 0.5 ml mixture(RNase and PI) and incubated for 30 min at 37 °C in dark place. Flow cytometer (BD Biosciences) was used to detect the red fluorescence and scattered light at 488 nm wavelength. The analysis of DNA content and scattering light was processed by using the ModFit software (Verity Software House, USA). All experiments were repeated for three times.

Cell apoptosis analysis: HSC-T6 cells were collected from suspension by centrifugation, and binding buffer (400ul) was added to resuspend the cells(about $1*10^6$/ml). Then, 5ul Annexin V-FITC (5 min, 2–8 °C, dark) and PI were added (5 min, 2–8 °C, dark), successively. When the preparation was completed, cells suspension were detected by flow cytometer (BD Biosciences) within 1 h and