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Basic Study

Mechanism and therapeutic strategy of hepatic TM6SF2-deficient non-alcoholic fatty liver diseases *via* in-vivo and in-vitro experiments

TM6SF2 gene and NAFLD

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Abstract

BACKGROUND

Lack of effective pharmacotherapies for nonalcoholic fatty liver disease (NAFLD) is mainly attributed to insufficient research on its pathogenesis. At present, the pathogenesis of TM6SF2-deficient NAFLD remains unclear, resulting in no therapeutic strategy for TM6SF2-deficient patients.

AIM

In this paper, we aimed to investigate the role of TM6SF2 on fatty acid metabolism in the background of fatty liver, and proposed the possible therapeutic strategies of NAFLD caused by TM6SF2 deficiency.

METHODS

Applying western blotting, immunohistochemistry (IHC) and qPCR, liver samples collected from both NAFLD mouse models and human subjects (80 cases), and RNA-seq data retrieved from GEO database were used to evaluate the expression of TM6SF2. Knockdown and overexpression of TM6SF2 was performed for clarifying the mechanistic basis of hepatic lipid accumulation in NAFLD. MK-4074 administration served as the therapeutic intervention to evaluate its effect on NAFLD caused by TM6SF2 deficiency.

RESULTS

Hepatic TM6SF2 Levels are elevated in both NAFLD patients and mouse NAFLD models. Overexpression of TM6SF2 can reduce the hepatic lipid accumulation, suggesting the protective role of TM6SF2 in a high-fat diet condition. Down-regulation of TM6SF2, simulating the condition of TM6SF2 E167K mutation, increases intracellular lipid deposition due to dysregulated fatty acid metabolism, being characterized by enhanced fatty acid uptake and synthesis, accompanied by impaired fatty acid oxidation. Due to the potential effect of TM6SF2 deficiency on lipid metabolism,

applying acetyl-CoA carboxylase (ACC) inhibitor (MK-4074) could reverse the NAFLD phenotypes caused by TM6SF2 deficiency.

CONCLUSION

TM6SF2 serves as a protective role in the high-fat diet (HFD) condition, its deficiency enhanced hepatic lipid accumulation through dysregulated fatty acid metabolism and MK-4074 treatment could alleviate the NAFLD phenotypes caused by TM6SF2 deficiency.

Key Words: Transmembrane 6 superfamily member 2; Nonalcoholic fatty liver disease; Fatty acid metabolism; Treatment; MK-4074

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Core Tip: In this paper, we found the overexpression of TM6SF2 in the cases of NAFLD. Overexpression of TM6SF2 can reduce the hepatic lipid accumulation, suggesting the protective role of TM6SF2 in a high-fat diet condition. Meanwhile, there exists an imbalance among the processes of uptake, synthesis and intracellular expense of fatty acids in TM6SF2-deficient mice models. Targeting this phenomenon, we proposed the possible therapeutic strategies of NAFLD caused by TM6SF2 deficiency.

INTRODUCTION

The prevalence of NAFLD is rapidly increasing, which is now more than one quarter of the entire population in the world [1]. Approximately 6–30% of NAFLD patients evolved from simple steatosis (SS) to steatohepatitis, being characterized by excessive hepatic lipid accumulation, inflammation, and hepatocyte damage [2, 3]. A

long-term steatohepatitis can cause liver fibrosis, and its adverse outcome may include liver dysfunction and hepatocellular carcinoma (HCC) [4, 5]. The prognosis of NAFLD and its related complications vary among individuals given that genetic variants matter in occurrence and progression of NAFLD [6]. At present, the development of genome-wide association studies (GWASs) and high-throughput technologies have allowed for the in-depth analysis of the genetic risk factors for NAFLD [7, 8]. Identification of genes with risk single nucleotide polymorphisms (SNPs) have allow to screen individuals with genetic predisposition to NAFLD [9] and several studies have reported some potential SNP loci (e.g., PNPLA3 rs738409, MBOAT7 rs641738 and PPARGC1A rs2290602) associated with the susceptibility and progression of fatty liver [10-12].To further illuminate the mechanistic basis of the link between the potential SNPs and the susceptibility of NAFLD, functions of many candidate genes have been deeply dug into, including transmembrane 6 superfamily member 2 (*TM6SF2)*.

TM6SF2 is a multi-transmembrane protein, expressed predominantly in the intestine and liver, implying a metabolic related function [13]. Our previous study and others demonstrated that the rs58542926 Locus of TM6SF2 gene conferred susceptibility to NAFLD [14-16]. The nonsynonymous variants of TM6SF2, rs58542926 (E167K), would lead to the protein misfolding, acceleration of protein degradation and therefore a reduction of TM6SF2 protein level and gene function [15]. Recently, a bulk of research focused on the influence of TM6SF2 deficiency or its E167K mutant on cholesterol metabolism and its association with reduced VLDL content that caused a decrease in hepatic triglyceride (TG) output [17-19]. Considering that liver is the central hub for lipid metabolism, there exist a balance among the processes of uptake, synthesis and intracellular expense of fatty acids under normal circumstances. When this balance is broken, hepatic lipid accumulation may occur [20]. For now, the regulatory roles of TM6SF2 in fatty acid metabolism in the context of NAFLD remain largely unknown. Therefore, our research aimed at elucidating the influence of TM6SF2 on fatty acid metabolism in the experimental NAFLD models.

In the present study, we revealed that hepatic TM6SF2 expression was markedly elevated in both NAFLD patients and mouse models. Our results suggests that TM6SF2 elevation is a compensatory response during NAFLD. Physiologically, reactive overexpression of TM6SF2 can protect the liver and reduce the hepatic lipid accumulation under the condition of HFD. Loss of TM6SF2 would exacerbate hepatic lipid accumulation. Further investigation revealed that the enhanced processes of uptake and synthesis of fatty acids and impaired oxidation process were observed in the context of hepatic TM6SF2 deficiency, suggesting that TM6SF2 deficiency caused a pathogenic link between the metabolic dysfunction of fatty acid and hepatic steatosis in NAFLD. The elucidation of metabolic alterations suggested the way of therapeutic intervention, our results showed that liver-specific acetyl-CoA carboxylase (ACC) inhibitor (MK-4074) would block the enhanced synthesis of fatty acid, improve fatty acid β-oxidation and then reverse the NAFLD phenotypes caused by TM6SF2 deficiency. Collectively, this study hinted a protective role of TM6SF2 in a HFD condition, revealed a pivotal role of TM6SF2 in fatty acid metabolism and suggested a way of therapeutic intervention for TM6SF2-deficient exacerbated NAFLD.

MATERIALS AND METHODS

Cell culture

Knockdown of TM6SF2 gene was mediated by the lentiviral vector pLenti6.3-MCS-TM6SF2-EGFP (sh-TM6SF2) or pLenti6.3-MCS-EGFP (sh-Ctrl). The target sequences for sh-TM6SF2: TGACCTGGCCCTTGTCATATA. After two or three generations of antibiotic screening, the expression of TM6SF2 was evaluated through immunoblotting. All cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Cat. No: C125C5, NCM Biotech, Suzhou, China) at a 37˚C, 5% CO_2 condition. Cells were cultured under 1% O_2 and 5% CO_2 condition for 24 h to simulate a hypoxia condition. As for starvation condition, cells were cultured in DMEM containing 0.1% FBS for 24 h.

Western blot

Cells in a 6-cm dish with 80% confluence were lysed with 250 ul of RIPA Lysis Buffer, centrifuged at 13000 rpm. The quantification of supernatant collected was assessed by BCA method (Beyotime, Jiangsu, Chang). After electrophoresis and proteins was transferred to a 0.45μm PVDF membrane according to the standard immunoblotting protocol. The membrane was blocked in 5% fat-free milk and then maintained in primary antibodies for 12 h at 4°C After washing, the membrane was incubated with HRP-labeled goat anti-rabbit (mouse) antibody at 25°Cfor 60 min and visualized with ECL reagent (Millipore, MA, USA). The supplementary material listed antibodies used in this study.

Clinical Specimens

Liver specimens were collected from Shanghai General Hospital. Parts of liver were snap frozen in liquid nitrogen and then transferred to -80°C refrigerator for the detection of mRNA levels, and some were stored in formaldehyde solution for later pathological examination. When 5% or more of hepatocytes had steatosis, the diagnosis of NAFLD was confirmed. The diagnosis of tissue sections was reviewed independently by two liver pathologists. This study was approved by the Ethics Committee of Shanghai General Hospital and proceeded strictly in accordance with the declaration of Helsinki. All patients or their family members have been fully informed and signed the written consents.

Immunohistochemistry.

The paraffin-embedded liver tissue was deparaffinized with standard protocols and rehydrated. After antigen retrieval, the tissue was blocked and incubated with the anti-TM6SF2 (1:200) antibody overnight at 4°C Then the slides were washed and incubated with biotin-labeled goat anti-mouse IgG (H+L) at 37°Cfor 15mins and developed with DAB work solution. Images were obtained by a Leica microscope (Germany).

Real-time RT-PCR assay

A total of 50 mg of liver samples from mice or human was lysed with RNAiso Plus reagent (Takara Biotechnology, Otsu, Japan) and total RNA were extracted according to the standard protocol. The reverse transcription of 500ng RNA was performed using the RNA PCR Kit (Takara Biotechnology) and the resulting cDNA was used as the PCR template. Quantification of target gene expressions was assessed by LightCycler®96 (Roche, Switzerland) using a Hieff® qPCR SYBR Green Master Mix (No Rox) kit (Yeasen Biotechnology Co., Ltd, China). The mRNA level of *β-actin* was considered as the endogenous control. We chose the TM6SF2 Level of one patient as the reference, and the TM6SF2 Levels of all other patients were presented in several times of this reference. The primers used were listed in Supplementary material.

Experimental animals

The AAV system (type 8) expressing TM6SF2 shRNA (AAV-shTm6sf2) or overexpressing human TM6SF2 (AAV8-TM6SF2) was employed to regulate the levels of TM6SF2 in C57BL/6 mice. The corresponding controls are AAV-shNC and AAV8-vector, respectively. All mice were firstly under a normal chow diet (NCD) condition for 3 days, and then 100 ul of AAV8 virus (2×10^{11}) was injected into the tail vein. The transfection efficiency was determined by immunoblotting. Male mice weighing 19-22 g (aged 4-6 wk) were housed in a 23±2°C environment with a 12h light/dark cycle. All mice were allowed water ad libitum and fed a high-fat diet (HFD) or NCD continuously for 16 wk. As for MK-4074 treatment, mice were primarily fed an 8-week HFD for inducing NAFLD phenotypes and then were administered orally with MK4074 (10mg/kg/day) or placebo (normal saline) for additional 8 wk on the same diets.

Evaluation of serum parameters and hepatic lipid content

The concentrations of cytokines, metabolites, and hepatic enzymes in serum were determined using commercial kits (ab208348 for TNF, ab197742 for IL-1β, ab222503 for IL-6, JLC049 for CCL2, JLC5800 for CXCL10, ab180875 for acetoacetate, ab180876 for β-

hydroxybutyrate, C010-2-1 for AST and C009-2-1 for ALT) according to the manufacturer's instructions. The hepatic lipid content and malonyl-CoA levels were also measured using commercial kits (A110-1-1 for TG, A111-1-1 for TC, A042-2-1 for NEFA, JL47416 for malonyl-CoA) and normalized by the total protein. Details of all commercial kits used were listed in Supplementary material.

Microarrays

After palmitic acid (PA, 150 μM) stimulation for 24 h, total RNA was extracted from both TM6SF2-knockdown L02 cells and corresponding controls using RNAiso Plus (Takara Biotechnology). Microarray gene expression analysis was performed by illumina sequencing. We analyzed differentially expressed genes (DEGs) between sh-Ctrl and sh-TM6SF2 cells by using the "edgeR" package based on R platform[21]. Fold change >1.2 and adjusted *P* value < 0.05 were set as the screening cutoffs for the upregulated DEGs. These DEGs were further analyzed *via* DAVID 6.8 (https://david.ncifcrf.gov/) for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. The "ggplot2" package in R is used for result visualization.

Preparation of fatty acid solution

A 5 mmol/L stock solution of palmitic acid (PA, Sigma-Aldrich) was obtained by dissolving PA in 3% of bovine serum albumin (BSA, Sigma-Aldrich) medium by continuous stirring for ~4 h with a 60 °C water bath. The stock solution was then diluted by DMEM medium to achieve the designated concentration and 3% of BSA was used as a control. The concentrations of free fatty acid had been reported to range between 0.1 and 0.7 mmol/L [22-24].

Glucose and insulin tolerance test

Glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed as previously described [25]. After the above steps were finished, the glucose concentrations

of blood collected from the tail vain at indicated points were analyzed immediately by a glucose meter (Yuwell, China).

Nile red staining

After sh-ctrl and sh-TM6SF2 L02 cells were constructed, cells were incubated in DMEM medium contained 150 μM of palmitate acid (PA) for 24 h to generate a steatosis cell model. Fixed with 4% paraformaldehyde, cells were then incubated with 0.1μg/mL Nile Red for 15 min. After being washed with PBS, cells were incubated with DAPI (Solarbio) for additional 5 min. Images were taken with a Leica microscope (Leica, Germany) and quantified by Image J software.

Magnetic Resonance Imaging (MRI)

MRI imaging were performed on the 7.0 T Bruker BioSpec MRI scanner (Bruker, USA). Before imaging, mice fasted overnight to avoid the disturbance of stomach contents on liver imaging. The mice were first anesthetized with oxygen contained 2-3% isoflurane, and then placed in a prone position with their heads facing inward. Throughout the imaging process, mice were continuously provided with oxygen mixed with 1-3% isoflurane through the nosecone to maintain the respiratory rate at 50-70 breaths/minute. All mice underwent abdominal MRI to acquire the maximum cross-sectional area of liver. The region of interest was analyzed by Image J software, and the size of the cross-section area is represented by number of pixels.

Histological analysis

Liver sections were embedded in paraffin followed by staining with haematoxylin and eosin (H&E) and frozen liver sections were stained with Oil Red O (O8010-5g; Solarbio) for lipid visualization. Images were procured using a light microscope (Leica, Germany). Oil red O- stained sections are quantified by Image J software.

FAO measurement.

A total of 6,000 cells were seeded in a XF96 cell culture micro plate and each group was assayed in 5 or 6 repeated wells. After cell adherence (about 8 h), the growth medium was changed to substrate-limited growth medium (0.5 mmol/L of glucose, 1 mmol/L of GlutaMAX, 0.5 mmol/L of XF L-carnitine, 1% FBS, pH 7.4) for overnight incubation. Before the Seahorse Bioscience XF96 instrument (Agilent, USA) was started, the media was again change to palmitic acid measurement medium. Injectors added different inhibitors (oligomycin 2 μM, FCCP 2 μM and R/A 1.2 μM) to each chamber when the instrument began to measure the oxygen-change rate. The difference between the rate of oxygen consumption in each group revealed the level of fatty acid oxidation (FAO) based on palmitate substrates and the basal respiration, maximal respiration, spare respiratory capacity and ATP production were calculated.

Cell viability Assay

Cell viability was assessed by a CCK-8 (NCM Biotech, China). Sh-ctrl and sh-TM6SF2 cells were seeded in a 96-well plate at a density of 5,000 cells per well and the cell viability assay was performed after PA or fatty acid-free BSA treatment for 24 h. The absorbance (450 nm) was evaluated by the microplate reader (BioTek, USA). Each sample was assayed in 5 repeated wells and the experiment was performed three times independently.

Quantitation of intracellular TG levels in cell lines

After PA or BSA treatment for 24 h, cells were lysed on ice with RIPA buffer (Beyotime, Beijing, China) for 20 min and centrifuged at 13000 rpm for 20 min at 4°C. And then the supernatant was transferred to a new tube. TG (triglyceride) levels were evaluated by a TG detection kit (Nanjing Jiancheng, Jiangsu, China) and normalized by the total protein. The experiment was performed three times independently.

Bioinformatic analyses

Expression profiles of TM6SF2 gene in NAFLD cases retrieved from the Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo) database. The accession numbers were GSE130970, GSE48452, GSE83452 and GSE89632. The details of these cohorts were list in supplementary material.

Statistical analysis

All data were presented as means ± SD. Student's t-test was used to evaluate the difference between two groups. Differences among more than two groups were compared with one-way ANOVA followed by Tukey's multiple comparisons test. *In vitro* studies, all experiments were independently performed three times. SPSS software (version 25.0) was applied in all statistical analyses. $P< 0.05$ was considered statistically significant and denoted as * $P<0.05$, ** $P<0.01$, and *** $P<0.001$, n.s., not significant.

RESULTS

Hepatic TM6SF2 expression is upregulated in NAFLD patients and HFD-induced mice models.

To evaluate the expression of TM6SF2 in NAFLD cohort, the mRNA levels of *TM6SF2* were examined across 80 Liver samples of NAFLD patients and healthy subjects (HS, $n = 40$), which showed that the transcriptional levels of TM6SF2 were both elevated in patients with simple steatosis (SS) or non-alcoholic steatohepatitis (NASH) (Figure 1A, SS: $n = 20$; NASH: $n = 20$). Additionally, we retrospectively analyzed another 4 transcriptomic data retracted from GEO database (GSE13970, GSE48452, GSE83452 and GSE89632, Figure 1B), which contained transcriptional profiles of both heathy subjects and NAFLD patients. Consistently, patients with SS or NASH displayed higher hepatic *TM6SF2* mRNA levels than the healthy subjects. Immunohistochemistry (IHC) staining indicated TM6SF2 displayed a tendency of upregulated TM6SF2 expression from normal liver to severe simple steatosis (Figure 1C). We also noted an up-regulation of TM6SF2 protein in the liver samples of patients with SS or NASH (Figure 1D). To further confirm the overexpression of TM6SF2 under overnutrition, we then evaluated the changes of TM6SF2 expression in *in-vitro* NAFLD cell model and stimulated two cell lines, L02 and HepG2, with palmitic acid (PA), as it was reported that this *in vitro* model mimics steatosis *in vivo* [22-24]. The results showed PA stimulation increased the protein levels of TM6SF2 in a time- and dose- dependent manner

(Supplementary Figure 1A). Further, whether in protein or mRNA levels, the augmented TM6SF2 expression was also demonstrated in high-fat diet- (HFD-) fed mice for 12 wk (Supplementary Figure 1B). Taken together, these evidences revealed that the hepatic TM6SF2 Level was upregulated in NAFLD patients and HFD-induced mice models.

Overexpression of TM6SF2 mitigates hepatic lipid accumulation in HFD-induced mice models.

As we had found that the levels of TM6SF2 were increased in NAFLD subjects and in high-fat diet induced models, we next wanted to know the physiological significance of TM6SF2 elevation when NAFLD phenotypes occurs. To this end, we used the AAV8 vector to deliver human TM6SF2 (AAV8-TM6SF2) in the livers of mice (AAV8-vector was used as a control) and fed them with HFD for 16 wk (Figure 2A and 2B). The results showed that TM6SF2 overexpression in liver significantly reduced liver/body ratio and its impact on body weight was limited (Figure 2C and 2D). And hepatic TM6SF2 overexpression in mice showed lower levels of hepatic lipid contents as well as serum ALT and AST (Figure 2E and 2F). TM6SF2-overexpression groups relative to controls displayed equally in body size (Figure 2G), but demonstrated a minor fatty liver as well as a smaller cross-sectional area of fatty liver (Figure 2H). Hepatic lipid accumulation was also ameliorated in AAV8-TM6SF2 mice as indicated by pathological examination (Figure 2I). We also observed that TM6SF2 knocked-down cells reconstituted with the wild type TM6SF2 showed decreased intracellular lipid accumulation and improved PA-induced cell death (Supplementary Figure. 2A-C). These evidences suggested that the elevation of TM6SF2 was a potential reaction to antagonize the NAFLD phenotypes and served as a protective role during NAFLD.

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Knockdown of TM6SF2 promotes hepatic lipid accumulation and inflammation in HFD-induced mice model.

Pathologically, there exist genetic E167K mutation of TM6SF2 protein in human, those with TM6SF2 E167K mutation were associated with an increasing risk of NAFLD [26]. Ehrhardt N *et al* reported that E167K mutation caused a down-regulation of TM6SF2 in

protein level [27]. To simulate the E167K condition and explore the influence of TM6SF2 on lipid accumulation, we generated TM6SF2-knockdown cells in both L02 and HepG2 cell lines (Figure 3A), and these cells were incubated with PA (150μM) or BSA (fatty acid free) for 24 h. We found that TM6SF2-knockdown cells demonstrated higher levels of intracellular TG content (Figure 3B) and more severe lipid accumulation (Figure 3C). Lipid overload would induce endoplasmic reticulum stress (ERS) and cause cell death [28, 29], and the results of cell viability assay proved that TM6SF2 knockdown markedly exacerbated the PA-induced cell death (Figure 3D), which reflected the increase of lipid content in TM6SF2-knockdown group. To determine whether TM6SF2 deficiency exacerbate hepatic steatosis *in vivo*, we generated hepatic Tm6sf2-knockdown (hereafter referred to as AAV-shTm6sf2, AAV-shNC was used as a control) mice by injecting AAV vectors that carried Tm6sf2-targeting short hairpin RNA (shRNA), and mice were fed a HFD or NCD for 16 wk (Figure 4A and 4B). The HFD-fed AAV-shTm6sf2 mice, in comparison to control mice, displayed severe hepatocellular ballooning degeneration and lipid accumulation, as exhibited by H&E and Oil red O staining (Figure 4C), and these mice had higher levels of hepatic triglycerides (TG), total cholesterol (TC) and nonesterified fatty acids (NEFA) (Figure 4D). Similarly, AAV-shTm6sf2 mice had a higher liver/body weight ratio (Figure 4E) but exhibited a slightly higher body weight (Figure 4F) only at feeding initiation. In addition, hepatic TM6SF2-knockdown mice relative to controls displayed a modest increase in size (Figure 4G) but a significantly enlarged liver as well as a larger maximal cross-sectional area of liver (Figure 4H). However, these parameters did not show much difference between two groups when they were put in a normal chow-diet (NCD) environment (Figure 4D-4F).

It is noteworthy that hepatic steatosis is often accompanied by inflammation, and the boundary between the two phases (SS and NASH) of NAFLD is very blurred [30]. The hepatic TM6SF2-knockdown group displayed higher serum levels of inflammatory cytokines, such as TNF, IL-6, IL-1β, CCL2 and CXCL10, than the control mice after 16 wk of HFD feeding (Supplementary Figure 3A). Consistently, the hepatic expression of inflammation-related genes was also augmented in the AAV-shTm6sf2 group

(Supplementary Figure 3B). Meanwhile, AAV-shTm6sf2 group as compared to AAV-shNC group showed higher serum levels of alanine aminotransferase (ALT) and aspartate transaminase (AST, Supplementary Figure 3C) and more infiltrated neutrophils and macrophages (Supplementary Figure 3D). Together, these evidences showed that knockdown of TM6SF2 promotes hepatic lipid accumulation and inflammation in HFD-induced mouse model.

Hepatic knockdown of TM6SF2 enhances de novo lipogenesis

To explore the changes of biological processes in the context of TM6SF2 knockdown, triplicate samples of PA-treated TM6SF2-knockdown L02 cells and their controls were used for transcriptome analysis to acquire the upregulated differentially expressed genes (DEGs). KEGG pathway enrichment analysis was applied to explore the function of these DEGs, which highlighted the fatty acid metabolism was the key process that changed in TM6SF2-knockdown cells (Figure 5A) and genes associated with the process of fatty acid synthesis were found upregulated (Figure 5B). To confirm this, *in vitro* models of NAFLD were used to verify our finding. PA itself was a potent agonist of fatty acid synthesis [31]. PA administration activated fatty acid synthesis *in vitro*, as indicated by the increased protein levels of p-ACC, FASN, SCD-1 and SREBP-1c (Figure 5C). When comparing the levels of these proteins between TM6SF2-knockdown cells and corresponding controls, the results showed that these proteins were up-regulated in TM6SF2-knockdown cells, suggesting that PA-induced fatty acid synthesis was enhanced with the knockdown of TM6SF2 (Figure 5D). *De novo* lipogenesis mainly mediated by sterol regulatory elementbinding protein 1c (SREBP-1c) on transcriptional regulation of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). The mature form of SREBP-1c translocates to the nucleus and activates its target genes, such *Acaca* and *Fasn* [32]. And this translocation-to-nucleus phenomenon was also enhanced in TM6SF2-knockdown cells (Figure 5E). In addition, TM6SF2 knockdown increased the levels of hepatic malonyl-CoA, a metabolite of acetyl-CoA carboxylase [33], in HFD-fed AAV-shTm6sf2 mice (Figure 5F), which reflected that the activity of ACC was enhanced due to TM6SF2 deficiency. It is noteworthy that the transcriptional activity of hepatic

SREBP-1c was also influenced by circulating insulin status. We evaluated the levels of blood glucose and insulin in mice, which showed no differences in two groups with or without a HFD feeding (Supplementary Figure 4A). In addition, no obvious differences were noted in terms of glucose (GTT) and insulin tolerance test (ITT) (Supplementary Figure 4B). Together, these evidences suggested that the enhanced steatosis in hepatic TM6SF2-knockdown mice was unlikely induced by the alteration of insulin sensitivity. Moreover, we also noted that TM6SF2 knockdown did not exert an effect on fatty acid transport process, but caused an increase in the mRNA levels of molecules involved in fatty acid uptake (e.g., CD36 and FABP1) (Figure 5B). This increase was augmented when the TM6SF2-knockdown cells were under a starvation or hypoxia condition (Supplementary Figure 4C), as hypoxia and starvation has been reported to enhance lipid uptake [34-36]. In parallel, the levels of fatty acid synthesis- and uptake-related genes were elevated in the liver of AAV-shTm6sf2 mice compared with control mice (Figure 5G). Together, these evidences suggested that the unfavorable phenotype of fatty liver in hepatic Tm6sf2-knowndown mice may be associated with the enhanced de novo lipogenesis and lipid uptake.

TM6SF2 deficiency could lead to impaired fatty acid oxidation

It was previously reported that NAFLD caused by TM6SF2 deficiency was partially due to the impaired very low-density lipoproteins (VLDL) secretion, which is detrimental for the liver, as the VLDL secretion was considered a significant way of lipid export in liver [18]. Fatty acid β-oxidation (FAO), another way of energy expenditure [37], was rarely reported in the absence of TM6SF2 protein. Therefore, we evaluated and compared the expression of key molecules involving FAO. The results showed that the TM6SF2-knockdown cells exhibited the deceased levels of FAO-related proteins [38], including PPARα, CPT1A and ACOX1, with or without PA stimulation (Figure 6A), which is consistent with the results of transcriptomic data (Figure 5B). Furthermore, the PPARα signaling have been reported to function as a key factor in regulating FAO [39], we found that the down-regulated levels of FAO-related proteins in TM6SF2-knockdown cells could be reversed by PPARα activation through PPARα agonist

(GW7647) stimulation (Figure 6A). We next tested hepatic fatty acid oxidation *in vivo* by evaluating the levels of serum ketone bodies, β-hydroxybutyrate (β-OH) and acetoacetate, in both AAV-shTm6sf2 mice and control groups after a 16 h fast. The results showed that the levels of ketone bodies were significantly lower in Tm6sf2-knockdown group (Figure 6B), suggesting that Tm6sf2 deficiency reduces fatty acid oxidation. In addition, compared with the control group, TM6SF2-knockdown L02 cells had lower oxygen consumption rate (OCR) with palmitic acid as the only substrate (Figure 6C and 6D). Meanwhile, the CPT1A inhibitor etomoxir (ETO) could inhibit the process of FAO, and diminish the difference in both groups as well as the FAO-related proteins (Figure 6C-E). AAV-shTm6sf2 mice also showed lower expression of FAO-related genes in liver (Figure 6F). Together, these data indicated that TM6SF2 deficiency could impair fatty acid oxidation in cells.

MK4074 is effective in the treatment of NAFLD exacerbated by TM6SF2 deficiency.

Our above results showed that TM6SF2 deficiency could lead to abnormal fatty acid metabolism within cells, being characterized by enhanced lipogenesis and uptake, accompanied by impaired fatty acid β-oxidation (Figure 7). It is worth noting that the suppressed β-oxidation process may be associated with the increased malonyl-CoA content caused due to the enhanced activity of acetyl-CoA carboxylase (ACC) in this situation. Malonyl-CoA itself is the natural CPT1A inhibitor, which, as a result, would cause the decrease of β-oxidation in mitochondria [40]. Therefore, inhibiting the activity of ACC may be a promising method to alleviate fatty liver in mice with hepatic Tm6sf2 deficiency. To test this idea and rectify this metabolic abnormality, we thought a way of inhibiting ACC, this rate-limiting enzyme, by using a previously reported ACC inhibitor, MK-4074, which could simultaneously limit the de novo lipid synthesis pathway and increase the level of intracellular lipid oxidation, to explore whether the drug has therapeutic effect on Tm6sf2-deficient cases. To this end, both AAV-shTm6sf2 or control mice were firstly subjected to 8-week HFD feeding to induce NAFLD phenotypes, and then two group of mice received MK-4074 (MK-4074, 10 mg/kg/day)

or placebo (normal saline) treatment and fed with HFD for additional 8 wk (Figure 8A). Results showed that MK-4074 treatment could diminish the increase in the levels of malonyl-CoA (Figure 8B), as well as hepatic TG, TC and NEFA caused by TM6SF2 deficiency (Figure 8C), suggesting the success in blockade of SPREBP-1c/ACC pathway. The HFD-fed AAV sh-Tm6sf2- mice under MK-4074 treatment, in comparison to placebo treatment, also exhibited mild hepatic ballooning degeneration and steatosis (Figure 8D). At the same time, MK-4074 treatment improved the liver/body weight ratio (Figure 8E), although body weight improvement was rather limited (Figure 8F). In addition, the beneficial effect is also manifested *in vitro* experiments that MK-4074 could reduce lipid accumulation (Figure 9A and 9B) and improve PA-induced cell death in TM6SF2 knocked-down groups (Figure 9C). Meanwhile, MK-4074 could inhibit malonyl-CoA production (Figure 8B) and improve the decrease of FAO caused by TM6SF2 deficiency, as evidenced by the increased FAO-related protein levels and ketone body levels in TM6SF2-knockdown group (Figure 9D and 9E), which renders MK-4074 the prime option for treating this condition.

DISCUSSION

In the present study, we revealed that hepatic TM6SF2 expression is upregulated in both NAFLD mouse models and patients. TM6SF2 overexpression alleviates NAFLD phenotypes in HFD-induced models, suggesting the protective role of reactive TM6SF2 elevation under NAFLD circumstances. Simulating the situation of patients with E167K mutation, TM6SF2 knockdown exacerbated hepatic steatosis and inflammation. We noted that there was a dysregulated fatty acid metabolism process under the condition of TM6SF2 deficiency, being characterized by enhanced fatty acid uptake and fatty acid synthesis, accompanied by the impaired FAO process and insufficient ketone body production. Given the potential impact of TM6SF2 on fatty acid metabolism in NAFLD, exploring the effects of targeted NAFLD therapeutics on fatty liver are of the utmost importance, inspiring us to take the impact of genetic variation on fatty liver into consideration. Herein, we used a previously reported liver-specific ACC inhibitor, MK-

4074, which could reduce hepatic lipogenesis while increase FAO, to explore the therapeutic effect on the fatty liver caused by TM6SF2 deficiency (Figure 7). Our results show that MK-4074 has an encouraging effect on the treatment of TM6SF2 knockdown-induced NAFLD, as indicated by reduced hepatic lipid content, less liver/body ratio and alleviated PA-induced cell death, suggesting the effectiveness of MK-4074 in treating this condition.

Previous studies considered TM6SF2 a pivotal molecule in hepatic lipid output in form of VLDL-TG, and a bulk of studies focus on the association between the export of accumulated lipid and TM6SF2 E167K mutant [15, 18, 41]. The TM6SF2 E167K mutant may result in a misfolded protein that is easy to degrade within cells resulting in the decreased TM6SF2 Levels [42]. Liver is the pivotal hub for lipid metabolism, whose dysregulation could be a significant cause for NAFLD [43]. Therefore, we dissect the cause of TM6SF2 deficiency-induced NAFLD from another perspective, the dysregulated fatty acid metabolism. Our results revealed that knockdown of TM6SF2 significantly increased the expression of SREBP-1c and its target genes both *in vivo and in vitro*, suggesting abnormally enhanced de novo lipogenesis (DNL). Meanwhile, results showed that the levels of OCR were decreased in TM6SF2-knockdown groups, indicating that the FAO process was impaired under these circumstances. We further tested the transcriptional levels of FAO-related genes in hepatic TM6SF2-knockdown mice fed a HFD and showed lower levels of genes involving FAO comparing with the control mice on the same diet for 16 wk. Previous studies showed that the elevated levels of malonyl-CoA would suppress fatty acid oxidation by inhibiting CPT1A activity [44], and in our studies, the malonylCoA content is higher in AAV-shTm6sf2 mice comparing with the control mice, which implies the decreased FAO process is to do with the enhanced DNL due to TM6SF2 knockdown.

Currently, genetic technologies have already allowed for the identification of a subgroup of people with the increased risk of NAFLD or NAFLD-related complications, which are associated with specific pathophysiological characteristics [45]. In NAFLD field, there is no precise treatment for metabolic diseases with specific congenital

genetic variation. However, much efforts have been made to elucidate the mechanism of NAFLD caused by the genetic mutation, and implement precision medicine. For example, the human genetics of NAFLD offers the opportunity to apply the targeted therapy with the PNPLA3 I148M variant. As previously reported, this locus is the strongest genetic factor by increasing the susceptibility of NAFLD [10]. Recently, researchers used mice with PNPLA3 variant knock in and tried to improve NAFLD phenotypes by means of antisense oligonucleotides (ASO) to downregulate the PNPLA3 mutant protein [46]. Although the mechanisms are not fully understood, accumulating evidence reveals that the PNPLA3 I148M impairs lipid droplet remodeling in hepatocytes due to loss of lipase activity [47]. Inhibition of the expression of PNPLA3 148M led to a reduction in liver fat content in these engineered mice and, specifically, a beneficial effect in mitigating inflammation and fibrosis. If these applications are translated into humans, surely, after safety and reliability examination, downregulation of PNPLA3 148M may be the first case of human genetics-based targeted therapy. The importance would be that with this method, we will treat not just the phenotype of the disease, but also the cause.

As described in PNPLA3 mutant protein case, those evidence proved the possibility of a targeted therapy to eliminate the cause of fatty liver in patients carrying a specific risk allele. However, up to now, there is no report on the attempt for the treatment of fatty liver caused by TM6SF2 deficiency or its E167K mutant. Based on our data, mice with TM6SF2 deficiency showed enhanced de novo lipogenesis (DNL). Among the TM6SF2/SREBP-1c/ACC axis, ACC act as the rate-limiting enzyme for the conversion of acetyl-CoA to malonyl-CoA [48] The product, malonyl-CoA, was further catalyzed by FASN to form palmitate. Triglycerides is one of the many lipid species integrated by DNL-derived palmitate. Given that ACC is essential and pivotal in hepatic fatty acid metabolism, ACC inhibitors exhibit potential for mitigating hepatic steatosis in NAFLD. Independent studies reveal that blocking of DNL by ACC inhibition show a beneficial effect in reducing liver fat, steatosis and inflammation in NAFLD models [44, 49]. Among the ACC inhibitors, MK-4074, has recently been tested for NAFLD treatment in patients

and the effects were encouraging. MK-4074 treatment decreases DNL and enhances FAO in subjects under overnutrition. Based on its function, we hypothesized that it can be used to treat fatty liver caused by the reduction of TM6SF2, and *in vivo* and *in vitro* experiments had confirmed our hypothesis. Meanwhile, ACC inhibitors have recently been shown to effectively mitigate liver fibrosis in patients with NASH [49, 50]. The accumulating evidence have shown that TM6SF2 E167K is also associated with NASH and fibrosis [18, 51]. Whether ACC inhibitors can be applied to mitigate liver fibrosis in the NASH model with TM6SF2 deficiency or E167K mutant is unknown. This also indicates the direction of our future work.

CONCLUSION

In this paper, we have shown that dysregulated fatty acid metabolism occurs in the context of TM6SF2 deficiency under overnutrition. Therapeutics aimed at the abnormal fatty acid metabolism may be a promising strategy and hopefully improve the hepatic lipid profile of patients with TM6SF2 E167K variant in the clinical setting. Meanwhile, our study suggested that MK-4074 would be a potential drug to lower hepatic lipid content in TM6SF2-knockdown NAFLD mice model and further investigations were required to test whether this approach would lower hepatic lipid levels in NAFLD patients with E167K mutation.

ARTICLE HIGHLIGHTS

Research background

Previously, we have found that TM6SF2 E167K mutation is associated with the susceptibility of NAFLD in Chinese cohort, but the underlying mechanism is poorly understood.

Research motivation

Because existing evidence have shown that TM6SF2 gene mutation would reduce its expression in protein levels, we wanted to use NAFLD mice models to explore the

causes of NAFLD caused by TM6SF2 deficiency in the context of high-fat feeding, and test possible treatment strategies. These would benefit us the understanding of mechanisms of NAFLD happened in human being.

Research objectives

The mechanism and therapeutic strategy of hepatic TM6SF2-deficient non-alcoholic fatty liver diseases (NAFLD) *via* in-vivo and in-vitro experiments

Research methods

This study mainly involves the knockdown and overexpression of hepatic Tm6sf2 gene in NAFLD mice and cell models to explore its effects on liver pathological changes. RNA-seq, OCR technology, western blotting, and pathological examination were applied to investigate the underlying mechanisms of NALFD caused by TM6SF2 deficiency.

Research results

(1) Hepatic TM6SF2 expression is upregulated in NAFLD patients and HFD-fed mice.

(2) Overexpression of TM6SF2 mitigates hepatic lipid accumulation in HFD-induced mice models.

(3) Knockdown of TM6SF2 promotes hepatic lipid accumulation and inflammation.

(4) TM6SF2 deficiency promotes hepatic lipid accumulation through dysregulation of fatty acid metabolism

(5) MK-4074 administration may serve as the potential drug to improve NAFLD caused by TM6SF2 deficiency.

Research conclusions

In this paper, we have found that the reactive overexpression of Tm6sf2 gene in the HFD condition could alleviate hepatic lipid accumulation, loss of which accelerated the NALFD phenotypes under HFD feeding. We also found that that dysregulated fatty

acid metabolism occurs in the context of TM6SF2 deficiency under overnutrition. Therapeutics aimed at the abnormal fatty acid metabolism may be a promising strategy and hopefully improve the hepatic lipid profile of patients with TM6SF2 E167K variant in the clinical setting. Our study suggested that MK-4074 would be a potential drug to lower hepatic lipid content in TM6SF2-knockdown NAFLD mice model.+ADw-/p+AD4APA-/html+AD4-

Research perspectives

TM6SF2 serves as a protective role in the high-fat diet (HFD) condition, its deficiency enhanced hepatic lipid accumulation through dysregulated fatty acid metabolism and MK-4074 treatment could alleviate the NAFLD phenotypes caused by TM6SF2 deficiency. And the next step is to investigate whether MK-4074 have therapeutic effect in NAFLD patients harboring TM6SF2 E167K mutation.

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