The Role of TM6SF2 in Non-Alcoholic Fatty Liver Disease: From Mechanisms To Therapeutic Strategy

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Abstract

Background and aims

: Lack of effective pharmacotherapies for nonalcoholic fatty liver disease (NAFLD) is mainly attributed to an insufficient research on its pathogenesis. In this paper, we investigated 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

Liver samples collected from both NAFLD mouse models and human subjects, and RNA-seq data retrieved from GEO database were used to evaluate the expression of TM6SF2 in NAFLD. Knockdown of TM6SF2 was performed for clarifying the mechanistic basis of hepatic lipid accumulation in NAFLD. After confirming that TM6SF2 deficiency would cause an abnormality in fatty acid metabolism, 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. *In vivo* and *in vitro* experiments confirmed that TM6SF2 knockdown increases intracellular lipid deposition due to dysregulated fatty acid metabolism in the context of TM6SF2 deficiency, being characterized by enhanced fatty acid uptake and synthesis, accompanied by impaired fatty acid oxidation. Moreover, MK-4074 treatment could reverse the NAFLD phenotypes caused by TM6SF2 deficiency.

Conclusions

TM6SF2 deficiency enhanced hepatic lipid accumulation through dysregulated fatty acid metabolism and MK-4074 treatment could alleviate the NAFLD phenotypes 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 HSD17B13 rs72613567) 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, loss of which would exacerbate hepatic lipid accumulation. Further investigation revealed that the enhanced the 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 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 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% CO₂ condition. Cells were cultured under 1% O₂ and 5% CO₂ 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°C for 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. When 5% or more of hepatocytes had steatosis, the diagnosis of NAFLD was confirmed. 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°C for 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. The primers used were listed in Supplementary material.

Experimental animals

The AAV system (type 8) expressing TM6SF2 shRNA (AAV-shTm6sf2) was employed to regulate the levels of TM6SF2 in C57BL/6J mice (male). The corresponding controls are AAV-shNC. All mice were firstly under a normal chow diet (NCD) condition for 3 days, and then 100 μ l of AAV8 virus (2×10^{11}) was injected into the tail vein. The transfection efficiency was determined by immunoblotting. All mice weighing 19-22 g (aged 4-6 weeks) were housed in a $23 \pm 2^\circ\text{C}$ 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 weeks. 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 weeks 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-1 β , 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 μ M) 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 [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 mM 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 mM [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 vein

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 μ M of palmitate acid (PA) for 24 h to generate a steatosis cell model. Fixed with 4% paraformaldehyde, cells were then incubated with 0.1 μ g/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 mM of glucose, 1 mM of GlutaMAX, 0.5 mM 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 μ M, FCCP 2 μ M and R/A 1.2 μ M) 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.

Statistical analysis

All data were presented as means $\pm$ 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 expression of TM6SF2 was both elevated in patients with simple steatosis or NASH (Fig. 1a, SS: simple steatosis, n=20; NASH: non-alcoholic steatohepatitis, n=20). Additionally, we retrospectively analyzed 4 transcriptomic data retracted from GEO database (GSE13970, GSE48452, GSE83452 and GSE89632, Fig.1b). Consistently, the results showed that 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 (Fig. 1c). We also noted an up-regulation of TM6SF2 protein levels in the liver samples of patients with SS or NASH (Fig. 1d). We next 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 is 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 (Fig. S1a). Further, the augmented TM6SF2 expression was also demonstrable in high-fat diet- (HFD-) fed mice for 12 weeks (Fig. S1b). Taken together, these evidences revealed that the hepatic TM6SF2 level was upregulated in NAFLD patients and HFD-induced mice models.

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

To explore the influence of TM6SF2 on lipid accumulation, we generated TM6SF2-knockdown cells in both L02 and HepG2 cell lines (Fig. 2a), and these cells were incubated with PA (150 μ M) or BSA (fatty acid free) for 24 hours. We found that TM6SF2-knockdown cells demonstrated higher levels of intracellular TG content (Fig. 2b) and more severe lipid accumulation (Fig. 2c). Lipid overload would induce endoplasmic reticulum stress (ERS) and cause cell death [26, 27], and the results of cell viability assay showed that TM6SF2 knockdown markedly exacerbated the PA-induced cell death (Fig. 2d), 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 weeks (Fig. 3a, b). 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 (Fig. 3c), and these mice had higher levels of hepatic triglycerides (TG), total cholesterol (TC) and nonesterified fatty acids (NEFA), the major substrate source of hepatic steatosis (Fig. 3d). Similarly, AAV-shTm6sf2 mice had a higher liver/body weight ratio (Fig. 3e) but exhibited a slightly higher body weight (Fig. 3f) only at feeding initiation. In addition, hepatic TM6SF2-knockdown mice relative to controls displayed a modest increase in size (Fig. 3g) but a significantly enlarged liver as well as a larger maximal cross-sectional area of liver (Fig. 3h). However, these parameters did not show much difference in two groups fed a normal chow diet.

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 [28]. The hepatic TM6SF2-knockdown group displayed higher serum levels of inflammatory cytokines, such as TNF, IL-6, IL-1 β , CCL2 and CXCL10, than the control mice after 16 weeks of HFD feeding (Fig. 4a). Consistently, the hepatic expression of inflammation-related genes was also augmented in the AAV-shTm6sf2 group (Fig. 4b). Meanwhile, AAV-shTm6sf2 group as compared to AAV-shNC group showed higher serum levels of alanine aminotransferase (ALT) and aspartate transaminase (AST, Fig. 4c) and more infiltrated neutrophils and macrophages (Fig. 4d). 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 (Fig. 5a) and genes associated with the process of fatty acid synthesis were found upregulated (Fig. 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 [29]. PA administration activated fatty acid synthesis *in vitro*, as indicated by the increased protein levels of p-ACC, FASN, SCD-1 and SREBP-1c (Fig. 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 (Fig. 5d). *De novo* lipogenesis mainly mediated by sterol regulatory element-binding 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 as *Acaca* and *Fasn* [30]. And this translocation-to-nucleus phenomenon was also enhanced in TM6SF2-knockdown cells (Fig. 5e). In addition, TM6SF2 knockdown increased the levels of hepatic malonyl-CoA, a metabolite of ACC [31], in HFD-fed AAV-shTm6sf2 mice (Fig. 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 (Fig. S2a). In addition, no obvious differences were noted in terms of glucose (GTT) and insulin tolerance test (ITT) (Fig. S2b). 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) (Fig. 5b). This increase was augmented when the TM6SF2-knockdown cells were under a starvation or hypoxia condition (Fig. S2c), as hypoxia and starvation has been reported to enhance lipid uptake [32-34]. 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 (Fig. 5g).

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 [35], 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 decreased levels of FAO-related proteins [36], including PPAR α , CPT1A and ACOX1, with or without PA stimulation (Fig. 6a), which is consistent with the results of transcriptomic data (Fig. 5b). Furthermore, the PPAR α signaling have been reported to function as a key factor in regulating FAO [37], we found that the down-regulated levels of FAO-related proteins in TM6SF2-knockdown cells could be reversed by PPAR α activation through PPAR α agonist (GW7647) stimulation (Fig. 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 (Fig. 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 (Fig. 6c,d). 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 (Fig. 6c-e). AAV-shTm6sf2 mice also showed lower expression of FAO-related genes in liver (Fig. 6f). Together, these data indicated that TM6SF2 deficiency could lead to impaired fatty acid oxidation.

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

Our results showed that TM6SF2 deficiency could lead to abnormal fatty acid metabolism within cells, being characterized by enhanced fatty acid synthesis and uptake, accompanied by impaired fatty acid β -oxidation. It is worth noting that the suppressed β -oxidation process may be associated with the increased malonyl-CoA content caused due to enhanced ACC activity in this situation. Malonyl-CoA is the CPT1A inhibitor, which would cause the decrease of β -oxidation in mitochondria. The elucidation of metabolic alterations suggested the way of therapeutic intervention, our next investigated whether the use of liver-specific ACC inhibitor (MK-4074) could suppress the enhanced fatty acid synthesis and thereafter improve the decreased β -oxidation caused by TM6SF2 deficiency. 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 weeks (Fig.7a). Results showed that MK-4074 treatment could diminish the increase in the levels of malonyl-CoA (Fig.7b), as well as hepatic TG, TC and NEFA caused by TM6SF2 deficiency (Fig.7c). The HFD-fed AAV sh-Tm6sf2- mice under MK-4074 treatment, in comparison to placebo treatment, also exhibited mild hepatic ballooning degeneration and steatosis (Fig.7d). At the same time, MK-4074 treatment improved the liver/body weight ratio (Fig.7e), although body weight improvement was rather limited (Fig.7f). In addition, the beneficial effect is also manifested *in vitro* studies that MK-4074 could reduce lipid accumulation (Fig.8a, b) and improve PA-induced cell death in TM6SF2 knocked-down groups (Fig.8c). Meanwhile, MK-4074 could inhibit malonyl-CoA production (Fig.7b) 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 (Fig.8d, e), 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, and 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 (Fig. 9). The 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, 38]. The TM6SF2 E167K mutant may result in a misfolded protein that is easy to degrade within cells resulting in reduced TM6SF2 levels [39]. Liver is the pivotal hub for lipid metabolism, whose dysregulation could be a significant cause for NAFLD [40]. 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 in these circumstances. We further tested gene expression 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 weeks. Previous studies showed that the elevated levels of malonyl-CoA would suppress fatty acid oxidation by inhibiting CPT1A activity [41], 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 an increased risk of NAFLD or NAFLD-related complications, which is associated with a specific pathophysiological characteristic [42]. 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 causing susceptibility to 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 [43]. 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 [44]. 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 carriers with 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 have higher de novo lipogenesis. Among the TM6SF2/SREBP-1c/ACC axis, ACC act as the rate-limiting enzyme for the conversion of acetyl-CoA to malonyl-CoA [45]. 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 [41, 46]. 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. *In vivo* and *in vitro* experiments have confirmed our hypothesis. Meanwhile, ACC inhibitors have recently been shown to effectively mitigate liver fibrosis in patients with NASH [46, 47]. The accumulating evidence have shown that TM6SF2 E167K is also associated with NASH and fibrosis [18, 48]. 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.

Abbreviations

NAFLD, Nonalcoholic Fatty Liver Disease; TM6SF2, Transmembrane 6 Superfamily Member 2; SNPs, Single nucleotide polymorphisms; HS, Healthy subjects; SS, Simple steatosis; NASH, Non-alcoholic steatohepatitis; VLDL, Very low-density lipoproteins; ACC, Acetyl-CoA carboxylase; ALT, Alanine transaminase; AST, Aspartate aminotransferase; AAV, Adeno-associated virus; HFD, High-fat diet; NCD, Normal chow diet; TG, Triglyceride; TC, Total cholesterol; NEFA, Nonesterified fatty acid; PA, Palmitic acid; FAO, Fatty acid oxidation; BSA, Bovine Serum Albumin; MRI, Magnetic Resonance Imaging; Eto, Etomoxir; GEO, Gene Expression Omnibus; ASO, antisense oligonucleotides; DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Declarations

Acknowledgements

Not applicable.

Author contributions

Zuyin Li and Gang Wu performed the *in vitro* and *in vivo* experiments. Qiu Chen, Yupeng Wang, Guohe Song and Chao Xiao performed the GEO analysis. Guilong Deng, Ruitao Wang provided the technical supports. Xin Zhang interpreted the data. Zuyin Li wrote the manuscript. Xiaoliang Wang and Yulong Yang conceived and supervised the study. All authors critically reviewed and edited the manuscript.

Availability of data and materials

The data used and analysed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest

All authors declared that there was no conflict of interest.

Consent to participate

The study was conducted in accordance with the principles enshrined in the Declaration of Helsinki and the Good Clinical Practices and approved by the Ethics Committee of Shanghai General Hospital.

Consent for publication

All authors had access to the study data and reviewed and approved the final manuscript.

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Figures

Figure 1

Hepatic TM6SF2 expression is upregulated in NAFLD patients. a. Hepatic mRNA levels of TM6SF2 in liver specimens. Healthy subjects (HS, n = 40) and subjects with simple steatosis (SS, n = 20) or nonalcoholic steatohepatitis (NASH, n = 20). The expression of TM6SF2 was normalized to ACTB mRNA levels. b. Hepatic mRNA levels of TM6SF2 in individuals with or without NAFLD from four expression microarray series retracted from GEO database. c. Immunohistochemical staining with TM6SF2 was performed on individuals with or without NAFLD. Representative images of normal liver tissues versus tissues of simple

steatosis were shown. Scale bars: 50 μ m. d. Immunoblot analysis of TM6SF2 expression in liver specimens. HS (n = 10), SS (n = 5) and NASH, (n = 5). *, P < 0.05, **, P < 0.01, ***, P < 0.001.

Figure 2

Knockdown of TM6SF2 promotes intracellular lipid accumulation and lipid-overload induced cell death. a. The efficiency of TM6SF2 knockdown in two cell lines. b. Intracellular TG levels (n = 3) in TM6SF2-knockdown cells were examined after PA (150 μ M) or BSA (fatty acid free) treatment for 24 h. c. Nile red staining (left, nuclei labeled with DAPI, blue) and quantification of lipid accumulation (right) of sh-Ctrl or sh-TM6SF2 L02 cells after PA or BSA treatment. Scale bars: 50 μ m. d. TM6SF2-knockdown cells were incubated with PA (150 μ M) or BSA for 24 h. The cellular viability was examined (n=5). *, P < 0.05, **, P < 0.01, ***, P < 0.001.

Figure 3

Tm6sf2 deficiency exacerbates hepatic lipid accumulation and inflammation. **a**. Mice were injected with AAV-shNC and AAV-shTm6sf2 viruses via the tail vein and fed a HFD or NCD for 16 weeks ($n = 5$ mice per group). The efficiency of TM6SF2 knockdown in liver were shown. **b**. Schematic representation of animal experiments. **c**. Left, representative images of H&E-staining (left) and Oil red O-staining (right) of liver sections. Right, the NAS (left) and quantification of lipid (right, 8 fields of each mouse were examined)

were performed. d-f. Hepatic lipid contents (TG, TC and NEFA) (d), liver/body weight ratio (e) and body weight (f) were measured. g. Representative images of mice morphology. h. Top, representative photos of fatty livers (top) and livers subjected to MRI scanning (bottom) of mice were shown. Bottom, the maximal cross-sectional area of livers is quantified by the number of pixels. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

Figure 4

Knockdown of TM6SF2 promotes HFD-induced inflammation in liver. a-c. Mice were injected with AAV-shNC and AAV-shTm6sf2 viruses via the tail vein and fed a HFD or NCD for 16 weeks ($n = 5$ mice per group). The serum concentrations of inflammatory cytokines (a), hepatic mRNA levels of inflammation-related genes (b) and serum levels of ALT and AST (c) were shown. d. Representative images of immunofluorescent staining (left) and their quantification (right) were shown (8 fields of each mouse were examined). Scale bar: 50 μ m. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

Figure 5

The activity of fatty acid synthesis was enhanced in TM6SF2 knockdown group a. Pathway enrichment of the up-regulated DEGs by KEGG analysis. b. Microarray heatmap of genes involved in fatty acid metabolism in TM6SF2-knockdown cells. c. The SREBP-1c target protein levels in L02 and HepG2 cells after PA treatment. d. The SREBP-1c target protein levels in TM6SF2-knockdown cells after PA treatment. e. Immunoblot analysis of SREBP-1c in cytoplasmic and nuclear extracts. f. Hepatic malonyl-CoA contents of mice in two groups (n = 5 mice per group). g. The mRNA levels of genes involved in fatty acid synthesis (Srebf1, Fasn, Scd1, Acaca and Hmgcr) and uptake (Cd36, Slc27a1 and Fabp1). *, P < 0.05, **, P < 0.01, ***, P < 0.001.

Figure 6

TM6SF2 deficiency caused the downregulation of fatty acid oxidation in liver. a. Protein levels of PPARα, CPT1A and ACOX1 was analyzed in TM6SF2-knockdown cells after PA stimulation and their response to GW7647 (10 μM) treatment. b. Serum levels of β-hydroxybutyrate and acetoacetate in NCD- or HFD-fed AAV-shNC or AAV-shTm6sf2 mice (n = 5 mice per group). c-d. PA-dependent oxygen consumption rate (OCR) in sh-Ctrl and sh-TM6SF2 L02 cells with or without etomoxir (Eto, 40 μM) administration (c, n = 6). The basal respiration, maximal respiration, spare respiratory capacity and ATP production were calculated (d). e. Protein levels of PPARα, CPT1A and ACOX1 were determined in TM6SF2-knockdown cells with or without etomoxir treatment under PA (150 μM) stimulation. f. The hepatic mRNA levels of FAO-related genes in the indicated mice. *, P < 0.05, **, P < 0.01, ***, P < 0.001.

Figure 7

Therapeutic potential of MK-4074 on NAFLD caused by TM6SF2 deficiency a. Schematic representation of animal experiments. b-d. Two groups (AAV-shNC and AAV-shTm6sf2) of mice were fed a HFD for 8 weeks to induce NAFLD phenotypes and then each subgroup was dosed with MK-4074 (10 mg/kg/day) or placebo orally for additional 8 weeks. The hepatic malonyl-CoA levels (b) and lipid content (c) as well as results of H&E-staining and Oil red O-staining of liver sections (d) were shown. e-f. The liver/body

weight ratio (e) and the body weight (f) of HFD-fed AAV-shNC and AAV-shTm6sf2 mice with or without MK-4074 treatment (n = 5 mice per group) were shown. *, P < 0.05, **, P < 0.01, ***, P < 0.001.

Figure 8

MK-4074 can improve intracellular lipid accumulation, lipid overload-induced cell death, and fatty acids β-oxidation. a. Intracellular TG levels (n = 3) in TM6SF2-knockdown cells with PA (150 μM) stimulation were examined after MK-4074 (10 nM) treatment for 24 h. b. Representative Nile red staining images (left) and lipid droplet quantification (right) of sh-Ctrl or sh-TM6SF2 L02 cells after PA stimulation in response to MK-4074 (10 nM) treatment (10 fields of each sample were examined). Scale bars: 50 μm. c. The cellular viability was examined in TM6SF2-knockdown cells with or without MK-4074 treatment in response to PA-induced cell death (n=5). d. The sh-Ctrl and sh-TM6SF2 cells were cultured with PA (150 μM) for 24 h. Cells were then treated with MK-4074 (10 nM) for 12 h. The expression of FAO-related

proteins was determined. e. Serum levels of β -hydroxybutyrate and acetoacetate in the indicated mice with or without MK-4074 treatment. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

Figure 9

The role of TM6SF2 in non-alcoholic fatty liver disease: from mechanisms to therapeutic strategy

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