In vitro analysis of hepatic stellate cell activation influenced by transmembrane 6 superfamily 2 polymorphism

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Abstract. Non-alcoholic steatohepatitis (NASH) may progress via liver fibrosis along with hepatic stellate cell (HSC) activation. A single nucleotide polymorphism (SNP; *rs58542926*) located in transmembrane 6 superfamily 2 (*TM6SF2*) has been reported to be significantly associated with fibrosis in patients with NASH, but the precise mechanism is still unknown. The present study aimed to explore the role of TM6SF2 in HSC activation *in vitro*. Plasmids producing TM6SF2 wild-type (WT) and mutant type (MT) containing E167K amino acid substitution were constructed, and the activation of LX-2 cells was analyzed by overexpressing or knocking down TM6SF2 under transforming growth factor β1 (TGFβ) treatment. Intracellular α-smooth muscle actin (αSMA) expression in LX-2 cells was significantly repressed by TM6SF2-WT overexpression and increased by TM6SF2 knockdown. Following treatment with TGFβ, αSMA expression was restored in TM6SF2-WT overexpressed LX-2 cells and was enhanced in TM6SF2 knocked-down LX-2 cells. Comparing αSMA expression under TM6SF2-WT or -MT overexpression, expression of αSMA in TM6SF2-MT overexpressed cells was higher than that in TM6SF2-WT cells and was further enhanced by TGFβ treatment. The present study demonstrated that intracellular αSMA expression in HCS was negatively regulated by TM6SF2 while the E167K substitution released this negative regulation and led to enhanced HSC activation by TGFβ. These results suggest that the SNP in TM6SF2 may relate to sensitivity of HSC activation.

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Abbreviations: HCS, hepatic stellate cell; TM6SF2, transmembrane 6 superfamily 2; TGFβ1, transforming growth factor β1; αSMA, α-smooth muscle actin; SNP, single nucleotide polymorphism

Key words: hepatic stellate cell, liver fibrosis, non-alcoholic steatohepatitis, single nucleotide polymorphism, tm6sf2

Introduction

Non-alcoholic fatty liver disease (NAFLD) is becoming one of the most prevalent chronic liver diseases in modern countries, increasing rapidly as a result of recent upward trends in obesity and life-style changes (1). A subset of NAFLD patients go on to develop non-alcoholic steatohepatitis (NASH) by progression of steatosis and necro-inflammatory changes in the liver, leading to an increase in the incidence of hepatocellular carcinoma (2). Mortality in NAFLD patients has been reported to be independently associated with the stage of liver fibrosis (3), and it is important to prevent the progression of liver fibrosis in NAFLD patients. Recently, several drugs have been developed and have entered phase 2 or 3 clinical trials, but no effective drugs against NAFLD are yet available. Therefore, it is important to clarify the mechanism of liver fibrosis in NAFLD in order to identify therapeutic targets.

To identify clinical factors associated with the progression of liver fibrosis in NAFLD patients, several genome wide association studies (GWAS) have recently been performed worldwide. A single nucleotide polymorphism (SNP) at *rs738409* in patatin-like phospholipase domain containing *3* (*PNPLA3*) was identified as having strong associations with prevalence and disease progression in NAFLD and NASH (4-7). A SNP in transmembrane 6 superfamily 2 (*TM6SF2*) was also identified as a potential contributor to NAFLD pathogenesis (8,9). The SNP *rs58542926* in *TM6SF2* is significantly associated with

incidence of NAFLD and with fibrosis stage (10-13). TM6SF2 protein is highly expressed in the small intestine and liver and plays a role in lipid synthesis and secretion of triglyceride-rich lipoproteins in the liver (14-19). *TM6SF2 rs58542926* (C>T), a coding SNP that causes an amino acid substitution at codon 167 (E167K), is considered to lead to a loss of function and to accelerate hepatic steatosis (20). However, although lipids are metabolized in hepatocytes and may accumulate in these cells, liver fibrosis is strongly associated with hepatic stellate cells (HSCs) (21). The influence of the coding SNP in *TM6SF2* on the function of HSCs has not been clarified.

HSCs are normally activated in response to stimulation by inflammatory cytokines, such as transforming growth factor beta 1 (TGFβ1), and by pathogen-associated molecular patterns, such as lipopolysaccharides (21). Activated HSCs transform into myofibroblasts, and alpha-smooth muscle actin (αSMA) expression is upregulated in the transformed myofibroblasts (21,22). Activation of HSCs leads to secretion of extra-cellular matrix proteins such as collagen type 1 into the sinusoids, resulting in collagen accumulation and progression of liver fibrosis (21-23). Although the impacts of genetic factors on clinical features of NAFLD and hepatocyte functions have been analyzed, the impacts of genetic factors on HSCs have not been examined. In the present study, we explored the role of *TM6SF2* SNP *rs58542926* in liver fibrosis using an *in vitro* activated HSC model.

Materials and methods

Construction of TM6SF2 expression plasmids. Human *TM6SF2* mRNA was amplified from LX-2 cells and cloned into p3xFLAG-CMV-10 vector (Sigma-Aldrich). The cloned plasmid containing the wild-type CC genotype at rs58542926 in *TM6SF2* gene was designated as p3FLAG/TM6SF2-WT. Subsequently, a modified plasmid, designated as p3FLAG/TM6SF2-MT, was generated by introducing a C-to-T point mutation at rs58542926 in *TM6SF2* to create an amino acid substitution [glutamic acid (E) to lysine (K)] in the TM6SF2 gene using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies).

Cell culture. LX-2 cells from a human hepatic stellate cell line, which were provided by Dr Mutsumi Miyauchi (Hiroshima University, Hiroshima, Japan), were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37˚C and under 5% CO_2. Mycoplasma testing was done before and after the experiment.

Each TM6SF2 expression plasmid was transiently transfected into LX-2 cells by FuGENE HD Transfection reagent (Promega) in accordance with the instructions supplied by the manufacturer. Twenty-four hours after transfection, transfected cells were stimulated with 10 ng/ml of TGFβ1 for 48 h, and then the cells were harvested and stored at -80˚C until use.

Quantification of mRNA expression level. Total RNA was extracted from collected LX-2 cells using RNeasy Mini kit (Qiagen) and reverse-transcribed using ReverTra Ace (Toyobo Co., Ltd.) and random primer in accordance with the instructions supplied by the manufacturer. αSMA or *TM6SF2* mRNA levels were quantified from the resulting cDNA by quantitative PCR using the 7300 Real-Time PCR System (Applied Biosystems), with the expression of GAPDH serving as a control. Expression levels were compared using the Wilcoxon signed-rank test. Amplification was performed in a 25 μl reaction mixture containing 12.5 μl SYBR-Green PCR Master Mix (Applied Biosystems), 5 pmol of forward primer, 5 pmol of reverse primer, and 1 μl of cDNA solution. After incubation for 2 min at 50˚C, the sample was denatured for 10 min at 95˚C, followed by a PCR cycling program consisting of 40 cycles of 15 sec at 95˚C, 30 sec at 55˚C and 60 sec at 60˚C. The following primer sequences were used: αSMA; 5'-CTC ATTTTCAAAGTCCAGAGCTACA-3' and 5'-AGCGTGGCT ATTCCTTCGT-3', TM6SF2; 5'-TGAAGCCCACCACATAGC TG-3' and 5'-CGGTCTACAGCTTGTCCCAT-3', GAPDH; 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGT GATGGGATTTC-3'.

Automated capillary western blotting. LX-2 cells, transfected with TM6SF2 expression plasmids and treated with TGFβ1, were cooled on ice and dissolved with RIPA-like buffer [50 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% NP-40, 150 mM sodium chloride, and 0.5% sodium deoxycholate] containing protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were transferred onto capillary western immunoassay using Wes system (ProteinSimple). The proteins were detected with anti-TM6SF2 rabbit polyclonal antibody (Thermo Fisher Scientific, Inc.), anti-αSMA rabbit monoclonal antibody (Cell Signaling Technology Japan), or anti-GAPDH rabbit polyclonal antibody (Santa Cruz Biotechnology), followed by anti-rabbit immunoglobulin (GE Healthcare). Signal intensities were quantified using Compass software (ProteinSimple) and were corrected by GAPDH and analyzed by Mann-Whitney U test.

Knockdown of TM6SF2 by siRNA treatment. TM6SF2 siRNA was designed by siDirect (http://sidirect2.rnai.jp) using the TM6SF2 mRNA sequence (NM_001001524) as a reference. The designed siRNA sequence was as follows: 5'-AAAAUU CCGGUAUCUCUUCCU-3', 5'-GAAGAGAUACCGGAA UUUUGG-3'. Prepared siRNAs were transfected into LX-2 cells by electroporation using the Neon transfection system (Thermo Fisher Scientific, Inc.) at 1,100 mV for 30 msec followed by 24-h incubation with serum-free medium.

Immunocytochemistry. LX-2 cells that had been transfected with TM6SF2 expression plasmid or treated with siRNA were incubated for 48 h were fixed with 4% (v/v) paraformaldehyde and stained with anti-TM6SF2 antibody. The bound antibodies were detected with an Alexa 594-conjugated antibody against rabbit IgG (1:2,000) (Molecular Probes). Nuclei were counterstained with bisbenzimide H 33258 (Hoechst 33258; Abcam). The stained cells were examined using a Fluoview FV10i microscope (Olympus Co.). Fluorescence intensities of TM6SF2 were compared using the Mann-Whitney U test.

Statistical analysis. All experiments were performed in triplicate wells. All data are expressed as the mean ± standard deviation (SD) and are presented relative to control. Pairwise differences between groups were examined for statistical significance using the Mann-Whitney U test. Univariate or

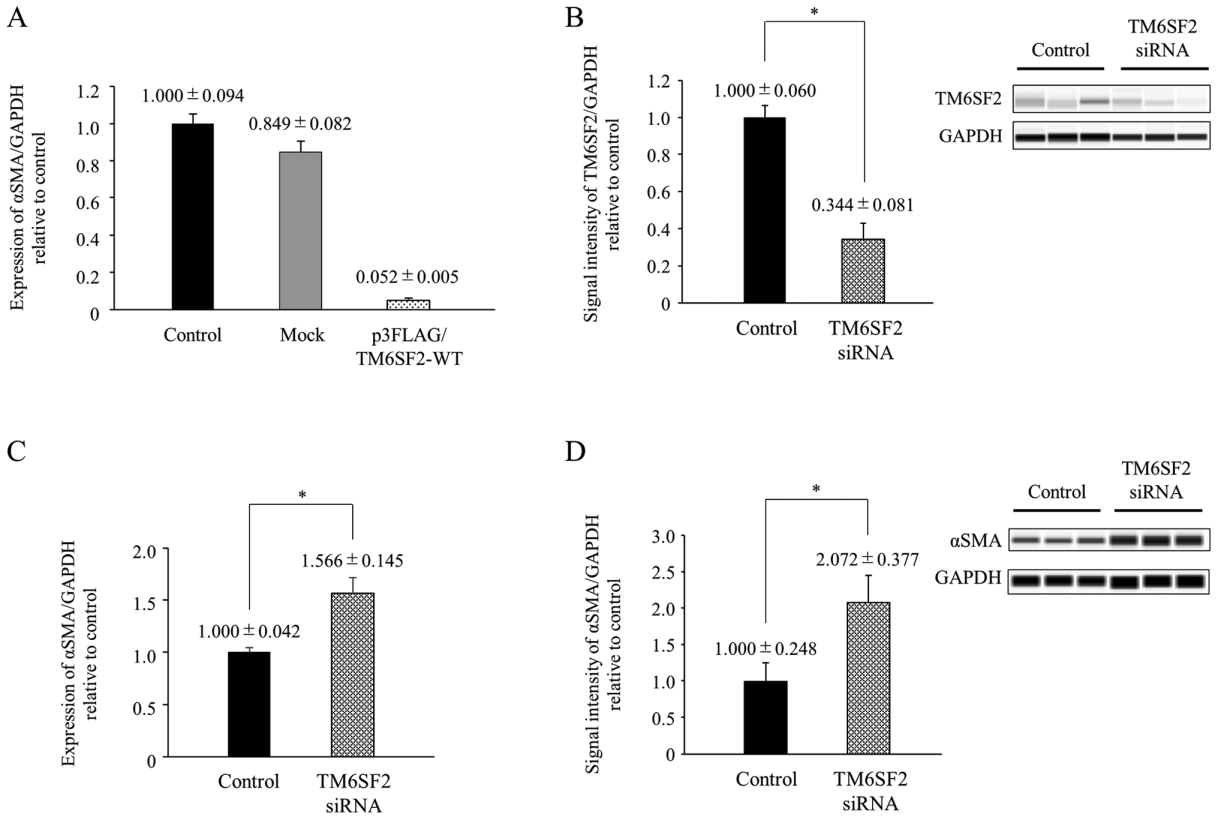

Figure 1. TM6SF2 regulates αSMA expression in LX-2 cells. (A) The cloned TM6SF2 expression plasmid (p3FLAG/TM6SF2-WT) and empty vector (Mock) were transiently transfected into LX-2 cells followed by 24 h of incubation. Intracellular αSMA expressions were measured by quantitative PCR. The expression of GAPDH served as a control. Experiments were performed in triplicate wells. (B) Non-treated and TM6SF2 knocked-down LX-2 lysates were transferred onto a automated capillary western blot analysis. Anti-TM6SF2 antibody or anti-GAPDH antibody were applied, followed by anti-rabbit immunoglobulin. Signal intensity was corrected by GAPDH and is shown in the bar graph. (C) Intracellular αSMA expression, measured by quantitative PCR, was compared in non-treated and TM6SF2 knocked-down LX-2 cells. GAPDH expression was used as a control. Experiments were performed in triplicate wells. (D) Non-treated and TM6SF2 knocked-down LX-2 lysates were transferred onto an automated capillary western blotting system. Anti-αSMA antibody or anti-GAPDH antibody were applied, followed by anti-rabbit immunoglobulin. Signal intensities were corrected by GAPDH and are presented in the bar graph. Experiments were performed in duplicate wells. *P<0.05. TM6SF2, transmembrane 6 superfamily 2; TGFβ1, transforming growth factor β1; αSMA, α-smooth muscle actin; si, small interfering.

multivariable differences among three or more groups were estimated using one-way or two-way ANOVA with Tukey's post-hoc multiple comparison test. P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp.).

Results

TM6SF2 regulates αSMA expression in LX-2 cells. To analyze the impact of *TM6SF2* on HSC activation, p3FLAG/TM6SF2-WT plasmid was transiently transfected into LX-2 cells, and the induction of alpha-smooth muscle actin (αSMA) level was compared. Although αSMA level was not changed by transfection with empty vector (mock), *αSMA* mRNA expression was significantly suppressed in the presence of TM6SF2 expression plasmids (one-way ANOVA, P<0.05, respectively) (Fig. 1A).

To verify this result, we also analyzed the association between *TM6SF2* and *αSMA* by knocking down TM6SF2. The siRNA targeted to *TM6SF2* was transfected into LX-2 cells by electroporation, and intracellular αSMA level was compared 24 h after siRNA treatment. TM6SF2 protein expression in LX-2 cells was suppressed to 34.4% by treatment with siRNA (Fig. 1B), and immunostaining of TM6SF2 exhibited the same results (Figs. S1 and 2). αSMA expression in TM6SF2-knocked down cells was 1.5~2.0-fold elevated compared to control cells in both mRNA and protein levels (Fig. 1C and D). A similar tendency was also observed in intracellular collagen type 1 alpha 1 (*COL1A1*) expression measured by real-time PCR (Fig. S3). These results suggest that TM6SF2 downregulates αSMA expression in HSCs.

TM6SF2 suppresses αSMA induction by TGFβ1 in LX-2 cells. To analyze the influence of TM6SF2 on αSMA expression under TGFβ1 stimulation, changes in αSMA expression in LX-2 cells were compared after TGFβ1 treatment. LX-2 cells were transfected with p3FLAG/TM6SF2-WT plasmid. Twenty-four hours after transfection, the cells were treated with 10 ng/ml of TGFβ1 for 48 h, and intracellular αSMA induction was analyzed by quantitative PCR. *αSMA* mRNA levels in TM6SF2-overexpressed LX-2 cells were significantly suppressed and failed to increase to control levels following TGFβ1 stimulation (Fig. 2A). A similar tendency was also observed in the siRNA experiment. The αSMA expression level in TGFβ1-stimulated LX-2 cells were similar

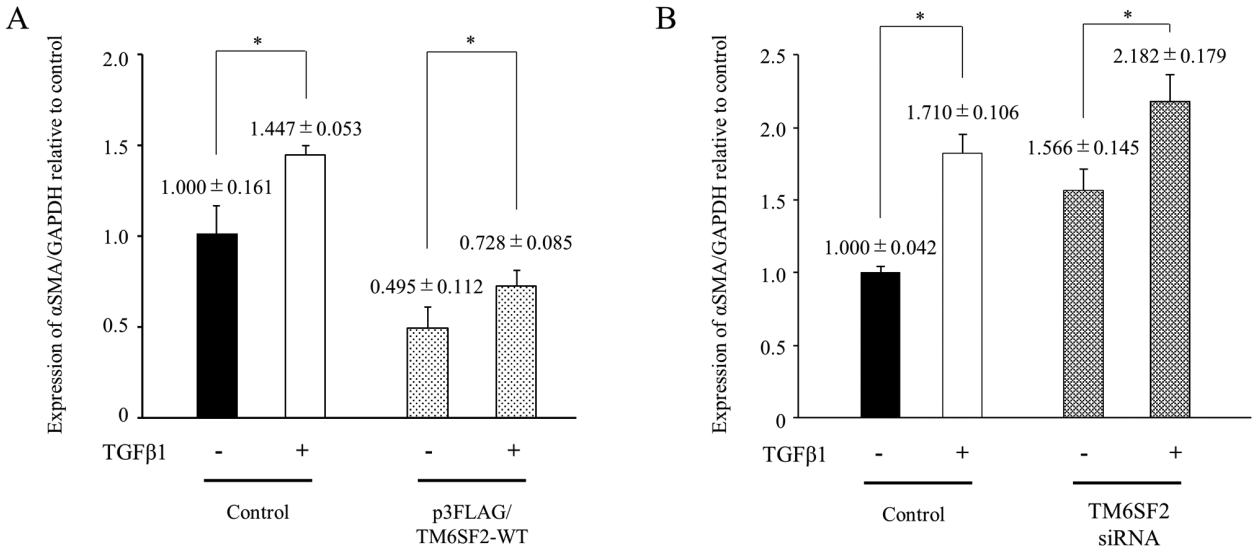

Figure 2. TM6SF2 suppresses αSMA induction by TGFβ1 in LX-2 cells. (A) The cloned TM6SF2 expression plasmid (p3FLAG/TM6SF2-MT) and empty vector (Mock) were transiently transfected into LX-2 cells followed by 24 h of incubation. LX-2 cells were stimulated with or without 10 ng/ml of TGFβ1 for 48 h. Intracellular αSMA expression was measured using quantitative PCR. The expression of GAPDH served as a control. Experiments were performed in triplicate wells. (B) Non-treated and TM6SF2 knocked-down LX-2 cells were stimulated with or without 10 ng/ml of TGFβ1 for 48 h and intracellular αSMA expression was compared using quantitative PCR. The expression of GAPDH served as a control. Experiments were performed in triplicate wells. *P<0.05. TM6SF2, transmembrane 6 superfamily 2; TGFβ1, transforming growth factor β1; αSMA, α-smooth muscle actin; MT, mutant type; si, small interfering.

in TM6SF2-knock down LX-2 cells, and the expression of αSMA was enhanced under TGFβ1 stimulation (Fig. 2B). These results suggested that HSCs could be additionally activated via TGFβ1 stimulation under lower expression of TM6SF2.

The impact of TM6SF2 phenotype on αSMA induction. To analyze the impact of the substitution at TM6SF2 amino acid 167, αSMA expression was compared between LX-2 cells transfected with p3FLAG/TM6SF2-WT plasmid and with p3FLAG/TM6SF2-MT plasmid. α*SMA* mRNA expression in cells transfected with p3FLAG/TM6SF2-WT plasmid was lower than that with p3FLAG/TM6SF2-MT plasmid (Fig. 3A; P<0.05). Similarly, αSMA protein expression was >50% suppressed following transfection with p3FLAG/TM6SF2-WT plasmid compared to transfection with p3FLAG/TM6SF2-MT plasmid (Fig. 3B; P<0.05).

LX-2 cells that had been transfected with or without TM6SF2 expression plasmid were stimulated by TGFβ1, and intracellular αSMA induction was analyzed by quantitative PCR. Although αSMA mRNA levels were suppressed by TM6SF2 expression, αSMA expression level in TM6SF2 E167K isoform (p3FLAG/TM6SF2-MT)-overexpressed LX-2 cells recovered and reached levels similar to those of control or mock cells after TGFβ1 stimulation (Fig. 3C). Cell transfection and TGFβ1 stimulation independently affected αSMA expression in LX-2 cells (two-way ANOVA; P<0.05), and, in particular, overexpression of p3FLAG/TM6SF2-WT plasmid significantly affected αSMA expression (Tukey's post-hoc multiple comparison test; P<0.05). A similar tendency was also observed in intracellular *COL1A1* expression estimated by real-time PCR (Fig. S4). These results suggest that basal αSMA expression in HSCs with TM6SF2 wild-type might be low but might be upregulated by TGFβ1 to a much higher level than HSCs with the TM6SF2 E167K isoform.

Discussion

NAFLD and NASH are progressive liver diseases characterized by accumulation of fat in human hepatocytes and an increased risk of cirrhosis or hepatocellular carcinoma. The number of patients is increasing worldwide, accompanied by recent upward trends in obesity, westernized high-fat oral intake, gut dysbiosis, inadequate exercise, and comorbid metabolic disorders like diabetes mellitus (2,24). To identify factors associated with NAFLD, clinical studies have concluded that the prevalence, prognosis, and progression or severity of disease is significantly associated with SNPs in *PNPLA3* (*rs738409*) and *TM6SF2* (*rs58542926*) (4,5,11,25,26). Several studies have shown that the *rs738409*SNP in *PNPLA3* causes a loss-of-function amino acid substitution (I148M) in *PNPLA3* that affects regulation of lipid droplets in human hepatocytes and retinol metabolism in human HSCs, resulting in positive modulation of HSC activation (27,28). However, the functional impact of the coding SNP in *TM6SF2* has not been sufficiently clarified. Although it has been reported that TM6SF2 is highly expressed in the liver, kidney, brain, and small intestine and that the E167K amino acid substitution TM6SF2 (rs58542926) interferes with localization to the endoplasmic reticulum due to protein misfolding (14,20), the association between the existence of the coding SNP in *TM6SF2* and activation of HSCs has not been fully elucidated.

We first analyzed the association between TM6SF2 and the activation of human HSCs. Intracellular αSMA mRNA expression in LX-2 cells was suppressed by TM6SF2 overexpression, and its expression was increased by knocking down TM6SF2 (Fig. 1A and D). Since similar results were observed in the other experiments (Fig. 2A and B), these data suggest that TM6SF2 negatively regulates HSC activation.

In the progression of liver fibrosis, it is well known that TGFβ1, secreted directly by HSCs or by activated Kupffer cells,

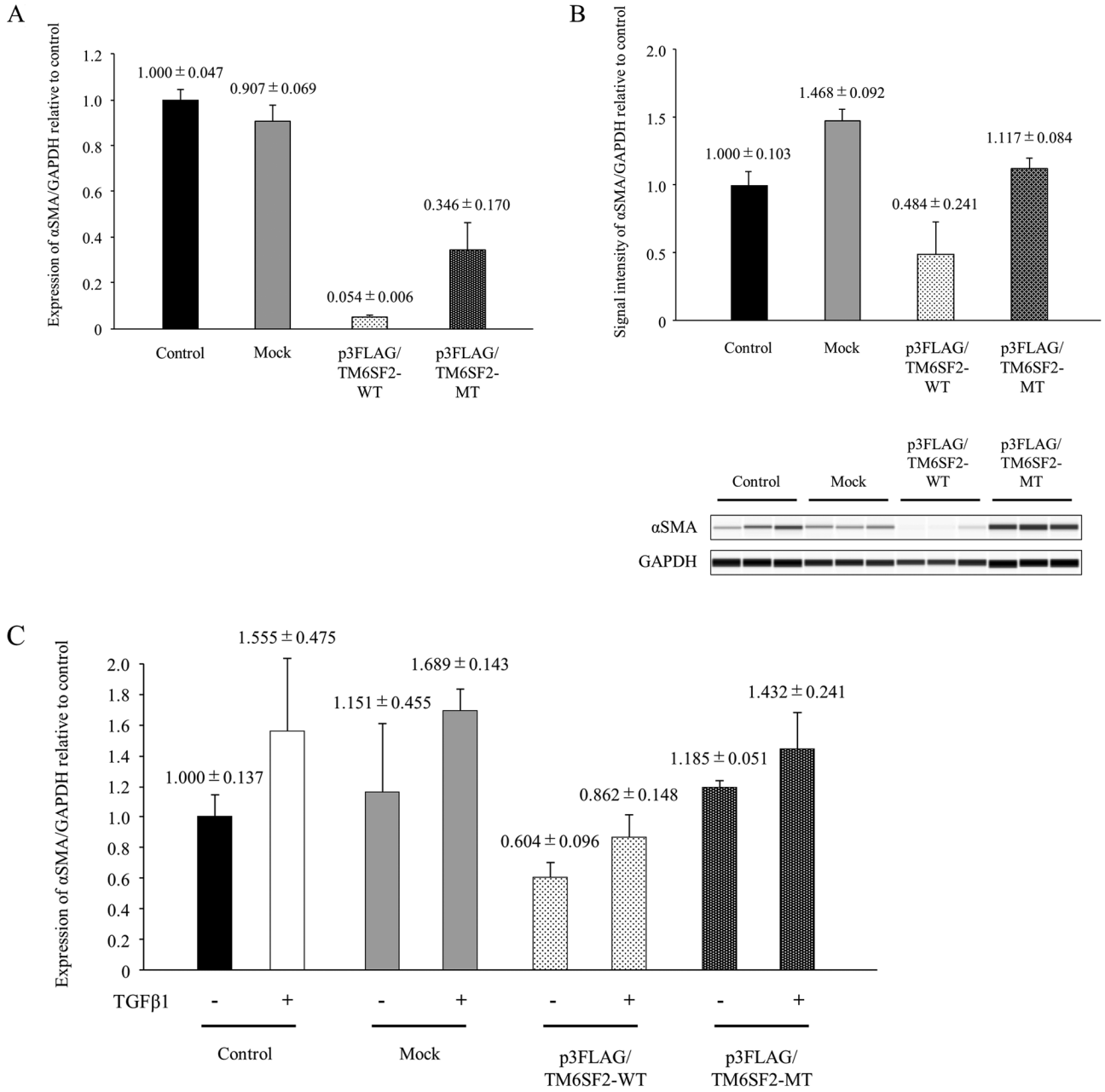

Figure 3. The impact of TM6SF2 phenotype on αSMA induction in LX-2 cells. (A) The cloned TM6SF2 expression plasmid consisting of p3FLAG/TM6SF2-WT and p3FLAG/TM6SF2-MT and empty vector (Mock) were transiently transfected into LX-2 cells, followed by 24 h of incubation. Intracellular αSMA expression was measured using quantitative PCR, with the expression of GAPDH serving as a control. Experiments were performed in triplicate wells. (B) Cloned TM6SF2 expression plasmids consisting of p3FLAG/TM6SF2-WT and p3FLAG/TM6SF2-MT and empty vector (Mock) were transiently transfected into LX-2 cells, followed by 24 h of incubation. LX-2 lysates were transferred onto a automated capillary western blotting system. Anti-TM6SF2 antibody or anti-GAPDH antibody were applied, followed by anti-rabbit immunoglobulin. Signal intensity was corrected by GAPDH, as shown in the bar graph. Experiments were performed in triplicate wells. (C) Cloned TM6SF2 expression plasmids consisting of p3FLAG/TM6SF2-WT and p3FLAG/TM6SF2-MT and empty vector (Mock) were transiently transfected into LX-2 cells, followed by 24 h of incubation. LX-2 cells were stimulated with or without 10 ng/ml of TGFβ1 for 48 h. Intracellular αSMA expression was measured using quantitative PCR, with GAPDH as a control. Experiments were performed in triplicate wells. TM6SF2, transmembrane 6 superfamily 2; TGFβ1, transforming growth factor β1; αSMA, α-smooth muscle actin; MT, mutant type; WT, wild-type.

could activate HSCs, triggering transformation of HSCs to myofibroblasts (22). Thus, we analyzed the impact of TM6SF2 on HSC activation via TGFβ1 signaling. Although intracellular αSMA expression in both control cells and TM6SF2 over-expressed cells was significantly upregulated by TGFβ1 treatment, αSMA expression in TM6SF2 overexpressed LX-2 cells was significantly lower than that in control LX-2 cells after TGFβ1 treatment (Fig. 2A). Similar results were observed in TM6SF2 knock down cells (Fig. 2B). A similar tendency was also observed in intracellular *COL1A1* expression measured using real time PCR (Figs. S3 and 4). These results indicate that lower TM6SF2 expression could activate HSCs and that TGFβ1 could enhance this HSC activation.

Subsequently, we analyzed the functional impact of the coding SNP in *TM6SF2 in vitro*. Normal HSCs have lipid droplets containing retinol. However, once HSCs are activated, lipid droplets are diminished, and retinyl ester is degraded in the endoplasmic reticulum (ER) in HSCs (29). Since the

amino acid substitution (E167K) in TM6SF2 (rs58542926 SNP) causes TM6SF2 to fail to localize to the ER (18,19), we propose that amino acid substitution E167K in TM6SF2 could induce HSC activation by disrupting homeostasis in the ER. When TM6SF2 wild-type or mutant type (E167K) were over-expressed in LX-2 cells, intracellular αSMA in LX-2 cells that overexpressed wild-type TM6SF2 decreased more than those that overexpressed mutant TM6SF2 (Fig. 3). Furthermore, αSMA expression in TM6SF2-mutant-overexpressed LX-2 cells increased to similar levels as control LX-2 cells after treatment with TGFβ1. Although the precise regulation of TM6SF2 in HSCs could not be determined in this study, our results suggest that the TM6SF2 E167K isoform affects HSC sensitivity by enhancing the response to TGFβ1.

In the present study, we demonstrated the impact of an amino acid substitution in TM6SF2 on liver fibrosis using LX-2 cells. Although the impact of this *TM6SF2* coding SNP on liver fibrosis might not be strong considering the hazard ratio calculated for this SNP by GWAS studies, we consider that these results could help to clarify the role of TM6SF2 and the impact of the *TM6SF2* SNP on the progression of liver fibrosis in NAFLD and NASH patients.

TM6SF2 negatively affects αSMA expression in HSCs, and the TM6SF2 E167K isoform associated with the *rs58542926* SNP might affect HSC activation sensitivity. These results suggest that TM6SF2 might play a role in the process of HSC activation and liver fibrosis in NASH.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SL, EM and KC conceived the study. GNM, AO, DM and HAC made contributions to the design of the experiment. SL and EM analyzed and interpreted the experimental data. TN, KO, YT, TU, KM, HF, MY, TK and AH were involved in analyzing the data and revising the manuscript. MT performed western blotting experiments and edited the manuscript. DM and AO performed part of the immunostaining experiments and checked gene expression analysis data. MI and HA designed the study and confirmed the quality of experimental data. CNH contributed to statistical analysis and proofreading. MT and CNH were major contributors in editing the manuscript. KC revised the manuscript and gave the final approval of the version to be submitted. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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