Review Article

TM6SF2 E167K Variant, a Novel Genetic Susceptibility Variant, Contributing to Nonalcoholic Fatty Liver Disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of liver dysfunction worldwide, and its prevalence is highly associated with genetic susceptibility. The transmembrane 6 superfamily member 2 (*TM6SF2*) E167K variant represents a general genetic determinant of hepatic triglyceride content and lobular inflammation, and its presence appears to be directly involved in the pathogenesis and development of NAFLD. Although this variant appears to be a novel powerful modifier in the development of NAFLD, whether it is associated with an increased risk of NAFLD-related liver fibrosis and hepatocellular carcinoma (HCC) remains to be determined. The aim of this review is to describe the functions of the *TM6SF2* E167K variant and its association with NAFLD, with particular emphasis on the underlying mechanisms of its role in the development and progression of NAFLD. Additionally, the links between the *TM6SF2* E167K variant and NAFLD-related liver fibrosis and HCC will be discussed.

Introduction

Nonalcoholic fatty liver disease (NAFLD) represents one of the most common chronic liver diseases and has emerged as a prevalent public health concern worldwide.[1,2] NAFLD can present as simple fatty liver, characterized by excess hepatic triglyceride content (HTGC) in the absence of excessive ethanol consumption,[3] and is generally considered a benign pathological process. However, approximately 20% of NAFLD patients display hepatocellular injury and lobular inflammation, designated nonalcoholic steatohepatitis (NASH). NASH may even progress to liver cirrhosis and/or hepatocellular carcinoma (HCC). These conditions have been demonstrated to confer increased liver-related mortality.[4–6] Furthermore, NAFLD, which may be a hepatic manifestation of metabolic syndrome,[7] can result in a variety of extrahepatic complications, including type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD).[5]

Generally, NAFLD is considered a complex disorder in which gene variants and environmental factors interact to determine disease phenotypes.[8–10] The predisposition to accumulate hepatic triglycerides varies considerably among individuals, partly as a result of genetic susceptibility.[11] Previously, genome-wide association studies (GWAS) have shown that a nonsynonymous single nucleotide polymorphism (SNP) in the patatin-like phospholipase domain-containing 3 (*PNPLA3*, also known as *adiponutrin*) gene, is significantly associated with the development and progression of NAFLD.[12–18] The associated polymorphism encodes an isoleucine to methionine substitution at residue 148 (I148M). However, NAFLD is not caused by individual SNPs or genes but by multiple susceptibility genes. A recent exome-wide association study published as a letter in *Nature Genetics* identified a potential association between a transmembrane 6 superfamily member 2 (*TM6SF2*) polymorphism (or rs58542926 c.449 C>T) and increased HTGC, as quantified by proton magnetic resonance spectroscopy (^{1}H-MRS).[19] The polymorphism resulted in a peptide in which glutamate is replaced with lysine at residue 167 (E167K). It has been reported that a variant within the *neurocan* (*NCAN*) gene (rs2228603 C>T), which is in strong linkage disequilibrium (D'=0.926, r^2=0.798) with the *TM6SF2* E167K variant, is also associated with radiologically and histologically characterized NAFLD in both GWAS[20,21] and candidate-gene studies.[22] Kozlitina and colleagues, for the first time, determined that the causative variant affecting HTGC was the *TM6SF2* E167K variant and not NCAN,[19] thereby explaining the association of the NCAN locus with altered lipid metabolism. Subsequently, several studies have investigated the link between the *TM6SF2* E167K variant and NAFLD.[23–25] This review summarizes current knowledge pertaining to the functions of the *TM6SF2* E167K variant and its association with

Keywords: Nonalcoholic fatty liver disease; *TM6SF2*; E167K variant; Polymorphism.
Abbreviations: ^{1}H-MRS, proton magnetic resonance spectroscopy; AAV, adeno-associated virus; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CVD, cardiovascular disease; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; GWAS, genome wide association studies; HCC, hepatocellular carcinoma; HTGC, hepatic triglyceride content; MI, myocardial infarction; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NCAN, neurocan; *PNPLA3*, patatin-like phospholipase domain-containing 3; SNP, single nucleotide polymorphism; T2DM, type 2 diabetes mellitus; *TM6SF2*, transmembrane 6 superfamily member 2; VLDL, very-low-density lipoprotein.
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NAFLD. In particular, we focus on the underlying mechanisms of the variant in the development and progression of NAFLD. In addition, the associations between the *TM6SF2* E167K variant and NAFLD-related liver fibrosis and HCC are discussed.

Function of *TM6SF2* gene and the *TM6SF2* E167K variant

TM6SF2, a gene with unknown biological function, is located on chromosome 19 and encodes a protein containing 351 amino acids, with 7–10 transmembrane domains predicted by protein pattern and domain prediction software.[26] *TM6SF2* is highly expressed in the liver, small intestine, and kidney, whereas its levels are relatively lower in other tissues.[19,27] Mahdessian *et al.* performed a protein subcellular localization study with confocal microscopy and observed that TM6SF2 protein was predominantly localized in the endoplasmic reticulum (ER) and the ER-Golgi intermediate compartment (ERGIC) in the human hepatoma cell lines, Huh7 and HepG2.[26]

Identification of the physiological functions of the *TM6SF2* gene is critical to understand the role of this gene in the development of diseases, such as NAFLD. However, the specific molecular functions of the *TM6SF2* gene remain unclear, and, despite extensive investigation, no functionally characterized proteins have been identified. The *TM6SF2* E167K variant (p.Glu167Lys substitution) has been demonstrated to reduce the expression of the TM6SF2 protein by 46% in Huh7 cells.[19] In a study of histologically proven NAFLD patients, carriers of the *TM6SF2* E167K variant had decreased mRNA and protein expression of TM6SF2 in the liver.[25] Moreover, functional studies showed that TM6SF2 activity is crucial for the secretion of very-low-density lipoprotein (VLDL)[19,26,28] and the activity of serum alkaline phosphatase (ALP).[19] Kozlitina and colleagues reported that recombinant adeno-associated viral (AAV) vectors expressing short hairpin RNAs mediated >90% hepatic inhibition of *TM6SF2* mRNA, accompanied with a 3-fold increase in HTGC and a 50% decrease in VLDL secretion, as well as a reduction in intestinal ALP activity.[19] Furthermore, a subsequent study confirmed that *TM6SF2* inhibition significantly reduced the expression of a number of genes (e.g. *PNPLA3, ACSS2, DGAT1,* and *DGAT2*) that play important roles in triglyceride synthesis and increased hepatic lipid droplet area and size.[26] Sanchez-Pulido and Ponting identified a novel functional domain, the EXPERA domain, which may possess catalytic activities as sterol isomerases.[29] In light of these findings, we speculate that the *TM6SF2* E167K variant may act as a loss of function mutation that enhances the accumulation of lipid content in the liver (Fig. 1). Briefly, the *TM6SF2* E167K variant is associated with a reduction in TM6SF2 function, which results in an increase in HTGC (by reducing VLDL secretion),[19,26,28] ALP activity,[19] expression of some lipid metabolism-related genes (e.g. *PNPLA3, ACSS2, DGAT1,* and *DGAT2*),[26] catalytic activities of sterol isomerases,[29] and/or other unidentified molecular mechanisms. However, the mechanism by which the *TM6SF2* E167K variant impairs VLDL secretion has not yet been determined.

Association between the *TM6SF2* E167K variant and NAFLD

In 2014, Kozlitina and colleagues explored for the first time genetic variants associated with predisposition to NAFLD in an exome-wide association study (the Dallas Heart

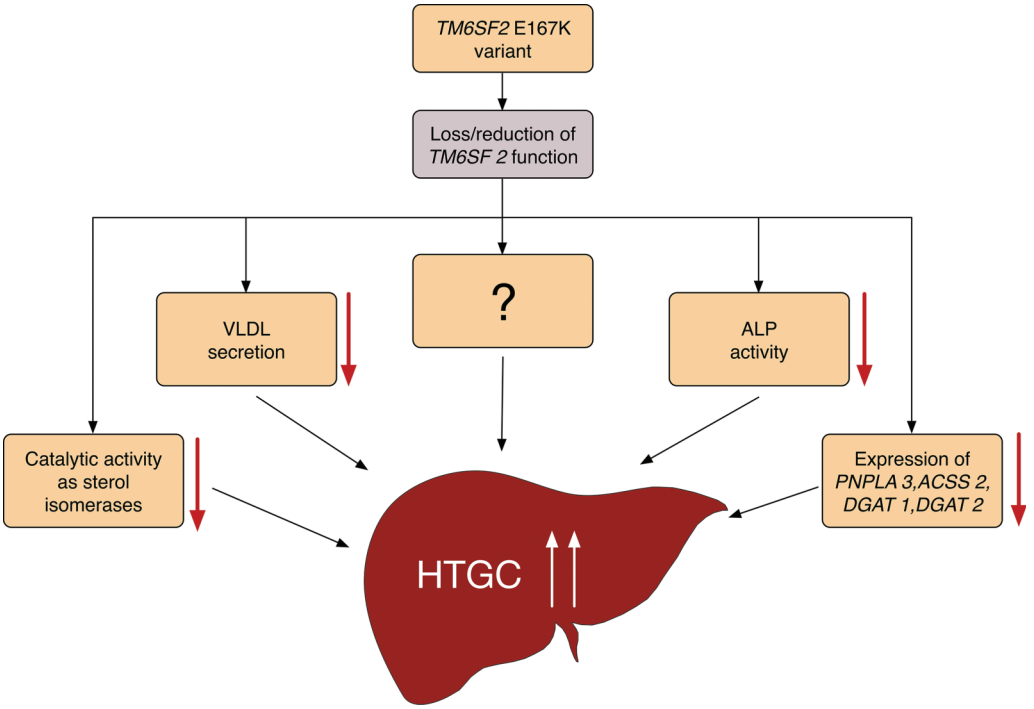

Fig. 1. Hypothetical molecular mechanism of hepatic triglyceride content accumulation associated with the *TM6SF2* E167K variant. *ACSS2*, acetyl-CoA short chain synthetase 2; ALP, alkaline phosphatase; *DGAT1*, diacylglycerol acyltransferase1; *DGAT2*, diacylglycerol acyltransferase2; HTGC, hepatic triglyceride content; *PNPLA3*, patatin-like phospholipase domain-containing 3; *TM6SF2*, transmembrane 6 superfamily member 2; VLDL, very-low-density lipoprotein.

Study).[19] They observed that the *TM6SF2* E167K variant, a nonsynonymous SNP, was a powerful determinant of HTGC in a multi-ancestry, multi-ethnic, and population-based cohort.[19] Interestingly, the association between the *TM6SF2* E167K variant and the hepatocyte triglyceride remodeling protein was independent of the effect of the *PNPLA3* I148M variant,[19] which was deemed a major common genetic modifier of NAFLD.[30] In addition, the *TM6SF2* E167K variant was strongly associated with increased levels of alanine aminotransferase (ALT). The former variant did not, however, affect aspartate aminotransferase (AST), suggesting that the variant was associated with liver injury, since ALT is one of the most accurate markers of liver dysfunction.[31]

Several subsequent studies have verified the association between the *TM6SF2* E167K variant and NAFLD in multi-ethnic groups, both in adults[23–25,32,33] and children (Table 1).[34] Wang *et al.*[24] carried out a case-control, community-based study of 768 Chinese NAFLD patients and healthy controls and demonstrated that, despite its low frequency, the *TM6SF2* E167K variant was significantly associated with NAFLD ($p<0.001$). This association remained significant upon occurrence of the *PNPLA3* I148M (rs738409) polymorphism ($p<0.001$) and the *NCAN* rs2228603 polymorphism ($p=0.001$); the latter was also identified as a risk factor of NAFLD.[20] Similarly, in an earlier study, Wong *et al.* reported that the *TM6SF2* variant was detected in four out of 920 Chinese subjects. These four subjects had increased hepatic fat content, and two of them had NAFLD.[32] These findings were replicated in a cohort of 300 Finnish subjects. The hepatic fat content in the analyzed subjects was significantly higher in carriers of the *TM6SF2* variant than in noncarriers.[33] In a study of 226 histopathologically proven NAFLD patients in Argentina, Sookoian and colleagues observed that the *TM6SF2* E167K variant was closely associated with the degree of hepatic steatosis, as measured by liver biopsy; and the effect was independent of age, sex, body mass index (BMI), and *PNPLA3* I148M polymorphism.[25] More recently, a study of 1,074 European Caucasian patients with histologically characterized NAFLD of different stages showed that the *TM6SF2* variant was associated with increased risk of pronounced steatosis (odds ratio [OR]= 1.379, 95% confidence interval [CI]: 1.019–1.865; $p=0.037$).[23]

Recently, three studies have reported an inconsistent association between the *TM6SF2* E167K variant and steatosis in the presence of chronic hepatitis C (CHC).[35–37] Coppola

Table 1. Studies evaluating the association between the *TM6SF2* E167K variant and non-alcohol fatty liver disease (NAFLD)

Author and year	Ref.	Population/ ethnicity/ country	N	Age	Diagnosis criteria	Key findings
Kozlitina *et al.*, 2014	19	European/ African/ Hispanic	2736	Adult	^{1}H-MRS	Increase in HTGC and serum ALT; reduced VLDL secretion and ALP activity
Wong *et al.*, 2014	32	Chinese	922	Adult	^{1}H-MRS	Increased HTGC but not associated with severe liver injury
Zhou *et al.*, 2014	33	Finnish	300	Adult	^{1}H-MRS	Increased liver fat content
Wang *et al.*, 2015	24	Chinese	768	Adult	Ultrasonography	Increased NAFLD risk, independent of the *PNPLA3* rs738409 and *NCAN* rs2228603 polymorphisms
Liu *et al.*, 2014	23	European	349	Adult	Liver biopsy	Associated with increased risk of greater steatosis
		European	725	Adult	Liver biopsy	Confers significant greater NAFLD-related, hepatic fibrosis/cirrhosis, independent of *PNPLA3* rs738409 polymorphism
		European Caucasian	99	Adult	Liver biopsy	Increased risk of NAFLD-HCC
Dongiovanni *et al.*, 2015	39	European	1201	Adult, pediatric	Liver biopsy	Increased susceptibility to NASH and advanced fibrosis, independent of the *PNPLA3* rs738409 but protects against cardiovascular disease
Sookoian *et al.*, 2015	25	Argentinean	361	Adult	Liver biopsy	Associated with the degree of liver steatosis but not lobular inflammation or fibrosis
Grandone *et al.*, 2015	34	Italian	1010	Pediatric	Ultrasonography	Associated with steatosis and higher ALT levels

ALP, alkaline phosphatase; ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; ^{1}H-MRS, hydrogen magnetic resonance spectroscopy; HTGC, hepatic triglyceride content; *NCAN*, neurocan; NAFLD, nonalcoholic fatty liver disease; *PNPLA3*, patatin-like phospholipase domain-containing 3; *TM6SF2*, transmembrane 6 superfamily member 2; VLDL, very-low-density lipoprotein.

et al. carried out a study in a cohort of 148 consecutive biopsy-proven CHC patients with different hepatitis C virus (HCV) genotypes and demonstrated that the score of liver steatosis (as assessed by a partially modified Kleiner scoring system for NAFLD[35,38]) was higher in patients with the *TM6SF2* E167K variant (1.9±1.3) than in patients with the *TM6SF2* 167E allele (1.1±1.1, p=0.02).[35] In agreement with Coppola's study, Milano and colleagues established a link between the *TM6SF2* E167K variant and severe steatosis (p=0.038). This finding was independent of age, gender, HCV-genotype 3, BMI, T2DM, alcohol intake, ancestry, and the *PNPLA3* I148M polymorphism.[36] However, in a large Caucasian cohort of 694 biopsied genotype 1 CHC patients, carriers of the *TM6SF2* E167K variant exhibited a similar distribution of steatosis severity compared to non-carriers (p=0.63).[37] This finding was in contrast to the two previous studies.[35,36] Several factors may contribute to the conflicting results in these studies, including the different HCV genotypes, the number of enrolled patients, and differences in the histological assessments.

In a study of 1,010 obese children, the *TM6SF2* E167K variant was reported to predispose children to NAFLD. In the same study, the *TM6SF2* E167K variant was significantly associated with steatosis and higher levels of ALT.[34] However, this association in children needs to be confirmed in well-designed studies that include larger and multiple ethnic pediatric NAFLD patients with matched healthy children.

TM6SF2 E167K variant in the progression of NAFLD/NASH

NASH is considered the potentially progressive and fatal form of NAFLD, which may result in dramatically increased risks of developing cirrhosis, HCC, and/or other end-stage liver diseases.[4] Therefore, the prognosis of NAFLD patients is largely determined by whether or not NASH can be controlled or even reversed. The detailed pathogenesis of NASH, however, remains unclear, and thus, strategic management of NASH is still lacking.

Recent studies have explored the association between the *TM6SF2* E167K variant and NASH.[23,39] Liu *et al.* confirmed in a study of 349 biopsy-proven patients that the *TM6SF2* E167K variant was associated with the severity of histological NASH (p=0.039) and a greater risk of NAFLD-related liver fibrosis (p<0.001). This result was independent of gender, age, BMI, T2DM, and the *PNPLA3 I148M* polymorphism.[23] Furthermore, in a large cross-sectional cohort of 1,201 subjects with histologically confirmed NASH, Dongiovanni *et al.* observed that carriers of the *TM6SF2* E167K variant had significantly increased susceptibility to NASH (OR=1.84, 95% CI: 1.23–2.79; p=0.003) and advanced fibrosis (OR=2.08, 95% CI: 1.20–3.55; p=0.008). These results were independent of the *PNPLA3* I148M variant.[39] However, the effect of the *TM6SF2* E167K variant on fibrosis severity was abolished after conditioning for NASH. In addition, this study also showed, for the first time, the associations of this variant with hepatocellular ballooning (p=0.036), lobular necroinflammation (p=0.040), and the full spectrum of fibrosis severity (p=0.022).[39]

The association between the *TM6SF2* E167K variant and fibrosis was not replicated in two recent studies.[25,32] Sookoian and colleagues, recruited 226 histopathologically proven NAFLD patients and demonstrated that the *TM6SF2* E167K variant was not associated with severity of fibrosis (OR=0.95; 95% CI: 0.66–1.36; p=0.77), lobular inflammation (OR=0.85; 95% CI: 0.60–1.20; p=0.34), or hepatocellular ballooning (OR=1.08; 95% CI: 0.78–1.50; p=0.64).[25] Furthermore, Wong *et al.* performed a study on 920 Chinese patients and reported that carriers of the *TM6SF2* E167K variant did not exhibit increased fibrosis relative to noncarriers. This was assessed using transient elastography.[32] It is possible that the inconsistent results may be partially due to the low frequency of the *TM6SF2* E167K variant in these two studies.[25,32] The frequency of the variant was 0.4% and 8.6%, respectively, and only four and 15 patients with fibrosis were carriers of the *TM6SF2* E167K variant, respectively. In conclusion, there is a minor association between the *TM6SF2* E167K variant and fibrosis. However, further well-designed studies that include larger numbers and multiple ethnic NAFLD patients are required to evaluate fully this association.

Even if there is a link between the *TM6SF2* E167K variant and NASH/fibrosis, the underlying specific mechanism by which the *TM6SF2* E167K variant plays a role in NASH/fibrosis remains unclear. Several studies have confirmed that dietary cholesterol and dysregulated cholesterol metabolism play a crucial role in the development and severity of hepatic inflammation and NASH.[41,42] However, another study demonstrated that accumulation of hepatic triglyceride may not be directly hepatotoxic in mice with methionine and choline diet-induced NASH.[40] In addition, Marí *et al.* reported that mitochondrial free cholesterol accumulation participated in the development of NASH as a first hit, by sensitizing hepatocytes to inflammatory cytokine-mediated death through the depletion of mitochondrial glutathione.[43] Free cholesterol has been shown to sensitize the main source of extracellular matrix in fibrosis,[44] hepatic stellate cells, to transforming growth factor β-mediated activation through enhancement of Toll-like receptor 4 signaling, resulting in exacerbation of hepatic fibrosis.[45] Based on the available findings, the *TM6SF2* E167K variant may contribute to the development and progression of NASH and/or NAFLD-associated hepatic fibrosis. This is likely to occur following the regulation of cholesterol metabolism and not the accumulation of triglyceride. However, whether progressive liver disease is related to cholesterol and/or triglyceride retention needs to be further investigated.

Finally, Liu and colleagues reported that the *TM6SF2* E167K variant also confers increased predisposition to NAFLD-related HCC (OR=1.922, 95% CI: 1.31–2.81; p<0.001).[23] However, this association was not apparent when risk factors, including gender, age, presence of T2DM, and cirrhosis, were considered (p=0.42). However, it should be noted that this study recruited only 99 Northern European Caucasian patients, where *TM6SF2* E167K is a low-frequency (*i.e.* minor allele frequency) variant. Thus, further investigation including a larger cohort of NAFLD-related HCC subjects is required to investigate the potential association.

Clinical implications and future research directions

As described above, a potential association between the *TM6SF2* E167K variant and NAFLD has been revealed.[19,23–25,32–34,39] Several studies have established that carriers of the *TM6SF2* E167K variant are protected from CVD.[28,39] Holmen and colleagues demonstrated that the *TM6SF2* E167K variant was associated with lower circulating lipoprotein levels.

The *TM6SF2* E167K variant also protected against myocardial infarction (MI) in a cross-sectional study (OR=0.87, 95% CI: 0.79–0.93, p=0.005).[28] Moreover, Dongiovanni *et al.* carried out a study in a large, prospective cohort and confirmed that the E167K carriers had a lower risk of developing carotid plaques (OR=0.49; 95% CI: 0.25–0.94) as well as a lower incidence of CVD (hazard ratio: 0.61; 95% CI: 0.39–0.95).[39]

The underlying mechanism linking the *TM6SF2* E167K variant to an increased risk of NAFLD, but decreased risk of CVD, remains to be determined. In the future, larger well-designed studies, including multiple ethnic groups, are required to confirm the association between the *TM6SF2* E167K variant and NAFLD and/or NAFLD-related metabolic syndrome (including CVD). Moreover, *TM6SF2* E167K transgenic mouse models may help to explore further the physiological and pathophysiological role of this gene in the development and progression of NAFLD, providing valuable experimental data for potential therapeutic targets.

Conclusions

The *TM6SF2* E167K variant, despite the low frequency of the minor allele, appears to be a novel powerful modifier associated with the development of NAFLD. Whether or not the variant is associated with an increased risk of NAFLD-related fibrosis and HCC remains to be determined. Identification of the *TM6SF2* E167K variant contribution to NAFLD should help in the development of new genetic predictors for early diagnosis of NAFLD, allowing for implementation of early preventive and therapeutic strategies for NAFLD in high-risk populations. However, further investigation is required to understand the underlying mechanisms involved in this process, thus facilitating the development of clinical applications.

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Conflict of interest

None.

Author contributions

Proposing the concept of the review (YNX), drafting and writing the manuscript (LZC), revising the manuscript with critical comments (HHXX, ZHL), approving the final version (SYX).

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