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***Basic Study***

# Effect of SPTLC1 on Type 2 diabetes mellitus

Effect of SPTLC1 on T2DM

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**Abstract**

BACKGROUND

Although numerous single nucleotide polymorphism in multiple genes involve in the risk of Type 2 diabetes mellitus (T2DM), the single gene defects of T2DM with strong family history is not clear yet. Serine Palmitoyltransferase Long Chain Base Subunit 1 (SPTLC1) are causative for Hereditary Sensory and Autonomic Neuropathy, which is clinical overlapping with diabetic peripheral neuropathy. Mice with adipocyte-specific deletion of SPTLC1 had impaired glucose tolerances and insulin sensitivity. Thus, it is necessary to investigate the *SPTLC1* mutations in adult-onset T2DM with strong family history.

AIM

To analyze the role of *SPTLC1* mutation on adult-onset T2DM with strong family history.

METHODS

By whole-exome sequence analysis of a patient with T2DM and his family members, an uncertain variant in *SPTLC1* was identified. Bioinformation analysis was used to evaluate the influence of mutation, rare variant gene-level associations for *SPTLC1* in T2DM, and the relationship between *SPTLC1* mRNA and T2DM in human islets from GSE25724. The effect of G371R of *SPTLC1* on the characteristics of inflammatory cytokines and apoptosis was also tested on HEK293 cells.

RESULTS

A single nucleotide variation in *SPTLC1* (c.1111G > A: p.G371R) was identified in a family with T2DM. The deleterious variant was predicted by FATHMM and M-CAP software. This pathogenicity might be derived from the different amino acid properties. In HEK 293T cells, p.G371R of *SPTLC1* induced the expression of TNF-α and the percent of apoptosis. Meanwhile, rare variant gene-level associations for *SPTLC1* also refer to

the high risk of T2M (the overall OR = 2.4968, $p$= 0.0164). Data from GSE25724 showed that *SPTLC1* mRNA was lower in pancreatic islets from T2DM human islets ($p$= 0.046), and was associated with the decreased level of *insulin* mRNA expression (Spearman $r$= 0.615, $p$= 0.025).

CONCLUSION

The study classified *SPTLC1* p.G371R mutation as the likely pathogenic mutation from an adult-onset T2DM patients with strong family history T2DM.

**Key Words:** Apoptosis; Diabetic peripheral neuropathy; Gene mutation; Inflammation; Serine Palmitoyltransferase Long Chain Base Subunit 1; Type 2 diabetes mellitus

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**Core Tip:** The single gene defects of type 2 diabetes mellitus (T2DM) with strong family history is not clear yet. We identified Serine Palmitoyltransferase Long Chain Base Subunit 1 (SPTLC1) p.G371R mutation as the likely pathogenic mutation from an adult-onset T2DM patients with strong family history. We also found that this mutation induced the expression of tumor necrosis factor-α and the percent of apoptosis in HEK 293T cells. Moreover, data from AMP T2D knowledge showed a positive correlation between rare variant gene-level associations for *SPTLC1* and the risk of T2M. This study provides a novel perspective to understand the pathology of T2DM.

## INTRODUCTION

Diabetes is a major global health issue that comes with severe complications, such as diabetic peripheral neuropathy (DPN), and higher mortality rates[1, 2]. Type 2 diabetes mellitus (T2DM), accounts for 90-95% of all diabetes, is described as a polygenetic multifactorial and heterogeneous disease[2], while maturity-onset diabetes of the young

(MODY) is caused by single gene mutations[2]. A growing number of studies indicated T2DM overlaps clinically and genetically with MODY[2,3]. For example, both T2DM and MODY present strong family history[2, 3]. And some patients with T2DM harbor single gene mutations that cause MODY, such as *HNF4A* p.T131I[2-4]. However, the potential genetic etiology of T2DM with strong family history is not clear yet.

Although numerous single nucleotide polymorphism (SNP) in multiple genes involve in the β-cell dysfunction, single gene defects may also influence risk of T2DM[2,5-7]. Whole genome sequencing has used to identify mutation in *WFS1, PAX6, APPL1* and *MAFA* gene as novel genetic cause of adult-onset familial diabetes[3]. Interestingly, among those gene, *PAX6* mutation have been reported to diminish glucose stimulated insulin release in human and to regulate pancreatic islet development[3]. Additionally, single gene defects were also reported in patients with T2DM and some hereditary disease[8,9]. For example, pathogenic mutations in *G6PC*, which is associated with postprandial hyperglycemia, was found in patients with T2DM and Glycogen storage disease type 1a (GSD1a)[9]. Thus, it is necessary to investigate the single gene mutations in T2DM with strong family history.

Serine Palmitoyltransferase Long Chain Base Subunit 1 (SPTLC1), the rate-limiting enzyme in sphingolipid biosynthesis, covers serine and palmitoyl-CoA to sphinganine (SA). Pathogenic variants in *SPTLC1* are causative for Hereditary Sensory and Autonomic Neuropathy (HSAN)[10, 11], Juvenile Amyotrophic Lateral Sclerosis[12, 13], macular telangiectasia type 2[11], or Charcot-Marie-Tooth disease[14]. Mice with adipocyte-specific deletion of *SPTLC1* had impaired glucose tolerances and insulin sensitivity[15]. Deoxysphingolipids, one of the catalytic products by SPTLC1, are cytotoxic for insulin-producing cells and neurons, and contribute to diabetes and DPN[16-20]. Therefore, we performed whole-exome sequencing (WES) combinate with functional study to identify a novel likely pathogenic variant in the *SPTLC1* gene (p.G371R) from adult onset T2DM with strong family history.

## MATERIALS AND METHODS

*Subjects*

Six participants from a China family with T2DM were enrolled and provided written informed consent for genetic analysis according to ethical guidelines of the Declaration of Helsinki (1964).

The proband of the family was a 52-year-old man (Family member III 1), who presented with a 10-year history of T2DM. The initiation symptoms of the index patient were polyuria, excessive drinking and eating, and weight loss. He began to feel fatigue of bilateral lower limb at age 44, and continued to progress to stabbing pain and numbing in a pair of feet. Sensory disturbance of this patients was confirmed through touching by cotton wool, 10-g monofilament, and electromyography. Notably, his pain could be reduced after controlling blood glucose during hospitalization. As shown in Table 1 and Figure 1, the patients' fasting and postprandial C-peptide levels at the time of diabetes onset were 1.04ng/mL (normal range 0.81-1.89ng/mL) and 2.39ng/mL, respectively, with hemoglobin A1c (HbA1c) 12.6%. Autoantibodies against glutamic acid decarboxylase (GADA), islet-cell (ICA), insulin (IAA), and insulinoma antigen-2 (IA-2A) were negative. During the past ten years, he always gave up on hypoglycemic treatment at home. Nevertheless, he did not present diabetic ketosis.

*Sequencing*

Genomic DNA samples were extracted from peripheral blood leukocytes. WES was analyzed in WeHealth Biomedical Technology Co.,Ltd (Shanghai, China) using 150 base pair paired-end sequencing on the Illumina HiSeq platform. Polymerase chain reaction (PCR) was performed to confirm the identified variant in Tsingke Biotechnology Co., Ltd (Beijing, China). Primer sequences are TGGGATACTGAGGTGAGAAGGG (Forward primer) and AGCTGCAATCTGGTCAAACTGA (Reverse primer). Sequences were compared to reference sequence NM_006415 of the *SPTLC1* gene by SeqMan Pro software.

*Mutation analysis*

The raw reads were aligned by Burrows-Wheler Aligner (BWA) tools, and duplicates were removed from the sorted alignment using Picard. Variants were classified according to the American College of Medical Genetics guidelines[21]. UniProtKB entry O15269 was used to annotate the STPLC1 protein. The structure of STPLC1 protein was built by using SWISS-MODEL.

**Bioinformation analysis**

The relative expression level of SPTLC1 in tissue specificity was obtained from ProteomicsDB (https: //www.proteomicsdb.org/) entry O15269. Rare variant gene-level associations for SPTLC1 in T2DM were analyzed in AMP T2D Knowledge Portal (www.type2diabetesgenetics.org), funding by the National Institutes of Health (NIH), the U.S. Food and Drug Administration (FDA), biopharmaceutical companies and non-profit organizations[6]. This project seeks to promote understanding and treatment of T2DM and its complications from 310 datasets.

The gene expression profile data (GSE25724) were obtained from Gene Expression Omnibus (GEO). This array data included 6 type 2 diabetic human islets (mean age and BMI were 71years and 26 kg/m$^2$, and three males) and seven non-diabetic islet samples (mean age 58years, mean BMI 24.8 kg/m$^2$, and four males)[22].

***Cloning***

The *SPTLC1* cDNA (NM_006415) was amplified and cloned into the vector FV149 with the use of TTGGTACCGAGCTCG (Forward primer) and GATATCTGCAGAATT (Reverse primer). The *SPTLC1* mutation (G371R) was introduced by site-directed mutagenesis with a C-terminally EGFP-tagged. All constructs were validated by sequencing. Cell lines expressing wild-type or G371R were created using LipofectamineTM 2000 in HEK 293T (Invitrogen). The ratio of plasmid and LipofectamineTM 2000 was 5μl: 4μg.

***Cell culture***

HEK 293T cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum and 1% Penicillin-Streptomycin solution at 37°C and 5% CO2. Before transfection, the cells were cultivated in serum-free DMEM for 2 hours. After 48h incubation time for the transfection, the cells and cultured supernatants were collected.

***Measurement of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)***

The concentrations of TNF-α and IL-1β in cultured supernatants were measured by commercial enzyme-linked immunosorbent assay kit (Elabscience Biotechnology Co., Ltd, Wuhan, China).

Real-time quantitative PCR (RT-qPCR) was used to detect the mRNA expression of TNF-α and IL-1β from HEK 293T cells. The forward and reverse primes of TNF-α (*Homo*) were TCAGAGGGCCTGTACCTCAT and GGAAGACCCCTCCCAGATAG, respectively. The forward and reverse primes of IL-1β (*Homo*) were CGAATCTCCGACCACCACTA and AGCCTCGTTATCCCATGTGT, respectively.

This reaction was performed in triplicate with 10ng cDNA in SYBR Green Master Mix and run on an ABI QuantStudio 6 system (Applied Biosystems). The mRNA expression levels were normalized by *Homo*-GAPDH.

***Assessment of cell apoptosis***

The apoptosis of HEK 293T cells after the transfection of wild-type or G371R was detected by AnnexinV-APC/7-AAD Apoptosis Detection Kit (Elabscience Biotechnology Co., Ltd, Wuhan, China). Briefly, HEK 293T cells were harvested after 48h and pelleted by centrifugation at 1500rpm for 5 min. The resuspended cells (1*105 cells/mL) were stained with 5μl 7-AAD and 1μl Annexin V-APC at room temperature. The percentage of cell apoptosis was determined by cytoFLEX platform (Beckmancoulter, USA).

***Statistical analysis***

The one-way *ANOVA* multiple comparisons were used for statistical analysis by SPSS 19.0 software. A two-tailed *p*-value < 0.05 was considered statistically significant.

## RESULTS

### *Description of the family*

III-1 is the proband of the pedigree, who suffered from T2DM at the age of 42yr and was diagnosed with DPN one year later. Surprisingly, the patient's mother, maternal aunt, and grandmother were also diagnosed with DM and DPN (Table 1, Figure 1 and Support material Table 1). Furthermore, the proband's son, sister, and a younger male cousin(III-11) also have DM, whose illness onset ages are 17,41and 22 yr, respectively. And the III-1also has obesity (Figure 2).

### *Identification of the SPTLC1 gene mutation*

WES analysis did not reveal the mutation of candidate genes of monogenic diabetes, such as *GCK*, *HNF1A*, *HNF4A*, *HNF1B*, *KCNJ11*, *INS*, and *ABCC8*, *et al*. However, we found a single nucleotide variation (SNV) in *SPTLC1*(NM-006415: exon12: C.1111G > A: p.G371R) in the proband (Figure 3A). Proband's mother, sister, and son were confirmed to carry this mutation by Sanger sequencing. However, his wife and father do not carry this mutation. Unfortunately, we don't have detailed data yet about II-4 or III-1.

The frequencies of this mutation in humans are less than 0.05% in dbSNP, ExAC, genom AD, or 1000 Genome databases. Although the interpretation of this mutation is likely benign from dbSNP, pathogenic variant effect on SPTLC1 protein was predicted by FATHMM and M-CAP software. This pathogenicity might be derived from the different amino acid properties (Figure 3C-D): A) the mutated residue is located in a domain that is important for the interaction with other molecules or the parts of the other protein, but is not essential for the catalytic site. b) the new residue was bigger and more hydrophilic than wild-type residue. c) the mutation introduces a positive charge, instigating repulsion between the mutant residue and neighboring residues (https: //www3.cmbi.umcn.nl/hope/report/625d75962412fdabe40cc1ce/). Thus, this

mutation was classified into uncertain significance (PM2+PP1+PP3) according to American College of Medical Genetics and Genomics (ACMG) criteria.

***The pro-inflammatory activity of SPTLC1 p.G371R***

Because the 371 amino acid residue is not essential for catalytic activation of SPTLC1 (https: //www.ncbi.nlm.nih.gov/Structure/icn3d/full.html?showanno = 1&mmdbid = 199148), we tested whether the mutation could give rise to the potential any other functions of SPTLC1. As shown in Figure 4, both mRNA and protein levels of TNF-α were slightly elevated in p.G371R expressing HEK 293T cells ($p< 0.05$). But the mutation of *SPTLC1* did not affect the expression of IL-1β.

***Pro-apoptosis of SPTLC1 p.G371R***

Flow cytometric analysis indicated that the percentage of apoptosis in p.G371R expressing cells was nearly three times than that of WT-expressing cells (Figure 5).

***Association between SPTLC1 gene rare variant and T2DM risk***

Of some interest, the data of 43125 humans from AMP T2D knowledge also showed a positive correlation between rare variant gene-level associations for *SPTLC1* and the risk of T2M (the overall OR = 2.4968, $p= 0.0164$) (Table 2).

***Effect of SPTLC1 gene on T2DM from GSE25724 data set***

SPTLC1 protein, localized to the endoplasmic reticulum, was found in widely cells and tissue. The median SPTLC1 protein expression in pancreatic islets ranks No.4 in all 39 human tissues (https: //www.proteomicsdb.org/). Importantly, data from GSE25724 showed that the relative expression of *SPTLC1* mRNA (ID-REF: 202278_s_at) was lower in pancreatic islets from 6 T2DM humans than 7 non-T2DM subjects ($p= 0.046$) (Figure 6). The lower expression of *STPLC1* mRNA was associated with a decreased level of *insulin* (INS, ID = REF: 206598_at) mRNA expression (Spearman $r= 0.615$, $p= 0.025$) (Figure 6). The receiver operating characteristic (ROC) curve indicated that the lower

level of *STPLC1* mRNA in islets could be used to diagnose T2DM with the area under the curve (AUC) 0.833 (95% confidence intervals (95%CI) 0.593-1.000, $p$= 0.046).

## DISCUSSION

Early genetic analysis based on familial linkage or Genome-wide association studies (GWAS) have revealed that T2DM is a disease of polygenic inheritance[1,5,6,23,24]. However, the role of single gene mutations in T2DM with strong family history are still unknown. In this study, we classified a novel likely pathogenic mutation in the *SPTLC1* gene from an adult-onset T2DM patients with strong family history by whole-exome sequencing (WES) combinate with functional study.

We initially identified a variant (p.G371R) in the *SPTLC1* gene as uncertain significance according to ACMG criteria in a family with dominant adult-onset diabetes (PM2+PP1+PP3). Nonetheless, uncertain genetic variants should not be considered benign because they may also confer a risk of related disease. It has been reported that in 36 diabetic patients carrying candidate variants, 27.9% were uncertain significance variants, which is more than likely pathogenic variants (20.9%)[25]. A recent study also reported that cardiomyopathy patients with uncertain variants in actionable cardiac genes had comparable left ventricular internal diameter-diastole and -systole than patients without medically actionable genes[26].

Furthermore, this study supports that p.G371R mutation is harmful to the function of SPTLC1 by functional study in HEK 293T cells (PS3). The pathogenic effect of p.G371R was predicted by FATHMM and M-CAP software (Figure 3). Additionally, some studies found the crucial role of SPTLC1, especially p.Y164F mutation, on the survival of bone marrow cells and adipocytes[27,28]. Consistently, this study also found that p.G371R of *SPTLC1* induced the expression of TNF-α and the percent of apoptosis in HEK 293T cells. (Figure 5). Thus, *SPTLC1* p.G371R may be a likely pathogenic mutation.

Moreover, there are some evidences to support the potential pathogenic role of SPTLC1 on adult-onset diabetes with strong family. Firstly, AMP T2D knowledge shows the rare variant of the *STPLC1* gene infer the high risk of T2DM by seven

different masks approaches (Table 2), which were developed to reveal the gene-level correlation of rare variants[6]. Based on this strategy, Flannick *et al*.[6]found that the most vital T2DM gene-level signals explain nearly 25% of the heritability of the strongest common single-variant signals.

Secondly, this study reported the pro-apoptosis and pro-inflammatory effect of *SPTLC1* p.G371R (Figure 4 and 5), which is consistent with the previous studies[27,28]. It has been confirmed that apoptosis of islets β-cells induced by inflammatory cytokines was the key molecular mechanism of diabetes mellitus[29]. Actually, the study found that lower expression of *SPTLC1* mRNA in pancreatic islets from diabetic patients was negatively associated with insulin levels (Figure 6). Interestingly, mice with adipocyte-specific deletion of *Sptlc1* exhibited insulin resistance and impaired glucose tolerance[15]. Cellular effects of SPTLC1 deletion appear to involve activation of endoplasmic reticulum (ER) stress[27], which leads to the progressive β-cell failure[29].

Thirdly, deoxysphingolipids, formed by the pathogenic mutation of *STPLC1*, participated in the development of T2DM and DPN[18-20]. It has been confirmed that the plasma concentration of deoxysphingolipids was significantly elevated in patients with metabolic syndrome, T2DM, and DPN[18, 19, 30, 31]. Importantly, deoxysphingolipids were independent risk factors for T2DM even after adjusting for glycated hemoglobin, age, and BMI[18, 19]. Lowering deoxysphingolipids by *L*-serine supplementation drastically improved the nerve conduction velocity in streptozotocin induced diabetic rats[17]. Some authors suggested that deoxysphingolipids caused the disassembly of actin stress fibers in Vero cells, increased the intracellular accumulation of filamentous actin in Ins-1 cells, and promoted dose-dependent apoptosis and impaired glucose-stimulated insulin secretion in insulin-producing cells[17, 20]. Thus, deoxysphingolipids activated metabolic stress and the apoptosis of the cells may be a possible bridge between SPTLC1 and T2DM and DPN.

Despite all our efforts to find the association between SPTLC1 and diabetes, some limitations which may reduce the credibility of the study still exist in this study. Firstly, p.G371R of the *SPTLC1* gene is not pathogenic or likely pathogenic according to the

criteria from ACMG, but, *in vitro* experiments showed the pro-apoptosis effect of this mutation. Importantly, several studies demonstrated that uncertain genetic variants also confer a high risk of related disease[25,26]. Secondly, some members of this family did not perform the genetic testing due to a lack of cooperation from these people. Thirdly, because the mutation of 371 amino acid residue is not essential for the catalytic activity site of SPTLC1, the profiles of sphingolipids and deoxysphingolipids were not analyzed in this study. Lastly, this study did not verify the pathological effects of the mutation of SPTLC1 on T2DM by animal models. Nevertheless, current literatures suggest that both *SPTLC1* deletion and higher deoxysphingolipids (formed by the pathogenic mutation of *SPTLC1*) induced the cells apoptosis and impaired insulin secretion.

## CONCLUSION

Taken together, the study classified *SPTLC1* p.G371R mutation as the likely pathogenic mutation from an adult-onset T2DM patients with strong family history by family study, bioinformation analysis and functional study. The study also found the inflammatory cytokines, especially TNF-α, and the increased apoptosis was the potential downstream pathway of p.G371R mutation of the *SPTLC1* gene. However, this theory needs more data to support. If so, this will provide a novel perspective to understand the pathology of T2DM and associated chronic complications.