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A case of NASH with genetic predisposition successfully treated with an SGLT2 inhibitor: a possible involvement of mitochondrial dysfunction

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Summary

In this study, we herein describe a 47-year-old Japanese woman who manifested inheritable non-alcoholic steatohepatitis (NASH) and severe dyslipidemia. Interestingly, her NASH progression was ameliorated by treatment with a sodium–glucose co-transporter 2 (SGLT2) inhibitor. This inheritability prompted us to comprehensively decode her genomic information using whole-exome sequencing. We found the well-established I148M mutation in *PNPLA3* as well as mutations in *LGALS3* and *PEMT* for her NASH. Mutations in *GCKR* may contribute to both NASH and dyslipidemia. We further mined gene mutations potentially responsible for her manifestations that led to the identification of a novel M188fs mutation in *MUL1* that may be causally associated with her mitochondrial dysfunction. Our case may provide some clues to better understand this spectrum of disease as well as the rationale for selecting medications.

Learning points

- While the *PNPLA3* I148M mutation is well-established, accumulation of other mutations may accelerate susceptibility to non-alcoholic steatohepatitis (NASH).
- NASH and dyslipidemia may be intertwined biochemically and genetically through several key genes.
- SGLT2 inhibitors emerge as promising treatment for NASH albeit with interindividual variation in efficacy. Genetic background may explain the mechanisms behind the variation.
- A novel dysfunctional mutation in *MUL1* may lead to metabolic inflexibilities through impaired mitochondrial dynamics and function.

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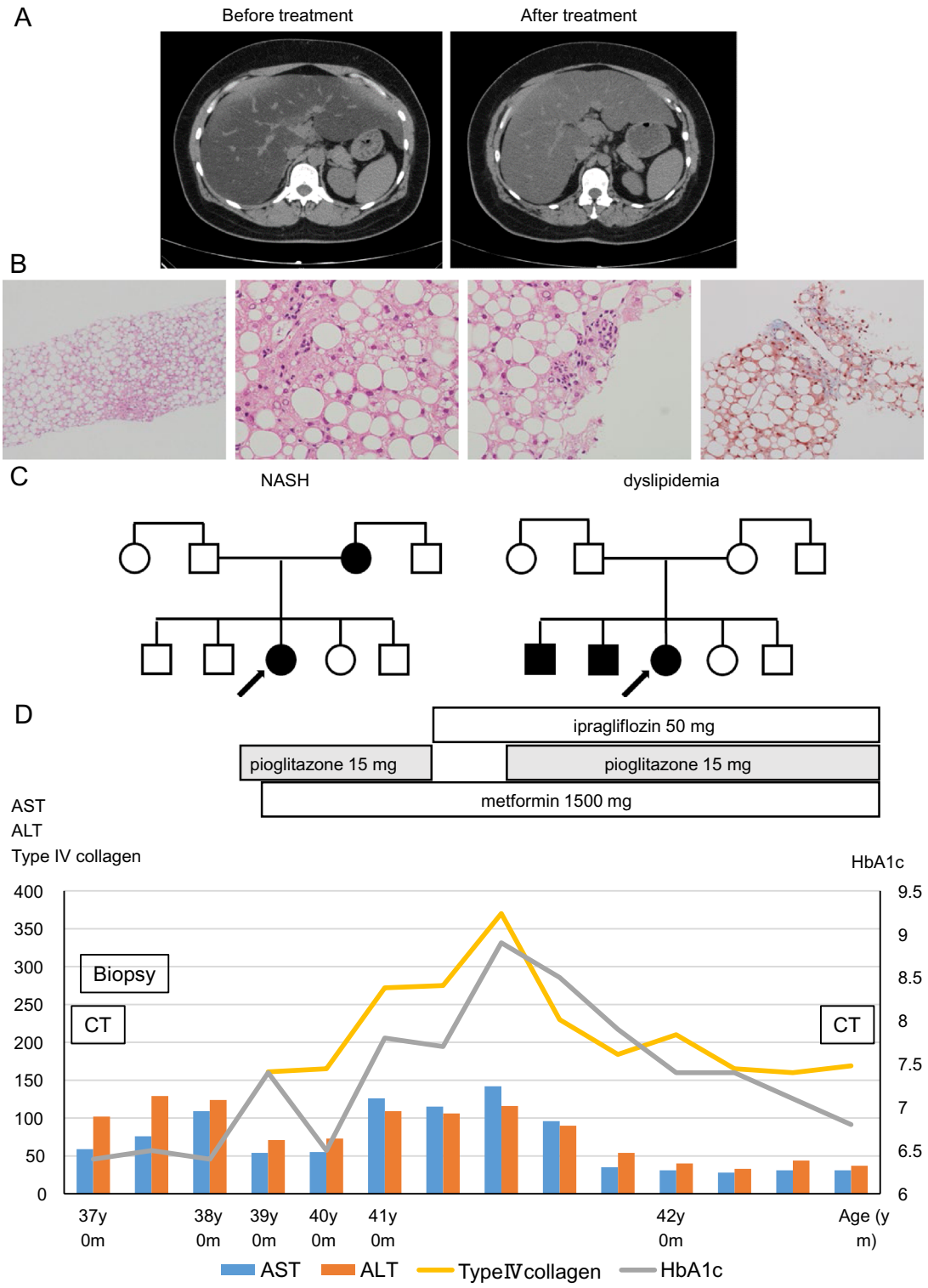

Figure 1
Clinical manifestations of the patient. (A) Changes of CT scan images induced by the SGLT2 inhibitor treatment (before (left) and after (right) administration of the SGLT2 inhibitor). (B) Histological analysis of the liver biopsy specimen. Left most: severe deposition of lipid droplets. Second from left: ballooning of parenchymal cells. Third from left: infiltration of inflammatory cells. Right-most: zone 3 perisinusoidal fibrosis. Hematoxylin and eosin staining except for right most (Azan staining). (C) The family trees showing heritability of NASH (left) and dyslipidemia (right). Filled symbols indicate symptomatic individuals and the arrow indicates the presented patient. (D) The influence of the medical treatments on parameters.

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Background

Metabolic steatosis or non-alcoholic fatty liver disease (NAFLD) is a spectrum of liver pathology where the pathological hallmark is neutral lipid accumulation in the parenchymal cells. A subset of cases exhibits progression from simple steatosis to steatohepatitis (non-alcoholic steatohepatitis, NASH), cirrhosis or even cancer formation, implicating a potential to pose a serious health threat. It has been frequently stated that NAFLD is a hepatic manifestation of the metabolic syndrome, and redefinition of NAFLD with a new terminology, metabolic dysfunction associated fatty liver disease (MAFLD), has been proposed with particular emphasis on metabolic aspects of the pathogenesis. Despite the urgent need to develop proper therapeutic approaches, the molecular bases of NAFLD/NASH remain enigmatic, and no existing animal model faithfully recapitulates human NASH, highlighting the importance of human clinical specimens to resolve this impasse.

In this circumstance, phenotype–genotype correlation in individuals with genetic predisposition to these pathologies offers valuable insights. Although the I148M substitution in *PNPLA3* has been most extensively investigated as a causal mutation for NASH (1), this mutation on its own may not be sufficient to cause NASH. This is in agreement with the well-accepted multiple hit theory where a wide range of insults including mitochondrial dysfunction are required for NASH progression (2). In addition, it has been repeatedly reported that the presence or absence of the polymorphisms in risk alleles may influence the efficacy of the treatment for NASH, implicating the importance to accumulate datasets containing phenotype including the responsiveness to therapeutic approaches along with comprehensive genetic information, where we may be able to find unidentified gene mutations to predict the pharmacological responses.

Case presentation

In this study, we describe a 47-year-old Japanese woman who had been receiving treatment for her type 2 diabetes in our outpatient clinic.

Investigation

Ten years ago, she underwent a liver biopsy since biochemical tests (type IV collagen 275 ng/mL, AST 115 U/mL and ALT 106 U/mL) as well as image-based assessments (the attenuated computed tomography (CT) number (~−25 Hounsfield unit (HU), Fig. 1A) indicated the existence of severe NAFLD. She was classified as obese class I (WHO classification) based on her BMI (34.8 kg/m^2). She did not have either alcohol consumption habits or any signs of hepatitis virus infection as well as autoimmune hepatitis. The histopathological analysis revealed all features of NASH (severe steatosis, inflammatory cell infiltration, ballooning of hepatocytes, slight perisinusoidal fibrosis that were scored based on NASH Clinical Research Network histological scoring system: steatosis 3, inflammation 1, ballooning 1, Fig. 1B) that can be classified into Matteoni's type 4. Intriguingly, her self-reported family tree indicated heritability of this clinical trait although we were not able to obtain detailed information due to the limited access to their family (Fig. 1C).

Table 1 Laboratory changes before and after ipragliflozin administration.

	Before	After
AST(IU/L)	115	31
ALT(IU/L)	106	37
Type IV collagen (ng/mL)	275	169
CT number (HU)	−25	15
HbA1c	7.7	6.8

Treatment

At the time of the diagnosis, her diabetes was not well-controlled (HbA1c 7.7%) with 1500 mg of metformin and 15 mg of pioglitazone.

Outcome and follow-up

Therefore, we additionally administered 50 mg of ipragliflozin, a sodium–glucose co-transporter 2 (SGLT2) inhibitor, that significantly improved biochemical parameters related to her liver functions (type IV collagen 169 ng/mL, AST 31 U/mL and ALT 37 U/mL) as well as hepatic lipid deposition (CT number ~15 HU, Fig. 1A) along with amelioration of her diabetes (HbA1c 6.8%). Of note, she lost 5 kg in a few months (BMI 32.7 kg/m^2) but gained

Table 2 Summary of the whole-exome sequencing in this study.

	Count
Total reads	84 600 562
Mapped reads	84 281 658
Not mapped reads	318 904
Reads in pairs	82 379 782
Broken paired reads	1 901 876

Table 3 Representative gene mutations identified in this study.

Gene	Zygosity	Read count	Read coverage	Mutation	Amino acid change	SNV
PNPLA3	Heterozygous	61	106	C>G	I148M	rs738409
PNPLA3	Heterozygous	82	173	G>T	G115C	rs2076212
PNPLA3	Homozygous	123	123	A>G	K434E	rs2294918
LGALS3	Heterozygous	38	87	C>A	P64H	rs4644
LGALS3	Homozygous	58	59	A>C	T98P	rs4652
LGALS3	Heterozygous	38	76	C>G	Q150E	rs199729394
PEMT	Heterozygous	21	43	C>T	V175M	rs7946
GCKR	Homozygous	99	99	T>C	L446P	rs1260326
APOE	Heterozygous	26	62	C>T	R176C	rs7412
Mul1	Heterozygous	126	259	del T	M188fs	

it back, and thus, her body weight was not overall affected by this additional medication, implicating that her NASH may be sensitive to SGLT2 inhibition in a body weight-independent manner (Fig. 1D, parameters are summarized in Table 1).

In addition, she had hypertriglyceridemia classified as Fredrickson/WHO type IV according to the lipoprotein electrophoresis that was not managed even with intensive medications. Her total cholesterol, LDL-cholesterol HDL-cholesterol and triglyceride levels were 171, 82, 34.5 and 426 mg/dL, respectively with 0.3 mg of pemafibrate, 1200 mg of ethyl icosapentate and 10 mg of ezetimibe. Serum lipoprotein concentrations were as follows: 106 mg/dL of apoB, 12.0 mg/dL of apoC2, 21.3 mg/dL of apoC3 and 5.6 mg/dL of apoE. Lipoprotein lipase (LPL) was neither absent nor decreased (95 ng/mL). Neither typical broad β-band nor chylomicronemia was observed on the agarose gel electrophoresis. The elevated apoB levels relative to LDL with the familial inheritance (Fig. 1C) support the diagnosis of familial combined hyperlipidemia.

Since these two lipid-related pathologies were obviously inherited in her family tree (Fig. 1C), we decided to decode the genetic signatures by whole-exome sequencing (parameters summarized in Table 2) and a

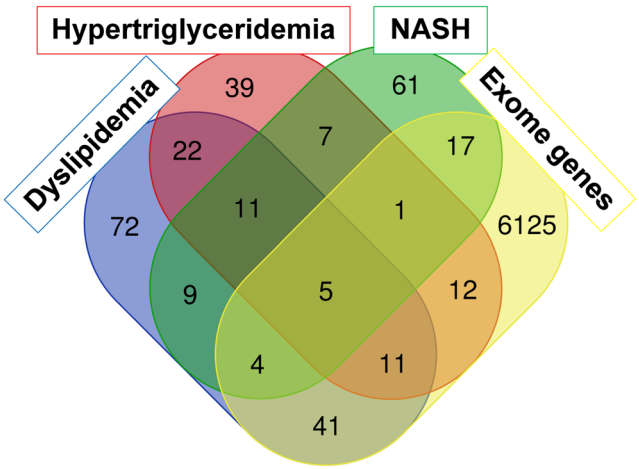

Figure 2
Extraction of the candidate genes. A Venn diagram illustrating the overlap between genes associated with diseases (dyslipidemia, hypertriglyceridemia and NASH) and those with non-synonymous mutations in the patient identified through our exome sequencing analysis (exome genes).

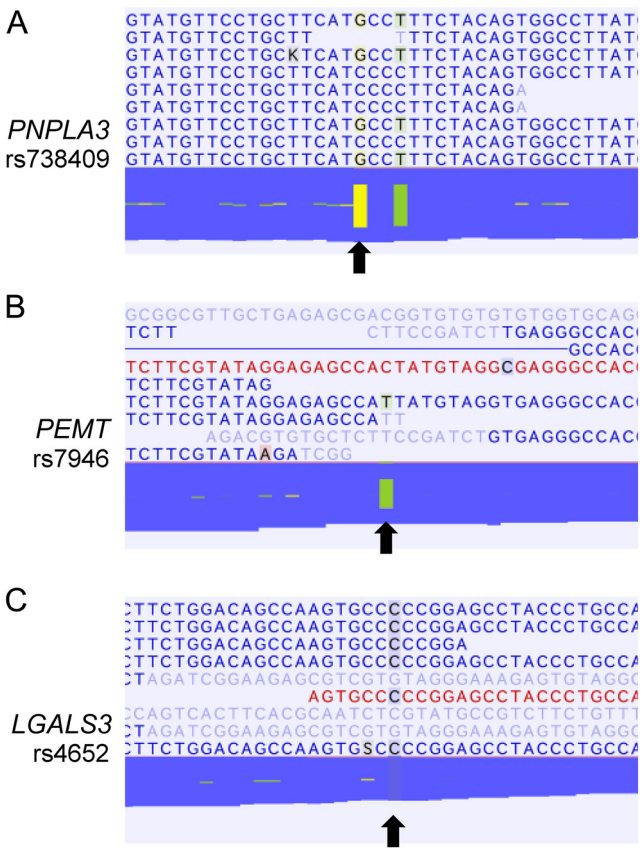

Figure 3
Candidate genes associated with NASH. Arrows indicate the non-synonymous mutations. (A) The heterozygous mutation in *PNPLA3* gene (rs738409). A synonymous mutation was observed right to the rs738409 mutation. (B) The heterozygous mutation in *PEMT* gene (rs7946). (C). The homozygous mutation in *LGALS3* gene (rs4652).

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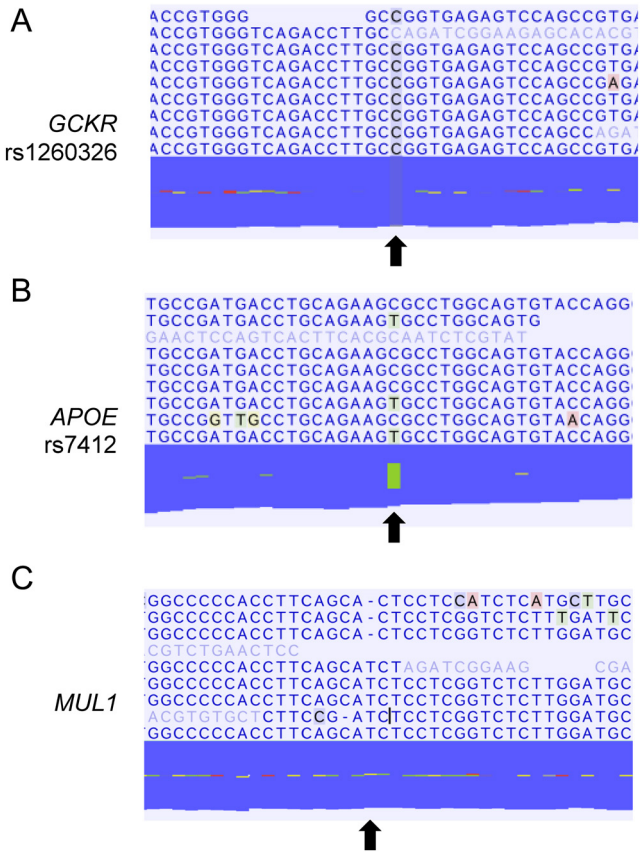

Figure 4
Candidate genes associated with dyslipidemia and the novel mutation in *MUL1* gene. (A) The homozygous mutation in *GCKR* gene (rs1260326). (B) The heterozygous mutation in *APOE* gene (rs7412). (C) The novel heterozygous mutation in *MUL1* gene.

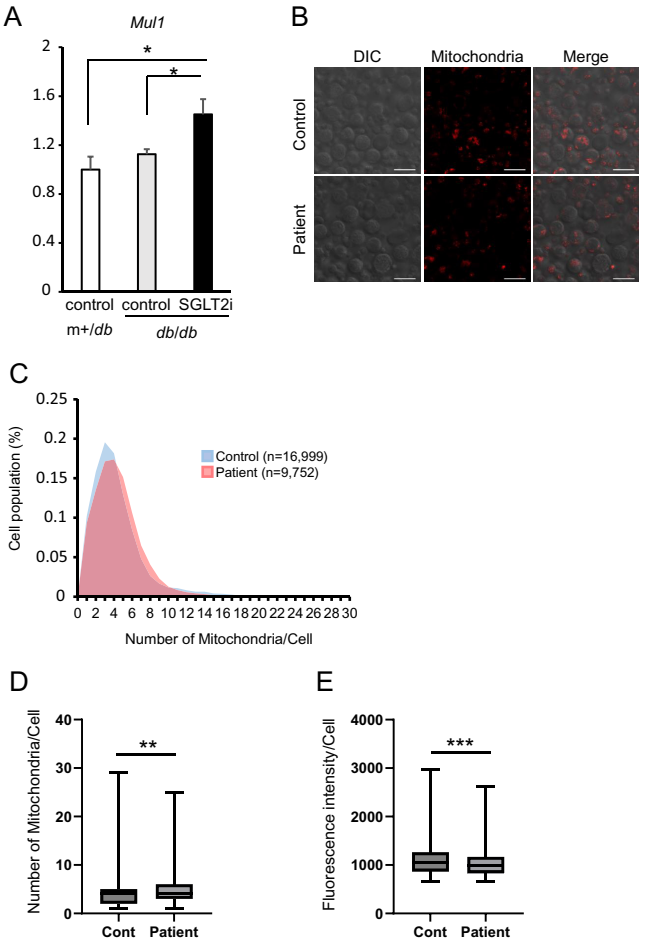

Figure 5
Analysis of the mitochondrial function. (A) The expression levels of *Mul1* gene in the liver of genetically obese *db/db* mice along with their control (n = 4). (B) Representative differential interference contrast (DIC) images of neutrophils stained with MitoTracker Red. (C) Distribution of the total number of mitochondria in individual cells. D,E. The number of mitochondria (D) and the fluorescent intensity indicating mitochondrial membrane potential (E) were analyzed. *, ** and *** denote $P < 0.05$, 0.01 and 0.001 by Student's t-test, respectively.

series of non-synonymous single nucleotide variants (SNVs) were identified (Table 3, Supplementary Table 1, see section on supplementary materials given at the end of this article). We examined the NCBI-Entrez Gene database and extracted genes annotated with functions related to either NASH, dyslipidemia or hypertriglyceridemia to narrow down the candidate gene mutations in this case (Fig. 2, Supplementary Table 1). We compared genes harboring non-synonymous mutation(s) in this case ('exome genes' in Fig. 2) with those disease-associated genes ('NASH', 'dyslipidemia' and 'hypertriglyceridemia' in Fig. 2) and identified overlapping genes for a closer investigation.

Discussion

Among the 27 NASH-related candidate genes, we identified a heterozygous I148M mutation in *PNPLA3* gene (rs738409) (1) (Fig. 3A and Table 3). We also identified heterozygous G115C (rs2076212) and homozygous K434E (rs2294918) mutations in this gene (Table 3). The former appears to be benign based on the preceding literature, while the latter was reported to play a critical role in the development of NASH in conjunction with the I148M mutation (3). Other than the PNPLA3 gene, we found a heterozygous V175M mutation in *PEMT* gene (rs7946) that was also causally implicated in the progression of NASH (4) (Fig. 3B and Table 3). In addition, we also found multiple mutations in *LGALS3* gene (heterozygous P64H (rs4644) mutation, homozygous T98P (rs4652) mutations (Fig. 3C) and heterozygous Q150E (rs199729394) mutation (Table 3)) and rs4644 and rs4652 were reported to be associated with NASH progression (5) although the evidence may be still premature. While the biochemical function of

PNPLA3 could be lipid mobilization, catalytic activity of PEMT is the conversion of phosphatidylethanolamine to phosphatidylcholine, a required step for the structuring of lipid droplets, and plays a critical role in the obesity-induced endoplamic reticulum (ER) stress and metabolic inflexibilities. LGALS3 (also known as galectin-3), a galactoside-binding lectin, modulates myriad biological processes and critically involves in the pathogenesis of obesity as well. These findings from molecular approaches at least in part support the association of these mutations with the progression of NASH.

Next, we investigated the 74 candidate genes for dyslipidemia/hypertriglyceridemia. The homozygous L446P mutations in *GCKR* gene (rs1260326) (Fig. 4A and Table 3) would at least in part explain her dyslipidemia (6). GCKR impairs or modulates glucokinase activity through a direct interaction and mutations in this gene have been reported to be associated with multiple metabolic abnormalities such as type 2 diabetes, atherosclerotic diseases, gout and importantly NAFLD (7). We also found a well-established risk allele, a heterozygous R176C mutation (rs7412) in *APOE* gene (Fig. 4B and Table 3) where the substitution of arginine to cysteine disrupts a salt bridge interaction with the LDL receptor-binding region (8).

Lastly, we attempted to identify unidentified mutations potentially explicable for her clinical manifestations. We took advantage of the responsiveness to the SGLT2 inhibitor for this screen, and *MUL1* was the most attractive since the expression levels of *Mul1* were increased by SGLT2 inhibitor treatment in the liver of genetically obese *db/db* mice, suggesting the increased *Mul1* may provide some metabolic benefits (9) (Fig. 5A). This approach revealed a novel frameshift mutation in *MUL1* gene (M188fs) that predictably produces truncated MUL1 protein composed of only 177 amino acids. The truncated MUL1 is predicted to lack the E3 ligase domain as well as the second transmembrane domain. Multiple SNVs nearby the M188 were predicted to be detrimental by polymorphism phenotyping (PolyPhen), suggesting the importance of this portion in MUL1. MUL1 has been reported to play a role in mitochondrial dynamics including mitophagy (10), and dysfunction of the mitochondrial quality control system is one of the molecular underpinnings of NASH (2). Therefore, we isolated peripheral neutrophils and stained them with MitoTracker Red to evaluate the mitochondrial morphology as well as the membrane potential (Fig. 5B). To obtain the quantitative data, we processed the imaging data with NIS-Elements AR imaging software. The neutrophils from the patient contained increased number of mitochondria compared to an aged-matched healthy volunteer (Fig. 5C and D), while the membrane potential of the patient's mitochondria was decreased (Fig. 5E). These data may suggest the impaired mitophagy associated with the heterozygous *MUL1* mutation led to accumulation of dysfunctional mitochondria in this patient that might have been mitigated by SGLT2 inhibitor treatment by activating residual MUL1 activity in this haploinsufficiency. This hypothesis needs to be sufficiently validated through basic experiments in the future.

The major limitation of this study is that we could not demonstrate the heritability of the mutations identified in this patient. Although the genomic sequencing of her parents should be most prioritized, we had limited access to them that abandoned our attempt to trace the genetic inheritance as well as obtain their detailed clinical manifestations.

In conclusion, we experienced a case of NASH who responded to SGLT2 inhibitor treatment and comprehensively analyzed her genome. Key genes to understand the complex manifestations would be *PNPLA3*, *PEMT*, *LGALS3*, *GCKR* and *APOE*. We also found a novel mutation in *MUL1*. The fact that only a subset of cases with severe fat deposition develops NASH implicates the importance of genetic susceptibility in this pathogenesis. Therefore, close inspection of a case with genetic information would offer bases to better understand the enigmatic pathogenesis. In addition, a significant heterogeneity in the efficacy of the SGLT2 inhibitor treatment in NASH patients may be explained at least in part by the genetic backgrounds. In this sense, this case study has instructive implications regarding how combined mutations in a specific set of genes could present clinical manifestations and responses to therapeutic approaches.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/EDM-22-0368.

Declaration of interest
All authors declare no conflict of interest.

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Patient consent

The patient provided written informed consent and this study was approved by the University of Tsukuba Hospital Ethics Committee with the protocol number R03-036.

Author contribution statement

RN and MS1 (Motohiro Sekiya) were in charge of the medical care for her diabetes and dyslipidemia. RN and MS1 extracted genomic DNA from patient's peripheral blood and analyzed the exome sequencing. YF helped preparation of the sequencing library. MS2 (Masashi Sato) and KF were in charge of the medical care for her liver dysfunction including the histological analysis. KH, YS, MSY and SC performed the exome sequencing analysis. ST, MT and HO analyzed LPL activity. YT, MY, YM, NM, MO, NO, DY, SM, YS, YO, HI, HS1(Hiroaki Suzuki) and HS2 (Hitoshi Shimano) supported the medical care and manuscript preparation. MS1 wrote the manuscript. HS2 supervised this project.

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