Dissecting the Impact of USP21 on Mouse Lipid Metabolism and Identifying Polymorphism-Associated Hypercholesterolemia in Outpatients

Sailakshmi Iyer , Naoko Hattori , Hiroshi Okuda , Takeya Nakagawa , Takahiro Maeda , Haruhiko Koseki , Takashi Ito *

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Article

Usp21 Knockout Cause Abnormal Lipid Metabolism in Mouse and Its Polymorphism Correlates with Hypercholesterolemia in Outpatients

Sailakshmi Iyer [1,†], Naoko Hattori [1,5,†], Hiroshi Okuda [1,†], Takeya Nakagawa [1], Takahiro Maeda [2,3], Haruhiko Koseki [4] and Takashi Ito [1,*]

[1] Department of Biochemistry, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki Japan.
[2] Department of Community Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan.
[3] Department of Island and Community Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan.
[4] Laboratory for Developmental Genetics, RIKEN Center for Integrative Medical Sciences, Yokohama, Japan.
[5] Department of Dermatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan
* Correspondence: 1-12-4 Sakamoto, Nagasaki, Nagasaki 852-8523, Japan TEL:81-95-819-7037,7038; E-mail: tito@nagasaki-u.ac.jp
† These authors contributed equally to this manuscript.

Running Title: *Usp21* KO causes hypercholesterolemia.

Abstract: USP21, a member of the ubiquitin protease family, plays a vital role in various biological functions. However, the effects of USP21 dysfunction have yet to be fully understood. Here, we generated *Usp21* knockout (KO) mice and conducted microarray to identify genes that *Usp21* regulates. Its results raised several genes based on their biological characteristics, and RT-qPCR confirmed their expression. KO mice exhibited significant elevations in the expression of the genes *Fabp7* and *Nlrc5*, which play a role in lipid metabolisms, compared to wild-type mice. Blood tests showed no impairment of liver function but did reveal elevated levels of total cholesterol and free fatty acids in KO mice compared to wild-type mice. These data suggest that *Usp21* may play roles in lipid metabolism associated with *Fabp7* and *Nlrc5*. To clarify the involvement of USP21 in human hypercholesterolemia, we examined single nucleotide polymorphisms (SNPs) around *USP21* in non-hypercholesterolemic and hypercholesterolemic outpatients. We found that the rs11421 SNP downstream of *USP21* was associated with hypercholesterolemia. These data suggest that USP21 plays a role in mice and human lipid metabolism and that its polymorphism may be a diagnostic marker for human hypercholesterolemia.

Keywords: USP21; *Fabp7*; *Nlrc5*; hypercholesterolemia

Introduction

Ubiquitin ligases and deubiquitylases form a large family that catalyze the bonding between the carboxy terminus of ubiquitin and lysine residues and the hydrolysis of the isopeptide bond at the amino terminus, respectively (1). Among the deubiquitylase family, ubiquitin-specific peptidase 21 (USP21), a cysteine protease, is highly conserved among species (2) and is known to play a role in intracellular processes and diseases such as bladder carcinoma (3) and pancreatic ductal adenocarcinoma (4). In addition, USP21 is known to be involved in epigenetic regulation as well (2). Nakagawa et al. found that *USP21* increases after partial hepatectomy and catalyzes the hydrolysis of nucleosome ubiquitylated H2A, which aids in the di- and trimethylation process of H3K4 and initiates transcription of several genes associated with liver regeneration (2). In addition, overexpression of *USP21* in the liver has been shown to upregulate the *Serpina6* gene, which is

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downregulated during hepatocyte regeneration (2). In addition to the full-length *USP21* consisting of 565 amino acids in humans and *Usp21* consisting of 566 amino acids in mice, Okuda et al. found a short variant of *Usp21* in mice. This variant is caused by alternative splicing of exon 2, resulting in the deletion of 87 amino acids from the amino terminus of the long isoform of *Usp21*. *Usp21* short variant lacks nuclear export signal and thus localizes to the nucleus better than the *Usp21* long variant (5). Despite these findings, the impact of *USP21* dysfunction on disease remains to be elucidated.

Hypercholesterolemia, also referred to as dyslipidemia, results due to elevated levels of cholesterol in the blood. One of the genetic factors is the low-density lipoprotein (LDL) receptor, which is involved in lipid metabolism and whose mutations are implicated in the development of atherosclerotic cardiovascular diseases (ASCVD) (6). However, it is a rare disorder and does not explain most of the hypercholesterolemia.

In this report, we generated *Usp21* knockout (KO) mice and identified genes regulated by *Usp21*. The genes Fabp7 and Nlrc5 showed significant elevation in KO mice compared to wild-type mice. Blood tests revealed that KO mice exhibited elevated total cholesterol levels and free fatty acids. In humans, we examined SNPs around *USP21* in non-hypercholesterolemic and hypercholesterolemic outpatients and found an association between hypercholesterolemia and the rs11421 SNP downstream of *USP21*. These data suggest that *USP21* plays a role in lipid metabolism and that its polymorphism could serve as a diagnostic marker for hypercholesterolemia and a potential target for therapy.

Materials and Methods

Generation of USP21 KO mice

Usp21$^{-/-}$ conditional KO mice are created in the ERT-Cre/loxP system by using tamoxifen (TAM). Upon administration of TAM the ERT2 promoter is activated and the Cre recombinase enzyme is produced. This enzyme recognizes loxP short sequences and the sequence is excised (7). Administering TAM to genetically modified mice with loxP in the middle of the *Usp21* sequence causes *Usp21* to be knocked out and hence *Usp21* KO mice are created. All the mice were fed a normal diet (ND) and maintained under a 12h/12h light and dark cycle.

DNA electrophoresis

The RNA was isolated from the wild-type (WT) and KO mice and amplified using *Usp21*-specific primers by RT-PCR. RNA was extracted using the RNeasy Plus kit (Qiagen, Germany). The *Usp21* KO was confirmed with agarose gel electrophoresis. *mGapdh* was used as a reference gene. The primers are listed in supplementary table 1.

Microarray and Enrichment Analysis

RNA microarray was carried out using Sure Print G3 Mouse GE 8x60K chip (Agilent Technologies, CA) according to the manufacturer's protocol. The RNA was extracted from the WT and KO mice. Total RNA was extracted using RNeasy Plus kit (Qiagen, Germany) and was quantified using Agilent 2100 Bioanalyzer (Agilent Technologies, CA) before performing microarray. The expression of the target genes was normalized to *mGapdh* expression. The *Usp21* KO and WT were considered for the study. The genes that were differentially regulated as a result of *Usp21* KO were analyzed using the Partek ® Genomics Suite ® (Partek, MO). All the microarray data can be found online in the NCBI GEO submission (GSE244360).

qPCR

The total RNA was extracted using the ISOGEN II reagent (NIPPON GENE, Japan). cDNA was synthesized using 0.5 µg total RNA as a template with oligo (dT) primer (Life Technologies, CA), random hexamers (Takara Bio, Japan), and M-MuLV reverse transcriptase (New England Biolabs, MA). Real-time RT-qPCR was performed with an ABI PRISM 7900HT (Applied Biosystems, MA)

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with SYBR green as a reporter, cDNA as a template and gene-specific primers. The target gene expression levels were standardized to *mGapdh* expression levels. All the primers designed for the experiment are listed in supplementary table 1.

Mice serum analysis

Serum samples were collected from sacrificing 10-11 weeks of WT mice, *Usp21* KO mice, and *Usp21* heterozygous (HE) mice fed with ND. 0.5-1.0ml of blood was taken from the inferior vena cava. The collected blood was allowed to coagulate by keeping it at room temperature for 30 minutes to 2 hours. The coagulated blood is then centrifuged at 3000rpm for 10-30 mins at room temperature. The supernatant separated as a result of centrifugation is the serum which is then cryopreserved to analyze the levels of triglycerides (TG), free fatty acids (FFA), total cholesterol (T-CHOL), aspartate aminotransferase (AST), and alanine transaminase (ALT). All the mice data are represented in supplementary table 2.

SNP selection and genotyping

All the individual samples are from participants who attended an annual health check-up. This annual check-up program is conducted by the local government and directed by the Ministry of Health, Labor and Welfare in Japan. And this study was approved by the Ethics Committee of Nagasaki University Graduate School of Biomedical Sciences (project registration number 14051404). Written consent forms were available in Japanese to ensure comprehensive understanding of the study objectives, and informed consent was provided by the participants. (Supplementary table 3). A 100kb sequence upstream and downstream of *USP21* was identified from the NCBI database GENBANK (GRCh37.p9) and input to GENETYX-MAC software to identify the restriction enzyme sites. All the SNPs within the restriction enzyme site around the *USP21* region were identified. A total of 22 SNPs were selected. A total of 67 patient samples were collected, of which 41 had lower concentrations of LDL cholesterol (<50mg/dL), and the remaining 26 displayed higher LDL cholesterol levels (>160mg/dL). All the patient's cDNA was amplified by PCR with primers specific for each SNP. The primers for all 22 SNP are mentioned in supplementary table 4. The PCR was performed using the GoTaq DNA polymerase (Promega Cat.No.M3008, WI). The PCR master mix included 5xGoTaq buffer 2µl, 2.5mM dNTP 0.8µl, 10µM forward primer 0.5µl, 10µM reverse primer 0.5µl, Go Taq enzyme 0.05µl, cDNA (about 100ng/µl) 0.1µl and distilled water to make up the total reaction volume to 10µl. The fragments were amplified at 95°C for 1min for the melting step, followed by 35 cycles of amplification (95°C for 1min, 55°C 1min, 72°C 30sec) and terminal extension at 72°C for 5min. Next, the PCR products were cut with specific restriction enzymes (PCR product 5µl, 100x BSA 0.1µl, 10x enzyme buffer 1µl, restriction enzyme 0.3µl and distilled water to make the volume up to 10µl). The samples were incubated for 1 hour at 37°C and 3µl of the restriction enzyme treated sample was run on 3% agarose gel electrophoresis at 170V for 30mins. For one of the SNPs (rs2301286), sequencing was done to confirm the results or restriction enzyme cut. as the gel electrophoresis results were not clear. Sequencing was performed using the Big Dye™ Terminator v3.1 (Thermo Fisher Scientific Inc, MA) according to manufactural protocol, and analyzed with ABI PRISM 3130xl genetic analyzer (Applied Biosystems, MA). The characteristics of the SNPs and the restriction enzymes used are mentioned in supplementary table 5. The frequency of the SNP alleles in patient's is mentioned in supplementary table 6.

Statistical analysis

Data are shown as means ± SD. Student's t-test was used to determine the statistical significance for the target genes subjected to RT-qPCR. One-way analysis of variance (ANOVA) and Tukey's post hoc test were used to determine the statistical significance between the mice genotypes and blood test parameters. The chi-square test was used to determine the statistical significance between the phenotypes and SNPs. P <0.05 was considered significant for all the test data. All the statistical analyses were performed using JMP Pro 17 software (SAS Institute, Cary, NC).

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Ethical Approval

All animal handling and experiments were conducted following the Guidelines for Animal Experimentation of Nagasaki University and with the approval of the Institutional Animal Care and Use Committee (approval number: 1204160979).

The experiments on participants who attended in an annual health check-up in this paper were approved by the Ethics Committee of Nagasaki University Graduate School of Biomedical Sciences (project registration number 14051404).

Results

Generation of Usp21 KO mice.

The KO models serve as a valuable tool in genetics to decipher a gene's biological role. Cre/loxP technology has generated conditional KO mice (7). For this study, the deletion of exon three and exon four generated the *Usp21* KO mice (Figure 1A). Exons 3 and 4 are known to encode essential parts of the USP21 USP domain (8). The *Usp21* with excised exons 3 and 4 is bred to Cre expressing transgenic mice strain to obtain *Usp21* KO mice (Figure 1A, B). To further confirm, sequencing analysis of *Usp21* KO mice confirmed the successful deletion of exons 3 and 4. Exons 3 and 4 are an 181bp long sequence, and the stop codon appeared immediately after entering exon 5. The DNA gel electrophoresis confirmed the *Usp21* KO (Figure 1C). Figure 1D depicts the generation of *Usp21* littermates. The littermates are generated by crossing B6 mice strains harboring WT *Usp21* and B6 & SW129 mix strains.

Fabp7 and Nlrc5 are upregulated in Usp21 KO mice

Genes up- and down-regulated more than 1.3-fold by KO of *Usp21* by microarray were analyzed by ANOVA in the Partek Genomics Suite, followed by Gene Ontology analysis (Figure 2A, B). A functional group with an enrichment score of more than three corresponds to an over-representation with a $P<0.05$. In upregulated genes, the functional group of Homeostatic process, Biological regulation, Developmental process, Biological process involved in interspecies interaction between organisms, Response to stimulus, Multicellular organismal process, locomotion, and Immune system process has an enrichment score of over three. In contrast, in downregulated genes, only the Cellular, Localization, and Metabolic processes have an enrichment score of more than three. The previous report that USP21 activates transcription by deubiquitinating ubiquitylated H2A can well explain the significant increase of upregulated genes by KO of *Usp21* in this report. Because we previously identified *USP21* as playing a role in liver regeneration (2), we try to find regulated genes related to the liver, such as liver regeneration, lipid metabolisms, and drug metabolisms. We focused on twenty-one genes described in Figure 2C. Among the genes that were analyzed, Fatty acid binding protein 7 (*Fabp7*), Cytochrome P450 family two subfamily b polypeptide 9 (*Cyp2b9*), Amyloid P Component, Serum (*Apcs*), C-X-C Motif Chemokine Ligand 13 (*Cxcl3*), Lipocalin 2 (*Lcn2*), Procollagen C-Endopeptidase Enhancer 2 (*Pcolce2*), Serum Amyloid A2 (*Saa2*), and Lysozyme 2, (*Lyz2*) were upregulated in the microarray (Figure 2C). To validate the microarray results, RT-qPCR confirmed their expression as described (Figure 2D, E). Among these genes, *Fabp7* and *Nlrc5* expressed more in KO mice compared to WT mice with $P<0.05$ (Figure 2D). Fatty acid binding proteins (FABPs), members of the intracellular lipid binding protein family are known to regulate lipid metabolism by increasing the fatty acid uptake (9), fatty acid oxidation (10), and by lipolysis (11). The astrocytes and oligodendrocytes in the brain mainly expressed FABP7(12) (13). Kupffer cells express FABP7 in the liver (14). A Study has shown that FABP7 might be involved in the fatty acid metabolism in sheep liver, thereby controlling the composition of fatty acid in the muscle (15). As for *NLRC5*, it is known to belong to the human Nod-like receptor (NLR) family. Of the 22 currently known human NLRs (16), obesity, inflammation, and immunity are associated with *NLRC5* (17, 18). SNPs in the *NLRC5* gene and its promoter regions link to alterations in TG, T-CHOL, and high-density lipoprotein cholesterol levels. (19). Additionally, *Nlrc5*$^{-/-}$ mice on an HFD exhibit more significant weight gain,

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waist circumference, large adipose tissues, and adipocyte size than female WT mice (20). These data show that *Usp21* might play a role in lipid metabolism associated with *Fabp7* and *Nlrc5*.

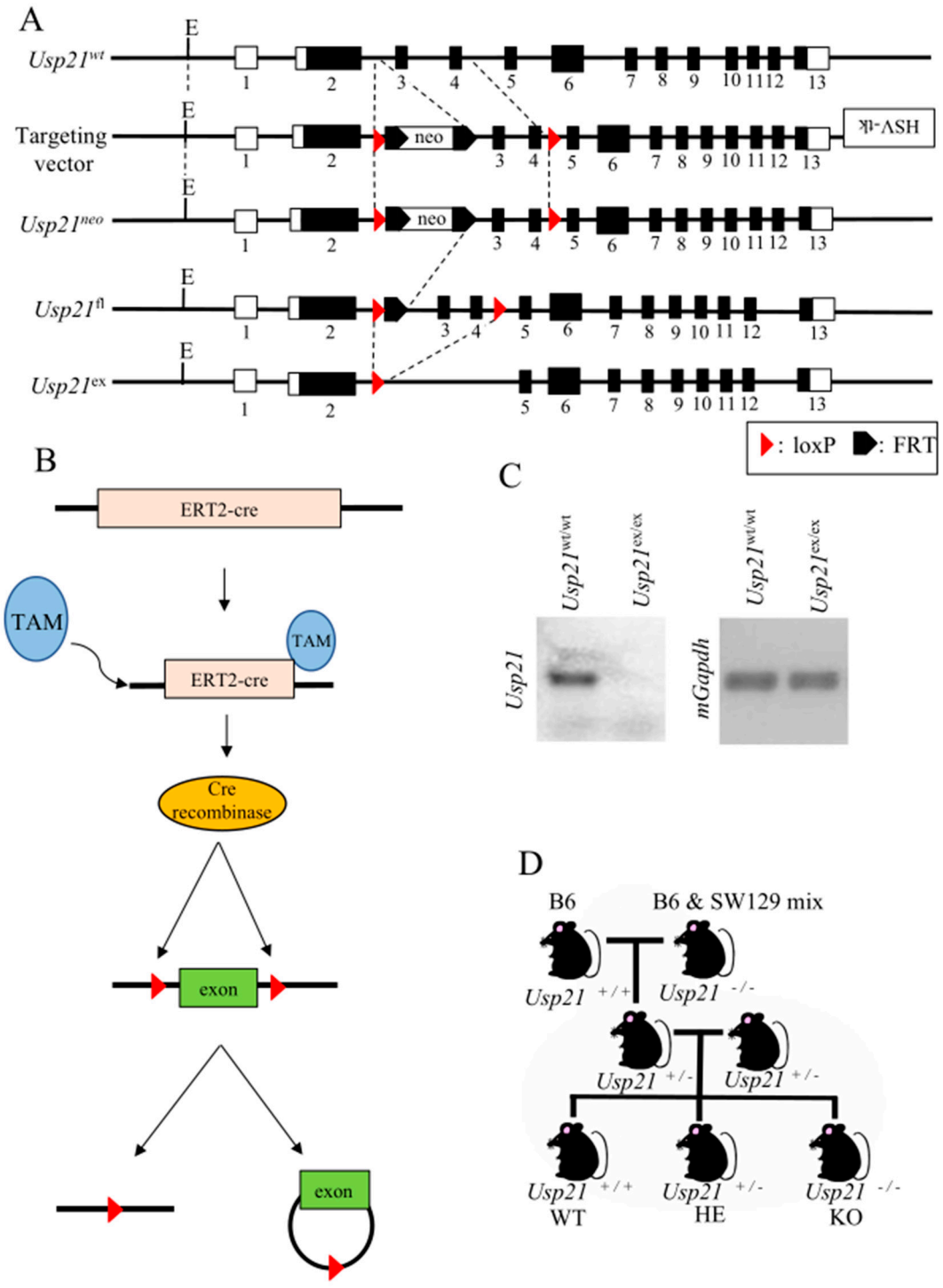

Figure 1. ***Generation of Usp21 KO mice and its confirmation.*** (A-B) Pictorial representation of the generation of *Usp21* conditional KO mice using the ERT2-Cre/loxP system. (C) DNA gel electrophoresis of *Usp21* $^{wt/wt}$ mice and *Usp21* $^{ex/ex}$ mice to check the deletion of *Usp21*. The *mGapdh* is used as the reference gene. (D) Schematic representation of the generation of *Usp21* KO mice littermates by mating B6 harboring *Usp21* WT gene with B6&SW129 mix having *Usp21* knocked out. ex, excision; fl, floxed; FRT, flippase recognition target sites; HE, heterozygous; HSV-tk, herpes simplex virus- thymidine kinase; KO, knockout; neo, neomycin resistance gene; TAM, tamoxifen; WT, wild-type.

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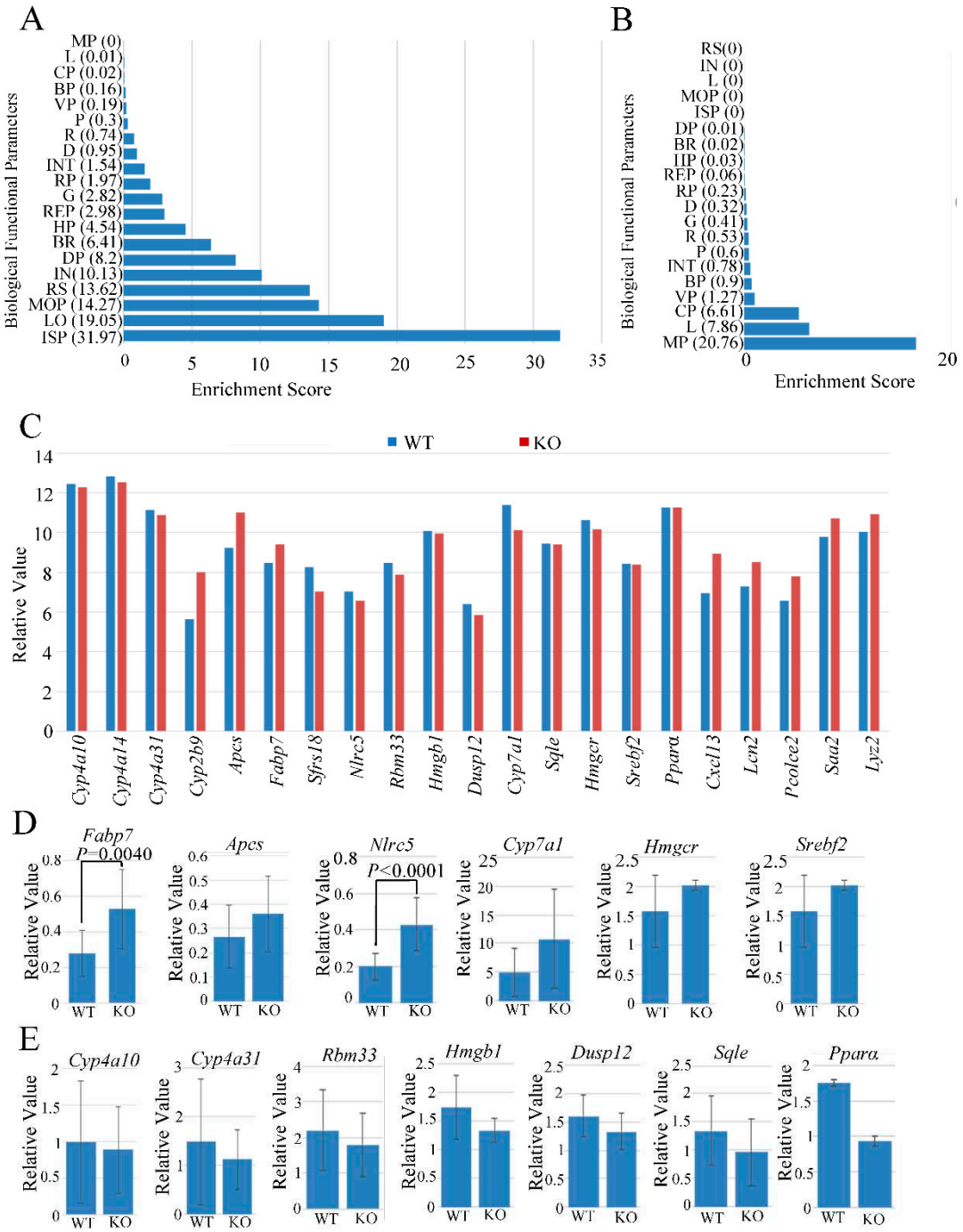

Figure 2. ***Fabp7 and Nlrc5 are upregulated in Usp21 KO mice.*** (A, B) Gene ontology analysis of upregulated genes and downregulated genes in *Usp21* KO mice. (C) Analysis of microarray results. (D, E) RT-qPCR results of (D) upregulated or (E) downregulated genes in *Usp21* KO mice. The values are normalized with the *mGapdh* expression. BR, Biological regulation; BP, Biological phase; CP, Cellular process; D, Detoxification; DP, Developmental process; G, Growth; HP, Homeostatic process; IN, Biological process involved in interspecies interaction between organisms; INT, Biological process involved in intraspecies interaction between organisms; ISP, Immune system process; KO, knockout. L, Localization; LO, Locomotion; MOP, Multicellular organismal process; MP, Metabolic process; P, Pigmentation; R, Reproduction; REP, Reproductive process; RP, Rhythmic process; RS, Response to stimulus; V, Viral process; WT, wild-type. Data are shown as mean ± SD. *P* values were tested with a student t-test. $P < 0.05$ is considered significant.

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Usp21 KO mice showed an elevation of serum FFA and T-CHOL.

A blood test determined TG, FFA, AST, ALT, and T-CHOL serum levels in the littermate of 10-11 weeks WT, KO, and HE mice (Figure 3A-E). The body weight (BW) was measured simultaneously (Figure 3F). The results showed no significant changes in the liver enzymes AST and ALT, confirming that liver function was not impaired. Although, the levels of FFA and T-CHOL were significantly elevated in KO and HE mice compared to WT with *P*-values (HE vs. WT; *P*=0.0126, KO vs. WT; *P*= 0.0013) and (HE vs. WT; *P*=0.0018, KO vs. HE; *P*<0.0001, and KO vs. WT; *P*<0.0001), respectively.

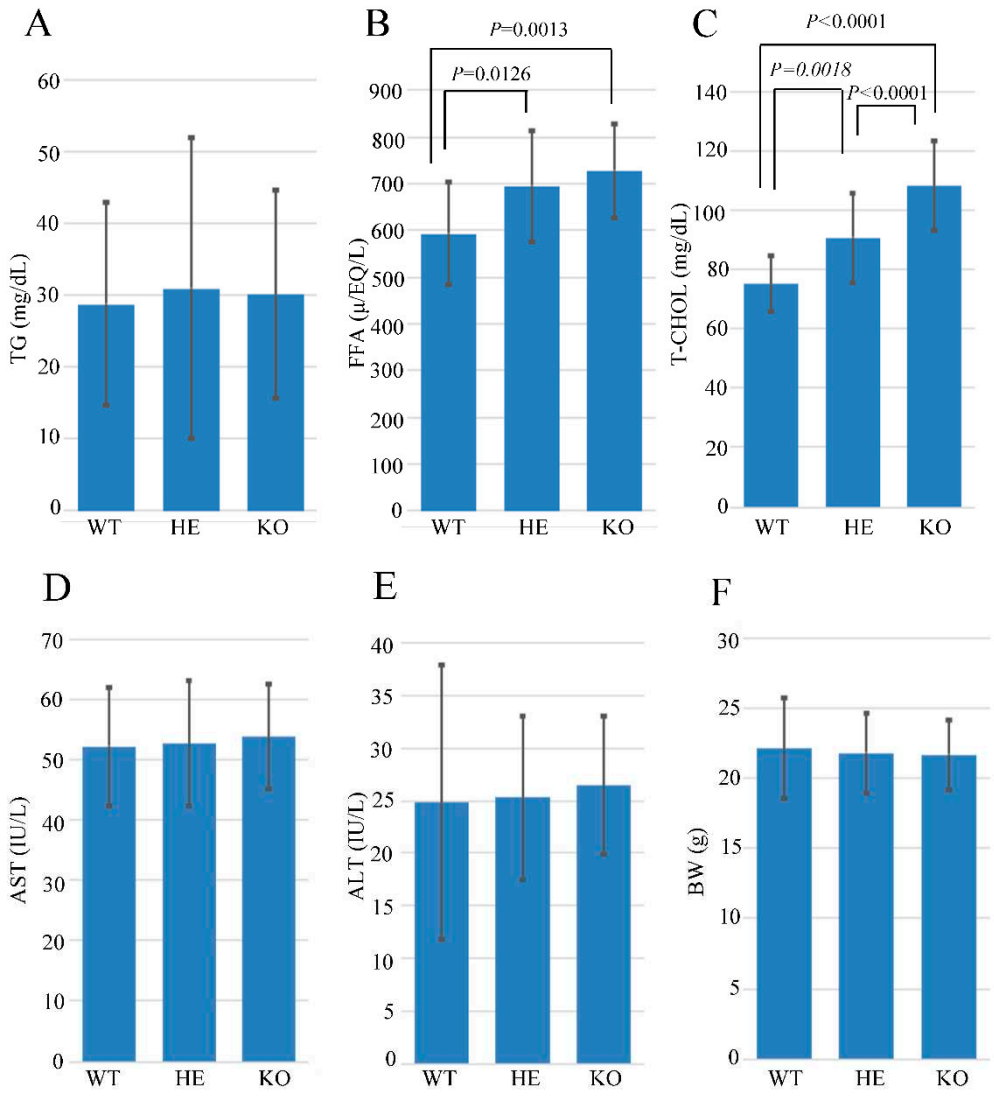

Figure 3. ***FFA and T-CHOL were elevated in Usp21 KO mice.*** (A- E) The blood test data of (A) TG, (B) FFA, (C) T-CHOL, (D) AST, and (E) ALT 10-11 weeks WT, HE, and KO mice. (F) BW in 10-11 weeks WT, HE, and KO mice. ALT, Alanine transaminase; AST, Aspartate aminotransferase; BW, Body weight; FFA, Free fatty acid; HE, heterozygous; KO, knockout; TG, Triglyceride; T-CHOL, Total cholesterol; WT, wild-type. Data are shown as mean ± SD. *P* values were tested with one-way ANOVA, followed by Tukey's test. *P* <0.05 is considered significant.

Concerning the fact that elevated level of FFA (21) and LDL cholesterol (6, 22, 23) is known to be associated with metabolic and cardiovascular diseases, our mice serves as an excellent model to clarify the precise role that TG and T-CHOL play in the development of metabolic syndromes and

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cardiovascular diseases and may provide alternative treatment strategies. In addition, *USP21* may serve as an essential diagnostic marker in the future.

rs11421 SNP is associated with hypercholesterolemia

To investigate whether the human USP21 gene is involved in hypercholesterolemia, we selected SNPs around the *USP21* gene based on GRCh37 and conducted an analysis (Figure 4A). After selecting the SNPs, we screened a sample of patients consisting of low LDL cholesterol (<50mg/dL) and high LDL cholesterol (>160mg/dL) for significant SNPs associated with serum cholesterol levels and performed a chi-square test to determine statistical significance. We found out of the 22 SNPs, the rs11421 SNP (Xsp I site ctag/ccag) showed statistical significance (Figure 4B, C). We observed a higher frequency of the ccag allele in high LDL cholesterol patients compared to low LDL cholesterol patients with a P-value of <0.0093. Therefore, the ccag allele of the SNP rs11421 is significantly associated with hypercholesterolemia. The simplicity of PCR amplification of SNP rs11421 followed by Xsp1 restriction enzyme cleavage contributes to the practical diagnosis of hypercholesterolemia in clinical settings.

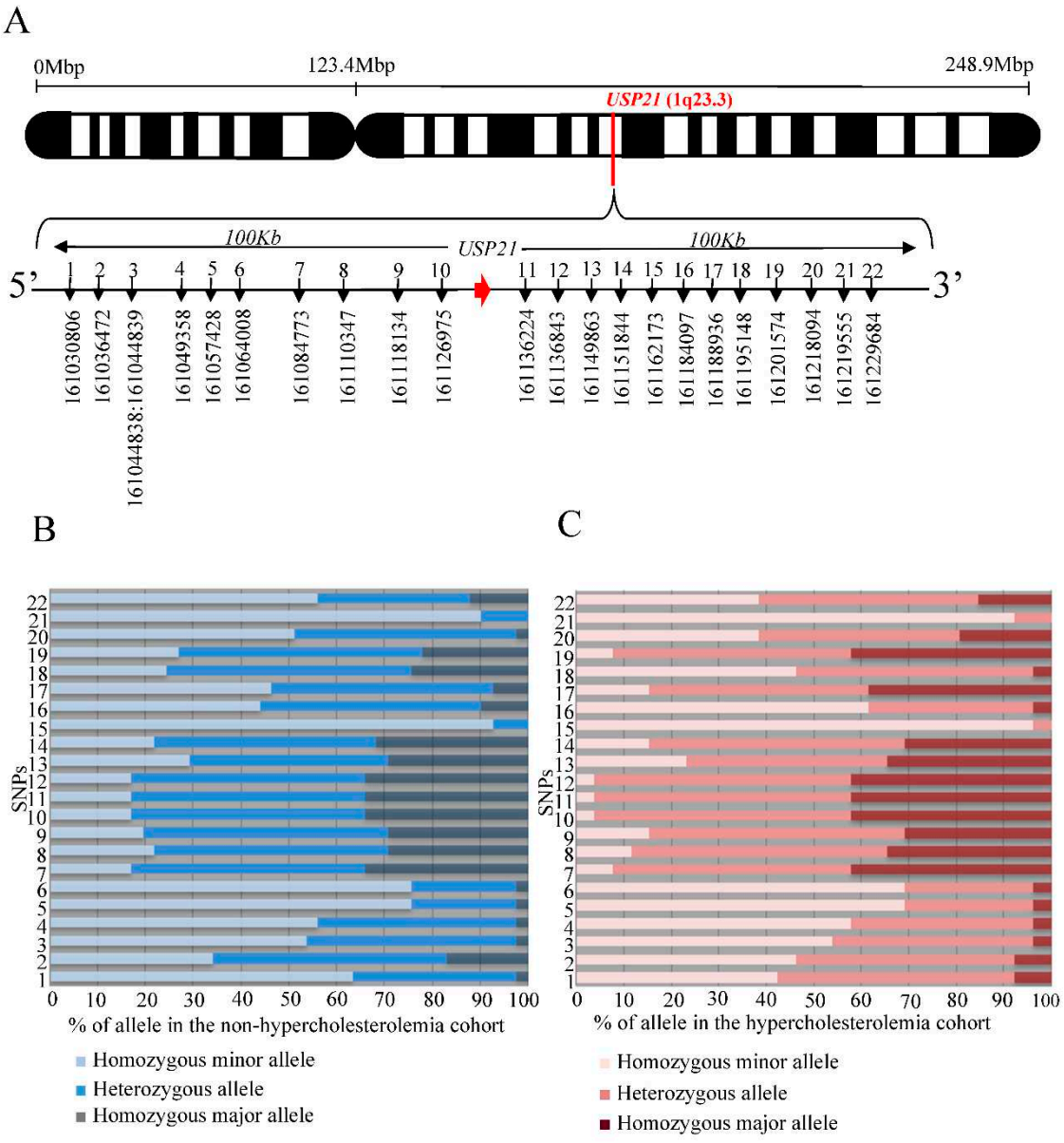

Figure 4. ***rs11421 is associated with hypercholesterolemia.*** (A) Schema of SNPs surrounding the human *USP21* gene. (B) Genotypes at each SNP locus in non-hypercholesterolemic outpatients. (C)

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Genotypes at each SNP locus in hypercholesterolemic outpatients. SNP, single nucleotide polymorphism.

Discussion

ASCVD continues to be the leading cause of morbidity and mortality. Hypercholesterolemia, significantly increased LDL cholesterol, is a well-established risk factor for ASCVD. Hypercholesterolemia has both inherited and acquired causes. The most common genetic condition is familial hypercholesterolemia, which results from mutations in the LDL-receptor gene (6). University College London's database records two thousand two hundred twenty-one unique LDL-receptor gene variants (24, 25). However, hypercholesterolemia without apparent genetic alteration has emerged as the most common type due to the sedentary lifestyle without exercise and increased intake of an HFD, including trans fatty acids. Therefore, the prediction of hypercholesterolemia and its management, including a healthy lifestyle, reducing HFD, and intake of foods with high fiber content, is more important than drug therapy after the onset of the disease (6). We discovered that SNP rs11421 close to *USP21* is significantly associated with hypercholesterolemia. Although it is unclear that USP21 causes hypercholesterolemia in humans, this SNP will help its prediction and management. Many studies have reported the association of *Fabp7* and *Nlrc5* in lipid metabolism. A study found that the mice fed with an HFD showed an upregulation of *Fabp7* and a downregulation of miR-21. By upregulating miR-21 and subsequently downregulating *Fabp7*, dietary lycopene prevented hepatic steatosis in high-fat mice (26).

Researchers have well-documented the role of NLRC5 in liver fibrosis (27) and hepatocellular carcinoma (28). NLRC5 expression was considerably higher in mice fed ethanol with elevated TG, T-CHOL, ALT, and AST levels than in control diet-fed animals' liver tissue. NLRC5 was also increased in mouse AML-12 hepatocyte cell lines when exposed to ethanol (29). These data suggest abnormal NLRC5 expression and its involvement in hypercholesterolemia. Further experiments involving *Fabp7* and *Nlrc5* KO can help elucidate these genes' effects and verify any correlation between these genes and hypercholesterolemia.

We found that *Usp21* KO mice showed an elevated expression of *Fabp7* and *Nlrc5* genes, previously reported in studies to be involved in lipid metabolism and lipid metabolism-related disorders such as obesity. In addition, the levels of FFA and T-CHOL were also significantly elevated in *Usp21* KO mice and HE mice compared to WT mice, suggesting that *Usp21* may contribute to lipid abnormalities. In addition, we found that the SNP rs11421 adjusting to *USP21* was significantly associated with hypercholesterolemia. This clinical linkage supports the data of *USP21* KO mice and may help predict and manage hypercholesterolemia.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Supplementary Table 1. List of primers for RT-qPCR. Supplementary Table 2. Mice characteristics and blood test data. Supplementary Table 3. Patients' characteristics. Supplementary Table 4. SNP-specific primers for PCR. Supplementary Table 5. Characteristics of SNPs. Supplementary Table 6. Frequency of which patients have SNP alleles.

Author Contributions: T. Ito, T. Nakagawa, N. Hattori, T. Maeda, and H. Koseki designed research; S. Iyer and N. Hattori analyzed data; S. Iyer, N. Hattori, and H. Okuda performed research; T. Ito, S. Iyer, N. Hattori, and H. Okuda wrote the paper; and All authors were involved in drafting and revising the manuscript.

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Data Availability Statement: The data supporting this study's findings are available in the methods and supplementary material of this article or in GEO. All the microarray data can be found online in the NCBI GEO submission (GSE244360).

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Conflicts of Interest: The authors declare that they have no conflicts of interest in this article.

Abbreviations

ALT, alanine transaminase; ANOVA, analysis of variance; *Apcs*, **Amyloid P Component, Serum;** ASCVD, atherosclerotic cardiovascular diseases; AST, aspartate aminotransferase; BW, body weight; *Cxcl13*, C-X-C Motif Chemokine Ligand 13; *Cyp4a10*, Cytochrome P450 Family 4 Subfamily A Polypeptide 10; *Cyp4a14*, Cytochrome P450 Family 4 Subfamily A Polypeptide 14; *Cyp4a31*, Cytochrome P450 Family 4 Subfamily A Polypeptide 31; *Cyp2b9*, Cytochrome P450 Family 2 Subfamily B Polypeptide 9; *Cyp7a1*, Cytochrome P450 Family 7 Subfamily A Polypeptide 1; *Dusp12*, Dual Specificity Phosphatase 12; *Fabp7*, Fatty Acid Binding Protein 7; FABPs, Fatty acid binding proteins; FFA, free fatty acids; HE, heterozygous; *Hmgb1*, High Mobility Group Box 1; *Hmgcr*, 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase; KO, knockout; LDL, low-density lipoprotein; *Lcn1*, Lipocalin 1; *Lyz2*, Lysozyme 2; ND, normal diet; *Nlrc5*, Nod-like receptor Family CARD Domain Containing 5; *Pparα*, Peroxisome Proliferator Activated Receptor Alpha; *Pcolce2*, Procollagen C-Endopeptidase Enhancer 2; *Rbm33*, RNA Binding Motif Protein 33; *Saa2*, Serum Amyloid A2; *Sfrs18*, Splicing Factor, Arginine/Serine-rich 18; SNP, single nucleotide polymorphism; *Sqle*, Squalene Epoxidase; *Srebf2*, Sterol Regulatory Element Binding Transcription Factor 2; T-CHOL, total cholesterol; TG, triglycerides; TAM, tamoxifen; *Usp21*, Ubiquitin Specific Peptidase 21; WT, wild-type

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