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Hypercholesterolemia induced by spontaneous oligogenic mutations in rhesus macaques (*Macaca mulatta*)

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Abstract

Background: A rhesus macaque with the fourth highest plasma cholesterol (CH) levels of 501 breeding macaques was identified 22 years ago. Seven offspring with gene mutations causing hypercholesterolemia were obtained.

Methods: Activity of low-density lipoprotein receptor (LDLR), plasma CH levels and mRNA expression levels of *LDLR* were measured after administration of 0.1% (0.27 mg/kcal) or 0.3% CH.

Results: Activity of *p.* (*Cys82Tyr*) of LDLR was 71% and 42% in the heterozygotes and a homozygote, respectively. The mRNA expression level of *LDLR* in the *p.* (*Val241Ile*) of membrane-bound transcription factor protease, site 2 (*MBTPS2*, S2P protein) was 0.83 times lower than normal levels. *LDLR* mRNA levels were increased for up to 4 weeks by administration of 0.3% CH before suddenly decreasing to 80% of the baseline levels after 6 weeks.

Conclusion: Oligogenic mutations of *p.* (*Cys82Tyr*) in *LDLR* and *p.* (*Val241Ile*) in *MBTPS2* (S2P) caused hypercholesterolemia exceeding cardiovascular risk levels under a 0.1% CH diet.

KEYWORDS
activity of LDLR, cholesterol, hemizygote, *LDLR*, *MBTPS2*, model animal, mRNA, S2P, sex-linked inheritance and recessive inheritance

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1 | INTRODUCTION

Ischemic heart disease is responsible for the highest number of human deaths in the world, causing 8.2 million deaths in 2019.[1] Hypercholesterolemia is a major factor underlying the development of arteriosclerosis, which can induce ischemic heart disease. Plasma cholesterol (CH) levels are affected mainly by five factors: biosynthesis of CH, absorption by the small intestine from food, endocytosis by low-density lipoprotein receptor (LDLR), efflux of CH as bile acids, and reabsorption of bile acids by the small intestine. Although methods such as medical treatment and apheresis of LDL have been developed to control CH levels, further investigations using model animals are required to develop new pharmaceutical products. The prerequisites for experiments using model animals are that they must have high accuracy and reproducibility, and extrapolation of the findings to humans must be possible.[2] In the present study, we report that rhesus macaques (*Macaca mulatta*, Zimmermann,1780) with hypercholesterolemia induced by spontaneous oligogenic mutations meet these requirements and are well-suited as model animals for studying the genetic mechanisms underlying the development of familial hypercholesterolemia (FH).

Since FH, which is caused by a mutation in the *LDLR* gene, was first identified by Brown and Goldstein,[3] 1261 pathogenic and pathogenic/likely pathogenic mutations of *LDLR* in humans have been identified; sequences for these mutations have been deposited in the Single Nucleotide Polymorphism Database at the National Center for Biotechnology Information.[4] Mutations in apolipoprotein B (ApoB) bound to LDLR,[5] the adaptor protein LDLRAP1 that is necessary for LDLR internalization,[6] and the protein convertase subtilisin/kexin type 9 (PCSK9), which escorts the LDLR-LDL complex for lysosomal degradation,[7] have been identified as the main factors affecting FH. Furthermore, mutations in the proteins that regulate the transcription levels of *LDLR* in response to the intracellular concentration of CH,[8] as well as proteins associated with the efflux of CH from cells,[9] have also been identified as factors that affect FH.

In sterol-depleted animal cells, sterol regulatory element binding protein (SREBP)-cleavage-activating protein (SCAP) transports SREBP from the endoplasmic reticulum (ER) to the Golgi complex.[10] The transported SREBP is digested by site 1 protease (S1P, encoded by the *MBTPS1* gene)[11] and site 2 protease (S2P, encoded by the *MBTPS2* gene),[12] both of which are located in the Golgi complex. The resulting N-terminal region moves to the nucleus where it binds to a sterol-responsive element sequence in the promoter region of *LDLR*, 3-hydroxy-3-methyl glutaryl coenzyme A reductase (*HMGCR*), which is a rate-limiting enzyme in CH synthesis, or to genes involved in fatty acid synthesis[13–15]; the subsequent transcription of these genes increases the intracellular concentration of CH or fatty acids. In sterol-overloaded cells, the protein of the insulin-induced gene (INSIG) binds to SCAP, prevents the transportation of the SCAP/SREBP complex, and decreases the transcription of *LDLR*, *HMGCR* and genes involved in fatty acid synthesis.[16,17]

Several genome-wide association studies (GWAS) on hypercholesterolemia have been published to date.[18–36]

If mutations associated with FH were identified in rhesus macaques, which have a similar metabolism to humans, then their discovery might contribute to medical treatment in humans. As macaques are primarily herbivorous and feed on insects containing CH only opportunistically, and since CH is not included in the chow that is provided to macaques in captivity, it was assumed that any genetic mutations related to hypercholesterolemia would remain in the captive population.

To date, a nonsense mutation in exon 6 of *LDLR* has been shown to induce FH in rhesus macaques,[37] and we previously identified a hypercholesterolemic individual among 501 breeding macaques.[38]

2 | MATERIALS AND METHODS

2.1 | Veterinary care

Rhesus macaques that originated in India were bred at the Primate Research Institute, Kyoto University, Japan, following the third edition of The Guide for the Care and Use of Laboratory Primates. Until 2005, a hypercholesterolemic family was bred in an open enclosure (30 head per 500 m^2) at the institute. The macaques were fed a formulated primate diet (Primate Diet AS, Oriental Bio Service Inc.) supplemented with wheat or dried soybeans in the open enclosure twice a week. After 2006, the macaques were maintained in individual cages that could be connected to form a larger double cage. Breeding cages with a floor area and height of 0.39 m^2 and 75.2 cm, respectively, were used to house macaques with a body weight (BW) of 3 to 10 kg, and cages 0.54 m^2 and 81.3 cm, respectively, were used to house macaques with a BW of 10 to 15 kg. The temperature was maintained at 27°C in summer and 20°C in winter, and humidity was maintained at 40–70%. The macaques received 40–50 kcal/kg/day by feeding twice daily and sweet potatoes every 2 days.

2.2 | Sample collection

Under the ordinary (control) diet, blood samples were collected at the time of their annual health examination in autumn under anaesthesia using a combination of ketamine (5 mg/kg), medetomidine (0.025 mg/kg) and midazolam (0.125 mg/kg); atipamezole (0.1 mg/kg) was administered as an antagonist to medetomidine at the end of the procedure. Blood samples from macaques administered a CH-supplemented diet (0.1% or 0.3% CH) were collected once a week without anaesthesia. These examinations and experiments were approved by the Animal Welfare and Animal Care Committee of the institute.

2.3 | Measurement of lipid levels

Plasma total cholesterol level (Total-C) was measured by the cholesterol oxidase and p-chlorophenol method using a Cholesterol

CII Test kit (Wako Pharmaceutical Co. Ltd., Osaka, Japan) and plasma high-density lipoprotein cholesterol (HDL-C) level was measured by the heparin/Mn+2 precipitation method using an HDL-Cholesterol Test kit (Wako Pharmaceutical Co. Ltd.). Plasma triglyceride (TG) was measured by the glycerol phosphate oxidase (GPO)-p-chlorophenol method using a Triglyceride G Test kit (Wako Pharmaceutical Co. Ltd.) from 1995 to 2005. Subsequently, these assays were performed by FALCO Biosystems Ltd. (https://www.falco.co.jp/) from 2006 to 2009, and Nagoya Rinshou Kensa Center (Nagoya Clinical Examination Center, Aichi, Japan; https://www.mrso.jp/aichi/nagoya-syowa/meirin-center/) in 2021. These suppliers measured Total-C, low-density lipoprotein cholesterol (LDL-C), HDL-C and TG using Determiner C-TC, Determiner L LDL-C, MetaboLead HDL-C and Determiner C-TG kits (all from Kyowa Medex Co. Ltd. [now Minaris Medical Co., Ltd.]) using an automatic analyser (LABOSPECT008, Hitachi Ltd.). For the animals fed a 0.1% cholesterol diet, blood parameters were measured using Determiner L TCII, MetaboLead LDL-C, MetaboLead HDL-C and Determiner L TGII kits, all from Kyowa Medex Co. Ltd., using the same automatic analyser.

The principle of the MetaboLead LDL-C test involves measuring cholesterol and cholesterol esters enzymatically after solubilizing LDL and intermediate-density lipoprotein (IDL) in a detergent. In the feeding study using a 0.3% cholesterol diet in 2016, the measurement of plasma cholesterol levels was performed by Fuji Film Monolith Co., Ltd. ([now Fuji Film VET Systems Co., Ltd.]; https://www.fujifilm.com/ffvs/ja/what-we-do/), who used an automatic lipoprotein analyser (HLC-729LPII, Tosoh Bioscience Co., Ltd.) fitted with an anion exchange column (TSK gel Lipopropak-AEXII). In this method, CH was measured enzymatically after the lipoprotein particles were separated by column chromatography. Then, the sum of the CH in the IDL and LDL was calibrated relative to the LDL-C measurements obtained previously using a MetaboLead LDL-C kit (Kyowa Medex Co. Ltd.).

2.4 | Measurement of LDLR activity

LDLR activity was measured by Bio Medical Laboratories Inc. (http://www.bml.co.jp/) using the same method employed for humans.[39,40] Since the specific gravity of rhesus macaque lymphocytes is 1.077, which is the same as that in humans,[41] red blood cells could be eliminated using the same method used for humans.[40]

2.5 | Administration of CH

The AS primate diet containing 0.1% or 0.3% CH was produced by Oriental Yeast Co., Ltd. The CH-containing diet was fed to the macaques for 10 weeks in both experiments. Plasma CH levels were measured once a week for 10 weeks after administering the CH diet, and one to 5 weeks after terminating the CH diet.

2.6 | Measurement of *LDLR* and *HMGCR* mRNA levels

The extraction and measurement of *LDLR* and *HMGCR* mRNA levels were performed by Adjusted Cell Experimental Laboratory Inc. (ACEL Inc., https://www.a-cel.co.jp). Total RNA was extracted using a NucleoSpin RNA Blood kit (Cat. No. 740200.10, Macherey-Nagel Inc.). The *LDLR* and *HMGCR* mRNA levels were measured by one-step real-time RT-PCR using QuantiTect Probe RT-PCR Kit (Cat. No. 20443, QIAGEN) and the Taqman probes Rh02828936_m1 for *LDLR*, Rh01103005_g1 for *HMGCR* and Rh02621745_g1 for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Applied Biosystems). The expression levels of these mRNAs were calculated by the ΔΔCt method with *GAPDH* used as an internal standard.

2.7 | DNA sequence analysis

2.7.1 | Short DNA sequence determination

DNA was prepared using a QIAamp DNA Micro Kit from buccal cells or blood (QIAGEN). The nucleotide sequence was determined directly from the fragments amplified by PCR. Sequencing reactions were performed using an ABI Big Dye Terminator v.1 and ABI PRISM™ 310–20 (Applied Biosystems) for *LDLR*, and an ABI Big Dye Terminator v. 1 and Genetic Analyser 3130xl (Applied Biosystems) for the other genes. The primer sequences are given in Table 1.

2.7.2 | Whole-genome sequence analysis

The whole genome sequence analysis of three macaques (No. 1557, No. 1784, and No. 1834) was performed by Takara Bio Co. Ltd. (https://catalog.takara-bio.co.jp). These samples were used to prepare a library using a TruSeq DNA PCR-Free LT Library Prep Kit (Illumina Co. Ltd.) and Agilent XT-Auto System (Agilent Technologies Ltd.). Clusters were generated using a HiSeq X Five Reagent Kit v2.5 and a cBot Cluster Generation System (Illumina Co. Ltd.). Determination of the whole sequence was performed using a HiSeq X Five Reagent Kit v2.5 (Illumina Co. Ltd.) and a HiSeq X Five Sequencing System (Illumina Co. Ltd.) by the paired-end method. The obtained sequences were then mapped to the reference sequence (Ensemble *Macaca mulatta*: Mmul_8.0.1 by Genedata Profiler Genome v10.1.14b, BWA-MEM v0.7.15, GATK v3.6 and picard v2.2.4).

The mutations that affect amino acid sequences were predicted using the software package, SnpEFF (Source Forge; https://pcingola.github.io/SnpEff/) and were shown as Annotation impacts. Using this software, genetic variants of genes were annotated, and functional effects of proteins were predicted, as follows. Frameshift mutations, stop codons and changes in splice sites, were assigned as being High. The missense mutations were assigned as being Moderate, and synonymous changes were

TABLE 1 Primers used in PCR and sequencing reactions.

Forward primer	Forward primer sequence	Reverse primer	Reverse primer sequence
LDLR Ex3-F	TGACAGGTCAATCCTGTCTCTTC	LDLR Ex3-R	AATAGTAAAGGCAGGGCCACACT
LDLR_Ex12-F312	GGCACGTGACCTCTCCTTAT	LDLR Ex12-R583	ATGACCAGTTTTCCGCATTC
LDLR_Ex14-F	TTTGTTGGCCGAAAACCTAC	LDLR Ex14-R	ACACAGAAACAAGGCGTGTG
LDLRAP1_Ex3-F285	GAACAGTGCAGTGTAGGCCA	LDLRAP1 Ex3-R558	CACCCAGGTAGGAAACGGTA
MBTPS2 Ex6-F314	CAGAATGCCCAAGTGTTGTG	MBTPS2 Ex6-R558	CCTCTGATGAAAGAATGCCC
EPHX2 Ex2-F266	TCCTGGCAGTATGCCTTTCT	EPHX2 Ex2-R560	TTATGGTGGGCCCTAAGTTC
ITIH4 Ex1-F371	CCCCACTTGCTCTCAAGTTC	ITIH4 Ex1-R556	GTTCTCTCATCCCCCAGCTC
ITIH4 Ex10-F272	TTCTCACCCTCATCCCAAAC	ITIH4 Ex10-R562	GCAGAGTTGGGGGTGTCATA
ABCG5 Ex5-F	TCTGAGTCATGTGGCAGACC	ABCG5 Ex5-R	CACTCCAACACCAATGCAAC
CETP Ex11-F	TGTCCTTCCCATTTCTGAGG	CETP Ex11-F	GCACCCCTCATTACTTGCAT
CETP Ex16-F	AGGGCTTGAGGCAGTGTTTA	CETP Ex16-R	ATACACATCCCTTCCCCCTC
CYP7A1 Ex5-F	GGAATCACTGAGGCTTTCCA	CYP7A1 Ex5-R	CGATTAAGGGGACAATCCCT
LRLPAP1 Ex1-F	TGGAGCAACTACAATTCCCA	LRLPAP1 Ex1-R	CTTCTCCCAAAGCTGGTTCA
SOAT2 Ex6-F505	GTGCCCATGTTTCTGTCCAC	SOAT2 Ex6-R	GTTCCCCAAATCCCTCCTAA
HMGCR Ex10-F	AATTTTGTGCCGTGTTGTGA	HMGCR Ex10-R826	ACACGGTGCCCAGAACTTAG
LRP2 Ex5-F	GCTCAGCATTTAGTGACTTGC	LRP2 Ex5-R	AATTGGCTCTCTCAAGCCAG
LRP2 Ex8-F	CTGTGGCCTGTAACTCTCTCTT	LRP2 Ex8-R	GGGTGCTTTGTTACGCAAAC
LRP2 Ex49-F	TGAGCTCTCGTGCTTTCTGA	LRP2 Ex49-R	TTGAGGCTTGCTGGGTAACT

assigned as being Low. The quality of the alteration was expressed by the Phred scale. If there were more than two alterations, then the first alteration was assigned a value of '1', the second alteration a value of '2', and the third alteration a value of '3'. In this way, the genotypes could be represented as 0/0, 1/0 and 1/1 if they were the same as the reference nucleotide, heterozygous mutations or homozygous mutations, respectively. We used only the data that passed through filtration, which was performed using the following criteria: Quality Depth (QD) <2.0, Fisher Strand (FS) >60, Strand Odds Ratio (SOR) >3.0, Root Mean Square Mapping quality <40, Mapping Quality Rank Sum Test <12.5 and Read Pos Rank Sum Test <−8.0 for SNPs, and QD <2.0, FS >200.0, SOR >10.0, Read Pos Rank Sum Test <−20.0 and Inbreeding Coefficient <−0.80 for INDELs.

Genes for which the initiation codon and/or the termination codon were not clear and the mutations identified as being stop codons or frameshift mutations were checked against the reference sequence of Mmul_10.

2.8 | Number of animals used in the studies

Details regarding the number of animals and the identities of important individuals are summarized in Table 2.

3 | RESULTS

Macaca mulatta No. 1304, which originated in India and had the fourth highest Total-C level (283 mg/dL) of the 501 macaques (average: 165 mg/dL) that were kept at the Primate Research Institute, Kyoto University,[38] had two mutations in the *LDLR* gene, c.245G > A, *p.* (*Cys82Tyr*) in exon 3 (rs879254448 Homo sapiens) and c.2006 T > G, *p.* (*Arg669Met*) in exon 14 (rs1073643100 *Macaca mulatta*) (Figure 1). By 8 years of age, this macaque had two offspring (No. 1557 and No. 1624), which also had very high Total-C levels (245 and 234 mg/dL, respectively). To investigate which mutation affected the high Total-C level in these animals, we examined the mutations by denaturing gradient gel electrophoresis (DGGE) (Figure 2). While the mutation in exon 3 *p.* (*Cys82Tyr*) was also observed in the two offspring with hypercholesterolemia, the mutation in exon 14 was not identified in these offspring (Figure 2 and Table 3). These findings showed that *p.* (*Cys82Tyr*) is the causative mutation of hypercholesterolemia in macaques. To date, a total of seven heterozygous macaques and one homozygous macaque have been identified in the population of the rhesus macaques that originated in India and which are maintained at the Primate Research Institute at Kyoto University (Figure 3A).

3.1 | Cholesterol levels under the normal diet

Since plasma CH levels change with age,[38] we measured the plasma concentrations of CH over time in the same macaques. Table 4 shows the average plasma CH levels (Total-C, LDL-C and HDL-C) and TG levels of seven individuals with heterozygous *p.* (*Cys82Tyr*), 15 to 18 individuals with normal *p.* (*Cys82*), and the average age when they were examined. The Total-C and LDL-C levels of the heterozygous and normal macaques differed significantly

TABLE 2 Number of animals used in studies.

		Mutant				
		LDLR (*p.Cys82Tyr*)				Normal
Experiment	Result	*n*	Sex	No. of important individuals	MBTPS2 (S2P 241)	*n*
Normal diet (CH-free)	Table 4	7				18
Activity of LDLR	Table 5	5[b, c]		No. 1557 (MS)	Val/Ile	2
				No. 1774	Val	
				No. 1784 (SHI)	Ile	
+0.1%CH diet	Figure 4 and Figure S1	3	Male	No. 1774	Val	3
				No. 1784 (SHI)	Ile	
				No. 1834 (SHI)	Ile	
+0.1%CH diet	Figure S1	4[a, c]	Female	No. 1557 (MS)	Val/Ile	
+0.3%CH diet for plasma CH	Figure 5 and Figure S2	4[c]		No. 1834 (SHI)	Ile	2
Whole Genome Analysis	Table 3	3		No. 1557 (MS)	Val/Ile	
				No. 1784 (SHI)	Ile	
				No. 1834 (SHI)	Ile	
Genotype	Table 3	8				4
+0.3%CH diet for mRNA	Figures 6, 7 and Figure S3	3[b]		No. 1557 (MS)	Val/Ile	
				No. 1774	Val	
				No. 1784 (SHI)	Ile	

Abbreviations: CH, cholesterol; SHI, Super High (CH) Individual; MS, The Mother of No. 1834 and the Syster of No. 1784; LDLR, Low-density lipoprotein receptor; MBTPS2, Membrane-bound transcription factor protease, site 2; S2P, site 2 protease.

[a]No.1304 was dead.

[b]No.1834 was dead.

[c]Includes the homozygote No. 2041.

($p < .001$), even when they were fed CH-free monkey chow, while the HDL-C and TG levels remained relatively stable. The differences between Total-C and LDL-C levels in the heterozygous and normal individuals were likely not due to age differences, as there was no significant difference in mean age at the time of the examinations.

3.2 | Activity of LDLR

The activity of LDLR was measured in four heterozygous macaques, one homozygous macaque and two normal macaques (Table 2). When the LDLR activity of the macaques was measured using human LDL and compared to normal human leucocytes, binding was typically three times higher than that observed in humans (Table 5). Since the LDLR activity of two normal macaques was used as a control, these activity values were comparable. The average LDLR activity was 71.5% (53–88%) in the heterozygous macaques and 42% in the homozygous macaque.

3.3 | Plasma CH levels after administration of the 0.1% CH or 0.3% CH diet

Since CH was not included in the ordinary monkey chow, we supplemented ordinary monkey chow with 0.1% CH (0.27 mg/kcal), which is equivalent to consuming 2.5 eggs in a daily diet of 2000 kcal in humans. Monkey chow containing 0.1% CH was fed to six heterozygous macaques (No. 1304 had already died.), one homozygous macaque and three normal macaques (Table 2).

ApoB is contained in the atherogenic particles, very-low-density lipoprotein (VLDL), IDL and LDL, with that in LDL accounting for over 90%. Since ApoA-1 promotes cholesterol efflux from peripheral cells and macrophages to form HDL particles and transports CH back to the liver, the ApoB/ApoA-I ratio is considered to be the best indicator for assessing the risk of myocardial infarction.[42,43] However, Millán et al.[44] proposed that the lipoprotein ratios, Total-C/HDL-C and LDL-C/HDL-C, are more clinically useful indicators than the isolated blood parameters of Total-C or LDL-C. Specifically, they proposed that the cut-off value for the Total-C/

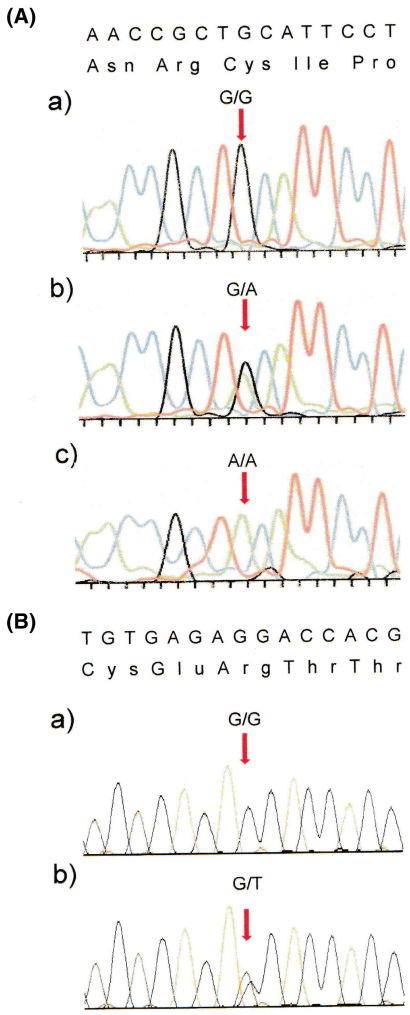

FIGURE 1 (A) DNA sequences of exon 3 in the *LDLR* gene of *Macaca mulatta*. a: Normal No. 1889, b: Heterozygous No. 2051 and No. 1304, and c: Homozygous No. 2041; (B) DNA sequence of exon 14 in the *LDLR* gene of *Macaca mulatta*. a: Normal No. 1774 and b: Heterozygous No. 1304.

HDL-C and LDL-C/HDL-C ratios for risk evaluation of cardiovascular disease in men and women are >5.0 and >3.5 (ApoB/ApoA-I >1.0), and >4.5 and >3.0 (ApoB/ApoA-I >0.9), respectively. We, therefore, adopted these values to evaluate the lipoprotein data in this study.

As shown in Figure 4A and Figure S1C, LDL-C was also significantly elevated only in two heterozygous monkeys (No. 1784 and No. 1834). However, because the HDL-C level of No. 1834 was approximately 50 mg/dL higher than that of No. 1784 (Figure 4C), the LDL-C/HDL-C ratio (5.7 Figure 4B) and Total-C/HDL-C ratio (6.1 Figure S1B) in one macaque (No. 1784) were higher than cardiovascular incidence risk levels in humans (3.5 and 5.0, respectively).[44]

Since the plasma LDL-C level of No. 1834 was high, albeit below cardiovascular incidence risk levels, administration of 0.3% CH was expected to increase LDL-C and exceed the risk level. In addition, monkey chow supplemented with 0.3% CH was fed to three heterozygous macaques (No. 1834, No. 1624, and No. 2051), a homozygous macaque (No. 2041) and two normal macaques (Table 2, Figure 5 and Figure S2). In female macaques No. 1624 and No. 2041,

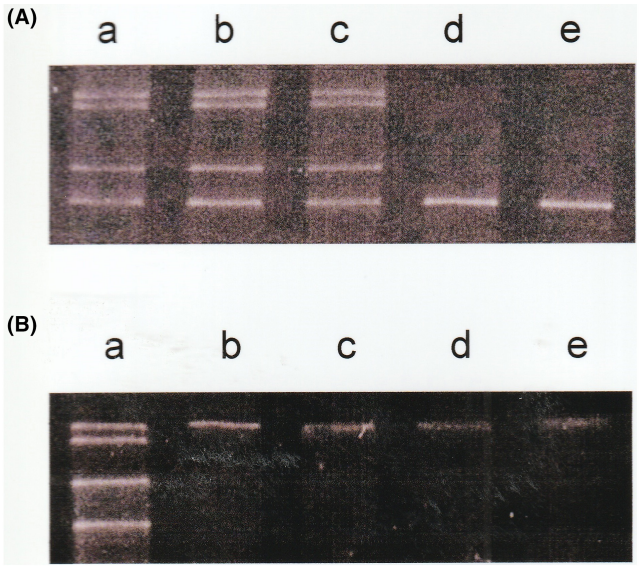

FIGURE 2 Denaturing gradient gel electrophoresis (DGGE) patterns of PCR products of exon 3 (A) and exon 14 (B) of the *LDLR* gene. a: No. 1304 (237), b: No. 1557 (245), c: No. 1624 (205), d: No. 1269 (178), and e: No. 1585 (140); values in parentheses show Total-C level (mg/dL) in animals aged approximately 10 years.

higher values of cardiovascular incidence risk were observed under the 0.1% CH-diet (Figure S1D). Further, since No. 2051 was the same age as No. 2041, whether the LDL-C of these macaques exceeded the risk level under 0.3% CH administration was also investigated. To better clarify the changes in the LDL-C/HDL-C and Total-C/HDL-C ratios in macaques on the 0.3% CH-diet, the values of these ratios obtained for the normal diet 1 week prior to the start of the experiment were subtracted from the LDL-C/HDL-C and Total-C/HDL-C ratios and standardized for everyone (Figure 5C and Figure S2C). The findings showed that the LDL-C/HDL-C and Total-C/HDL-C ratios in No. 1834 increased in two phases from the onset of the 0.3% CH-diet experiment. In the first phase, that is, for the 6-week period following the start of the experiment, there was a marked increase in both the LDL-C/HDL-C and Total-C/HDL-C ratios, but risk levels were not reached because of the high plasma HDL-C concentrations. After 6 weeks, in the second phase of the increase, the ratios increased up to 4.7 and 6.4, respectively (Figure 5B and Figure S2B), markedly exceeding the risk levels of 3.5 and 5.0, respectively. However, the plasma concentrations of LDL-C and Total-C in the other heterozygous macaques, No. 1624 and No. 2051, and the homozygous macaque, No. 2041, did not increase in the first phase and did not reach risk levels by 10 weeks of CH supplementation.

3.4 | Whole genome analysis of super hypercholesterolemic individuals (SHI)

To identify whether genes other than *LDLR* were causative genes of hypercholesterolemia in macaques, whole genome analyses were performed on two hypercholesterolemic macaques (No. 1784 and No. 1834) and one macaque whose plasma CH levels did not increase (No. 1557) (Accession Numbers from DNA Data Bank of

TABLE 3 Genotypes and maximum ratios of LDL-C/HDL-C after cholesterol administration.

								Indiv. No.	1	2	3	4	5	6	7	8	9	10	11	12
									*1304	1557	1624	1774	1784	**1834	2041	2051	1717	1778	1795	2033
								Sex	F	F	F	M	M	M	F	F	F	M	F	F
Gene name	Mutation No.	#Chrom	Position M mul 8.0.1	Exon	Ref	Alt	Amino acid substitution	Birth year	1994	2000	2002	2006	2006	2008	2013	2013	2005	2006	2007	2013
LDLR	rs879254448 (H. sap)	19	1.1E+07	3	G	T	*p.(Cys82Tyr)*		G/T	G/T	G/T	G/T	G/T	G/T	T/T	G/T	G/G	G/G	G/G	G/G
LDLR	rs288647932	19	1.1E+07	12	A	G	*p.(Ile598Val)*		G/G	A/G	G/G	G/G	G/G	G/G	G/G	G/G	A/G	G/G	G/G	G/G
LDLR	rs1073643100	19	1.1E+07	14	G	T	*p.(Arg669Met)*		G/T	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	T/T	T/T	G/G
LDLRAP1	rs301325078	1	2.4E+07	3	A	G	*p.(Asn102Ser)*		A/G	A/A	G/G	G/G	A/G	A/G	A/G	A/G	—	G/G	A/A	—
MBTPS2	rs30530257	X	2.1E+07	6	G	A	*p.(Val241Ile)*		G/A	G/A	G/G	G	**A**	**A**	G/G	G/A	G/A	G	G/G	G/G
EPHX2	rs289443791	8	2.8E+07	2	C	T	*p.(Thr51Ile)*		C/C	C/C	C/T	C/T	C/T	C/T	C/C	C/T	—	C/C	C/C	—
ITIH4	rs312121031	2	8.9E+07	1	A	G	*p.(Ser11Gly)*		A/A	A/A	A/A	A/A	A/G	A/G	A/A	A/A	—	A/A	A/G	—
ITIH4	rs302986364	2	8.9E+07	10	A	C	*p.(Glu432Asp)*		A/C	A/A	C/C	A/C	A/C	A/C	A/A	A/C	—	A/C	A/C	—
ABCG5	rs306501086	13	4.5E+07	5	G	A	*p.(Arg209Gln)*		A/A	A/A	G/A	G/A	G/A	G/A	A/A	G/A	G/A	G/A	G/G	A/A
CETP		20	4.1E+07	11	G	A	*p.(Gly357Ser)*		G/G	G/G	G/A	G/G	G/A	G/A	G/G	G/A	G/G	G/G	G/G	G/G
CETP		20	4.1E+07	11	A	C	*p.(Lys373Gln)*		A/A	A/A	A/C	A/A	A/C	A/C	A/A	A/C	A/A	A/A	A/A	A/C
CETP		20	4.1E+07	16	A	G	*p.(Gln516Arg)*		A/A	A/A	A/G	A/A	A/G	A/G	A/A	A/G	A/A	A/A	A/A	A/G
CYP7A1	rs194979464	8	5.7E+07	5	C	A	*p.(Asp376Glu)*		C/C	C/C	C/A	C/A	C/A	C/A	C/A	C/C	A/A	C/A	C/A	C/A
LRPAP1	rs1082776506	5	3829589	1	C	G	*p.(Leu16Val)*		G/G	C/C	C/G	G/G	C/G	C/G	G/G	G/G	C/G	G/G	G/G	G/G
LRPAP1	rs298735717	5	3829616	1	G	C	*p.(Gly7Arg)*		C/C	G/G	C/C	C/C	G/C	G/C	C/C	C/C	G/C	C/C	C/C	C/C
SOAT2	rs286820176	11	5.2E+07	6	C	G	*p.(Leu208Val)*		C/C	C/C	C/C	C/G	C/G	C/G	C/G	C/C	C/G	C/G	C/G	C/C
HMGCR	rs303122374	6	7.2E+07	10	A	T	*p.(Glu433Asp)*		A/A	A/A	A/T	A/T	A/T	A/T	A/A	A/T	A/T	T/T	A/T	A/A
LRP2	rs308676440	12	56252660	5	G	T	*p.(Trp166Leu)*		G/G	G/G	G/T	G/G	G/T	G/T	G/G	T/T	G/T	G/T	G/G	G/G
LRP2	rs307184314	12	56248964	8	A	G	*p.(Ile289Val)*		A/A	A/A	A/G	A/A	A/G	A/G	A/A	G/G	A/G	A/G	A/A	A/A
LRP2	rs194406702	12	56162232	49	G	T	*p.(Gln2944His)*		G/T	G/G	G/T	T/T	G/T	G/T	G/T	G/T	G/T	G/G	T/T	T/T
Max ratio of LDL-C/HDL-C after CH administration	0.1% CH									2.1	2.8	2.7	**5.7**	2.9	3.3	1.3	1.4	2.3	1.3	
	0.3% CH										2.7			**5.6**	2.3	2.2			1.0	2.2

Note: #Chrom: No. of chromosome in which the gene is located. Position M mul 8.0.1: Position of the mutation on the chromosome of the reference sequence (Ref Seq) of Ensemble Macaca mulatta v.8.0.1. Ref: The nucleotide in the Ref Seq. Alt: Altered nucleotide at the same position. Column Nos. 1–8 show the individuals who had a mutation in exon 3 in the LDLR gene. Column Nos. 9–12 show the individuals who had no mutation in the LDLR gene. Three individuals for which whole genome sequences were examined are indicated by column shading.

Abbreviations: CH, cholesterol; LDL-C, Plasma CH level in low-density lipoproteins; HDL-C, Plasma CH level in high-density lipoproteins; LDLR, Low-density lipoprotein receptor; LDLRAP1, Low-density lipoprotein receptor adaptor protein1; MBTPS2, Membrane-bound transcription factor protease, site2; EPHX2, Epoxide hydrolase 2; ITIH4, Inter-alpha-trypsin inhibitor havy chain family member 4; ABCG5, ATP binding cassette subfamily G member 5; CETP, Cholesteryl ester transfer protein; CYP7A1, Cytochrome P450 family 7 subfamily A member 1; LRPAP1, LDL receptor related protein associated protein 1; SOAT2, Sterol O-acyltransferase 2; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; LRP2, LDL receptor related protein 2.

*1304 was dead.

*1834 was dead.

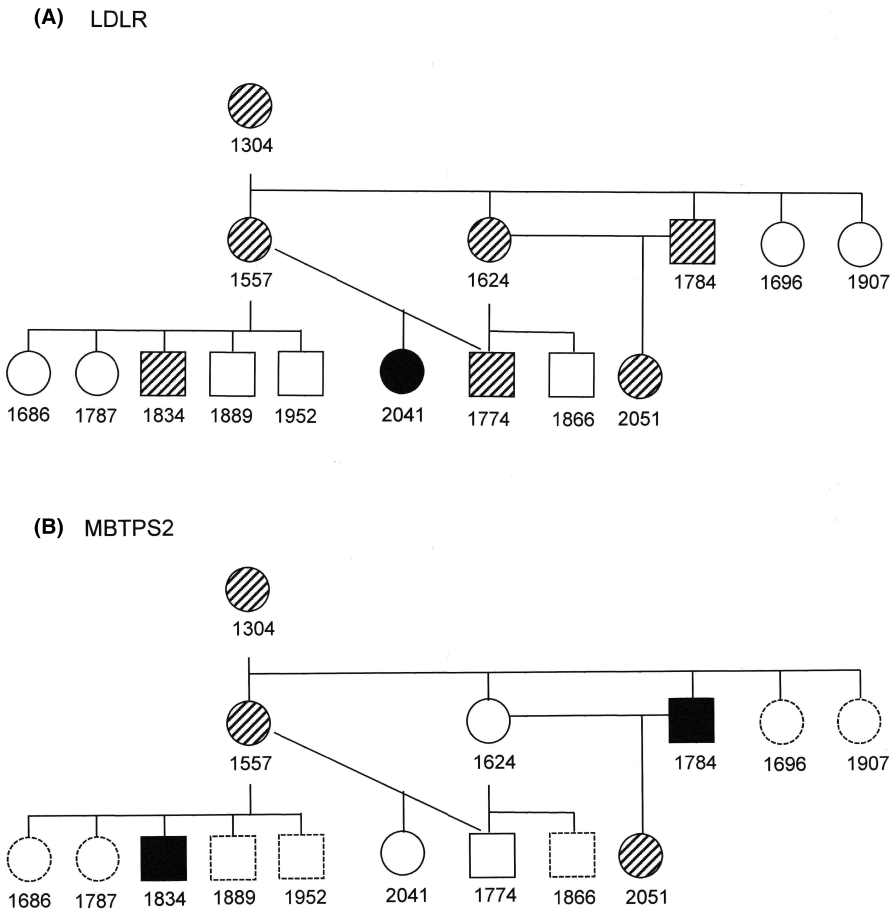

FIGURE 3 (A) Pedigree of the hypercholesterolemic rhesus macaque family. Oblique lines in symbols show macaques that are heterozygous for the *p. (Cys82Tyr)* mutation in the *LDLR* gene determined by sequence analysis. Solid symbols show homozygotes. Squares and circles indicate males and females, respectively. (B) Circles with solid borders show the individuals that were subjected to *MBTPS2* gene sequencing. Oblique lines in symbols show individuals that were heterozygous for *p.* (*Val241Ile*), and solid symbols show hemizygous individuals (*p.* (*Ile241*)).

TABLE 4 Comparison of plasma cholesterols and triglyceride levels between heterozygous individuals with the *p.*(*Cys82Tyr*) mutation in LDLR and normal subjects.

	Heterozygous (Tyr/Cys)			Normal (Cys/Cys)			Blood value	Age
	mg/dL	*n*	Age	mg/dL	*n*	Age	*t*-test	*t*-test
Total-C	226.6 ± 31.8	7	6.5 ± 4.6	175.9 ± 24.1	18	5.7 ± 2.3	$p < .001$	$p > .1$
LDL-C	135.2 ± 20.2	7		85.4 ± 27.1	15		$p < .001$	
HDL-C	90.8 ± 15.1	7		89.7 ± 15.2	17		$p > .5$	
TG	23.4 ± 9.8	7		24.6 ± 15.2	18		$p > .5$	
Total-C/HDL-C	2.5			2.0				
LDL-C/HDL-C	1.5			1.0				

Note: Blood cholesterol levels were measured several times in each individual, because these levels change with age. However, the mean age at the time of measurement was not significantly different.

Abbreviations: Total-C, Plasma total cholesterol level; LDL-C, Plasma cholesterol level in low-density lipoproteins; HDL-C, Plasma cholesterol level in high-density lipoproteins; TG, Plasma triglyceride level.

Japan (DDBJ) for the three macaques are DRR412789–DRR412791). Since No. 1784 and No. 1834 showed extremely high cholesterol levels after CH administration, we refer to these individuals as Super High Individuals (SHI). As No. 1557 is the mother of No. 1834 and the sister of No. 1784 (Figure 3), we refer to this individual as MS.

We selected potential causative genes from the literature review of Paththinige et al.[35] in which they reviewed the results of GWAS on hypercholesterolemia in humans.[18–34,36] In addition, we also surveyed reports on endocytosis of LDL,[45,46] control of *LDLR* transcription as it relates to SREBPs,[10,47–49] and genes related to the efflux of

TABLE 5 Activity of LDLR.

Indiv. No.	1304[b]	1557	1624	1774	1784	1834[c]	2041	1717	1795
Sex	F	F	F	M	M	M	F	F	F
Birth year	1994	2000	2002	2006	2006	2008	2013	2005	2007
AA at 82	Cys/Tyr	Cys/Tyr	Cys/Tyr	Cys/Tyr	Cys/Tyr	Cys/Tyr	Tyr/Tyr	Cys/Cys	Cys/Cys
LDLR activity compared to normal human		161	189	251	264		127	283	319
LDLR activity compared to that in normal *M. mulatta*		53	63	83	88		42	100[a]	100[a]

Abbreviations: AA, amino acid; LDLR, Low-density lipoprotein receptor.

[a]Average (283 + 319)/2 = 301 was converted into 100.

[b]Died in 2011.

[c]Died in 2018.

intracellular CH.[9] We surveyed a total of 112 genes that are related to hypercholesterolemia using Var_annotation.xlsx files, which were lists of the results for all variants obtained using the SnpEFF software package. We found one heterologous mutation in exon 12 of *LDLR* in No. 1557, but the same mutation was found in No. 1717 whose plasma CH levels were normal (Table 3). There was one nonsense mutation, 11 frameshift mutations, and no mutations at splice sites. However, it became clear that these mutations, except for the frameshift mutation in the patatin-like phospholipase domain-containing protein 5 (PNPLA5) (which is located at 10:85040507 in *Macaca mulatta* V.8.1.0 and at the same position as 10:7593591 in Macaca mulatta V.10), were in the introns in the Reference Sequence of *Macaca mulatta* Ver 10 (https://www.ncbi.nlm.nih. gov/assembly /GCF_003339765.1). We found one heterozygous frameshift mutation in *PNPLA5* in No. 1784, but No. 1834 was normal. Further, 426 synonymous mutations and 167 missense mutations were identified in the exons. We then reselected mutations using the following two conditions: (1) the same heterozygous mutation in the two SHIs, but not in the MS, or (2) the same homozygous mutation or hemizygous mutation in the two SHIs, but a heterozygous or no mutation in the MS. Mutations that met conditions (1) or (2) were found at 17 loci in 11 genes, as shown in Table 3.

If only one missense mutation was found in the two SHIs, then it would be the causative gene of hypercholesterolemia. PCR amplification and sequence determinations were performed for other individuals whose plasma cholesterol levels did not increase after supplementing their diet with CH (Table 3).

As shown in Table 3, only one hemizygous missense mutation –– c.721G>A in *MBTPS2 (p. (Val241Ile)* in site-2-protease (S2P) rs30530257) –– was found in the two SHIs. Since *MBTPS2* is located on the X chromosome, these male SHIs were hemizygous for this mutation. The heterozygous G/A mutation was found in four females including No. 1557, but the homozygote A/A was not found in females. If this mutation was a recessive variant, then it is noteworthy that it was the causative mutation of hypercholesterolemia in males. Interestingly, the mutation in *MBTPS2* was also observed in the same pedigree as that of *LDLR* (Figure 3B).

3.5 | Levels of LDLR and HMGCR mRNA during administration of 0.3% CH

S2P protein translated by *MBTPS2* produces the transcription factor, basic-helix–loop–helix-leucine zipper (bHLH-Zip) through the cleavage of SREBPs translocated to the Golgi complex when the intracellular concentrations of CH are low following S1P cleavage.[11,12] The produced bHLH-Zip enters the nucleus, binds to the sterol-responsive elements of *LDLR* and *HMGCR*, and activates the transcription of these genes.[13–15] To test the changes in the activity of S2P caused by the mutation in *MBTPS2*, we measured *LDLR* and *HMGCR* mRNA levels in macaques administered the 0.3% CH diet. The three macaques who had an A mutation (*p.* (*Ile241*), No. 1784), a G mutation (*p.* (*Val241*), No. 1774) and a G/A mutation (No. 1557) in *MBTPS2* were used for the experiment (Table 2). We were unable to measure the mRNA levels in No. 1834 because, to our regret, the animal died due to extensive haemorrhaging of the mucosae of the alimentary tract and petechial haemorrhaging of the epicardium of the right ventricle. Since these three macaques were heterozygous for the *p.* (*Cys82Tyr*) mutation in *LDLR*, Total-C and LDL-C levels were higher than they were in normal macaques. The results of plasma LDL-C and Total-C levels are shown in Figure 6A and Figure S3A. During the beginning of 0.3% CH administration until 4 weeks after the start of the experiment, plasma CH levels in No. 1784 (A (*p.* (*Ile241*)) *MBTPS2*) were higher than in No. 1774 (G (*p.* (*Val241*)) *MBTPS2*). At 6 weeks after the start of the experiment, the LDL-C levels in No. 1774 increased almost to the same level as that in No. 1784. As shown in Figure 6A and Figure S3A, LDL-C and Total-C levels in No. 1557 were too high and mRNA levels did not change throughout the experiment (Figure 7A). No. 1557 was 21 years old, which corresponds to an age of 63 years old in humans, so the data obtained from that individual were excluded. The other two males were 15 years old.

The *LDLR* and *HMGCR* mRNA levels are shown in Figure 7. During the first response phase (i.e. until 4 weeks after administration of 0.3% CH), the *LDLR* mRNA levels in No. 1784 (*MBTPS2* (A)) with *p.* (*Ile241*) in S2P were lower (Figure 7A, B), and plasma

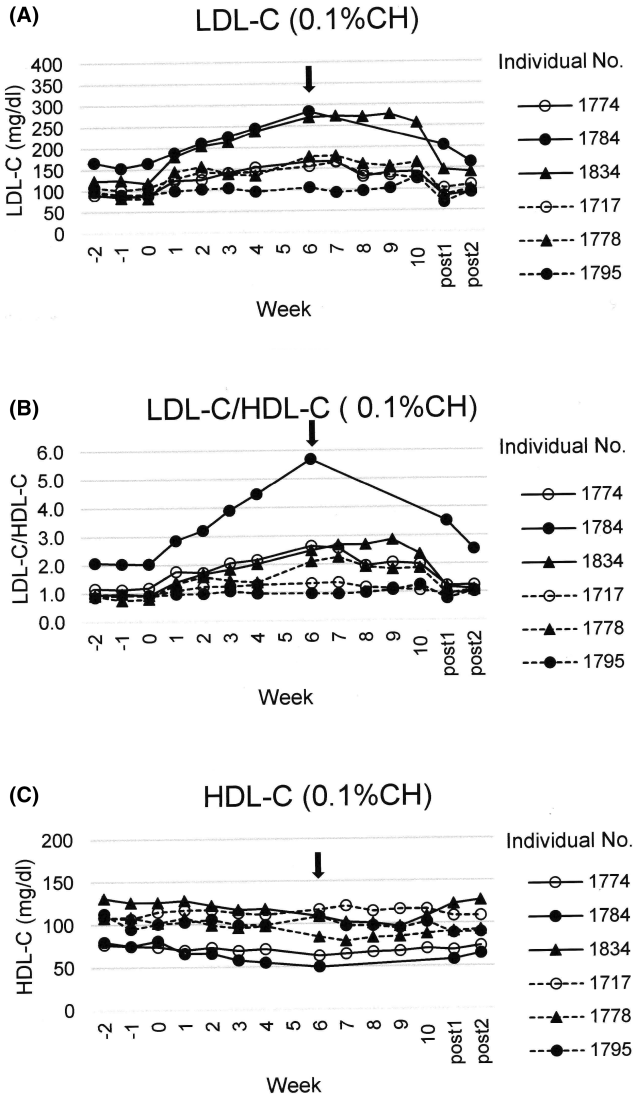

FIGURE 4 (A): Changes in plasma LDL-C levels of male macaques administered a diet containing 0.1% CH. The solid lines show individuals that were heterozygous for the *p.* (*Cys82Tyr*) mutation in *LDLR*, and the dashed lines show normal individuals. The arrow shows when feed containing CH was withheld from No. 1784 for reasons related to poor health. (B) Change in LDL-C/HDL-C under administration of 0.1% CH in the diet. The dash-dot line shows the risk level for developing cardiovascular disease. (C) Changes in plasma HDL-C levels. The line styles and their meanings in B and C are the same as in A. 'Post' means weeks after terminating CH supplementation.

LDL-C and Total-C levels were 50 mg/dL higher than in No. 1774 with (*p.*(*Val241*)) in S2P (*MBTPS2* (G)) (Figure 6A, B and Figure S3A, B). The *LDLR* mRNA level in No. 1774 increased until week four. Furthermore, when No. 1834 was administered 0.3% CH (Figure 5A and Figure S2A), the plasma LDL-C/HDL-C and Total-C/HDL-C ratios increased in two phases (Figure 5B, C and Figure S2B, C). Although the mRNA levels in No. 1834 could not be measured, it was estimated that they were similar to those observed in No. 1784, that is, a small increase in mRNA levels in the first phase followed by a sudden decrease in the second phase. Figure 7B shows the ratio

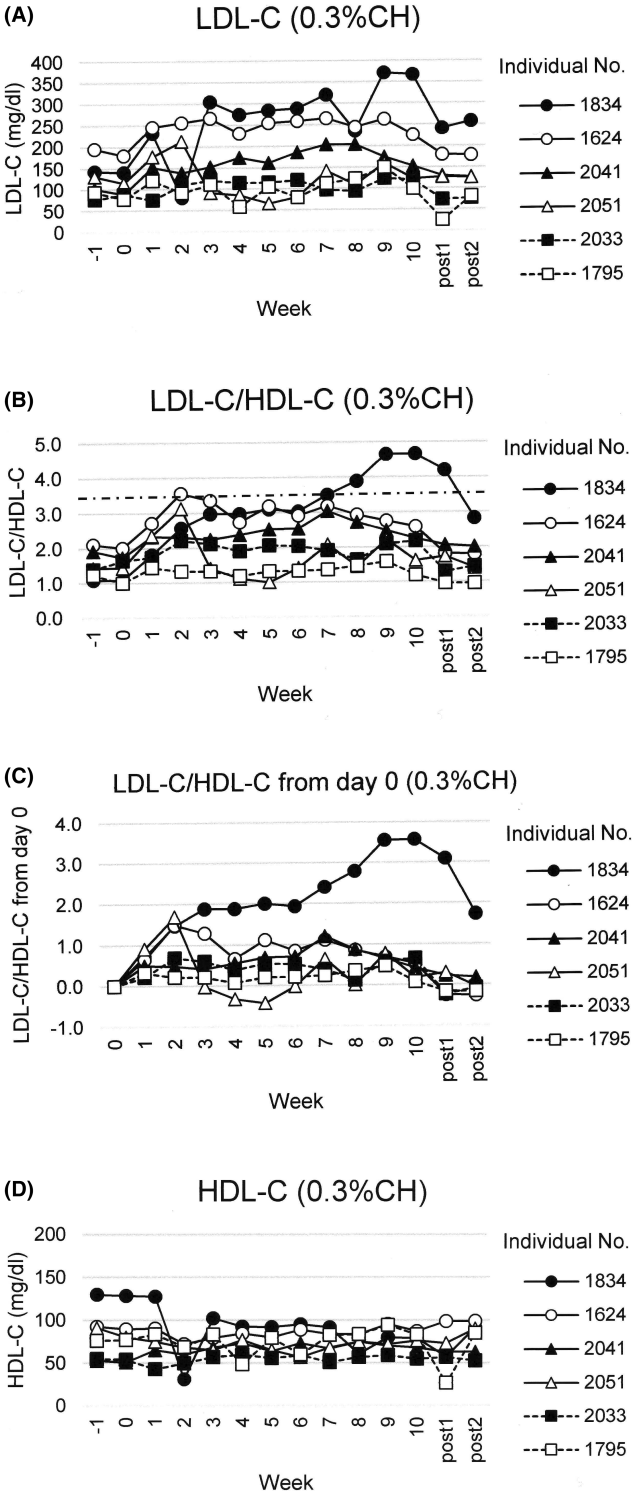

FIGURE 5 (A) Change in plasma LDL-C levels in macaques administered a diet containing 0.3% CH. No. 1624, No. 1834 and No. 2051 are heterozygous, No. 2041 is homozygous for *p.* (*Cys82Tyr*) in *LDLR* (solid lines). No. 1795 and No. 2033 are normal (dashed lines). (B) LDL-C/HDL-C levels in macaques administered a diet containing 0.3% CH. The dash-dot line shows the risk level for developing cardiovascular disease. (C) Difference in LDL-C/HDL-C levels from day 0 in macaques administered a diet containing 0.3% CH. (D) HDL-C levels in macaques administered a diet containing 0.3% CH. 'Post' means weeks after terminating CH supplementation.

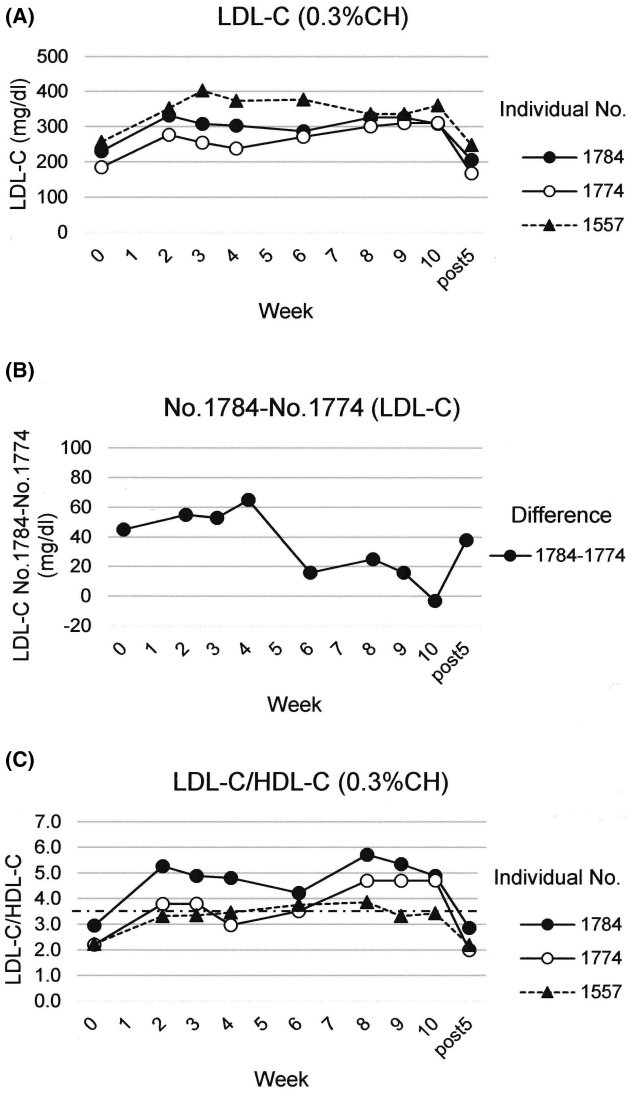

FIGURE 6 (A) Plasma LDL-C levels of three macaques with different types of *MBTPS2* (S2P protein). No. 1784 (male) is hemizygous for A (*p.* (*Ile241*)), No. 1774 (male) is normal G (*p.* (*Val241*)) and No. 1557 (female) is heterozygous for G/A (*p.* (*Val241/Ile241*)). (B) Difference between LDL-C levels in No. 1784 and No. 1774. (C) LDL-C/HDL-C levels in the same macaques. The dash-dot line shows the risk level for developing cardiovascular disease. 'Post' means weeks after terminating CH supplementation.

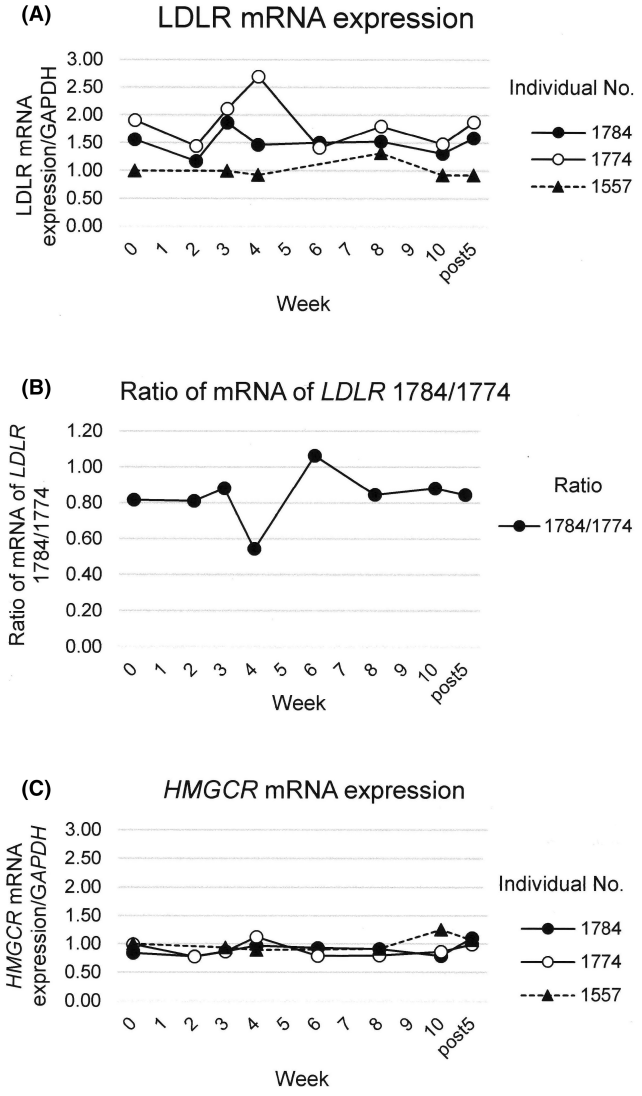

FIGURE 7 (A) The mRNA expression levels of *LDLR* in leucocytes of selected macaques. (B) Ratio of mRNA expression levels of *LDLR* in No. 1784 compared to those in No. 1774. (C) The mRNA expression levels of *HMGCR* in leucocytes of selected macaques. 'Post' means weeks after terminating CH supplementation.

of the mRNA expression level in No. 1784 to that in No. 1774; until 3 weeks, the mRNA level of No. 1784 was 80% that of No. 1774. The first increase in the *LDLR* mRNA level could be attributed to the plasma CH from the 0.3% CH diet being endocytosed by the LDLR at a lower level in No. 1784 than in No. 1774, resulting in plasma LDL-C and Total-C levels being 50 mg/dL higher in No. 1784 than in No. 1774 (Figure 6B and Figure S3B). Thus, the 20% decrease in *LDLR* mRNA levels due to the *MBTPS2* mutation (S2P *p.* (*Val241Ile*)) increased plasma LDL-C levels by 50 mg/dL (Figure 6B and Figure 7B). Although the differences in the LDL-C and Total-C levels between the two macaques appear small, they affect the risk factors. For example, even though the HDL-C concentration in No. 1784 was slightly lower than that in No. 1774 (differences ranged from 4 to 17 mg/dL), the LDL-C/HDL-C and Total-C/HDL-C ratios in a SHI (No. 1784) were around 5 and 6, respectively, which markedly exceeded the risk levels of 3.5 and 5.0, respectively (Figure 6C and Figure S3C). The first increasing phase observed in macaques on the 0.3% CH diet was comparable to the increase observed in macaques on the 0.1% CH diet, implying that oligogenic mutations could cause pathogenic hypercholesterolemia to induce cardiovascular disease.

The *HMGCR* mRNA level in No. 1774 was slightly higher than that in No. 1784 at week 4, before decreasing thereafter. However, the change was very small, which implies that the effect of bHLH-Zip binding SREBPs on the sterol-responsive element of *LDLR* is more pronounced than the contribution of the rate-limiting enzyme *HMGCR* on CH synthesis.

After 6 weeks of 0.3% CH administration, the *LDLR* mRNA level in both No. 1784 and No. 1774 decreased suddenly to below initial levels; however, the *LDLR* mRNA levels were restored after ceasing CH administration (Figure 7A). The administration of 0.3% CH to No. 1774 and No. 1784 was the first treatment (Table 2). In this treatment, LDL-C and Total-C levels increased to the same levels as those in No. 1834, even though levels in the other five macaques did not increase for 10 weeks (Figure 5 and Figure S2). However, in all the macaques examined, HDL-C levels did not change during 0.3% CH administration. These findings imply that the administration of 0.3% CH was too high to promote the take-up of CH by cells in the three macaques (i.e. No. 1784, No. 1834, and No. 1774). The mechanism involved in maintaining optimal intracellular CH concentrations (i.e. to prevent the influx of CH) was expected and *LDLR* mRNA levels decreased after 6 weeks of CH administration, resulting in *LDLR* mRNA levels that were slightly lower than those observed in macaques on the CH-free diet. As in the first phase of the animals' CH response, the *LDLR* mRNA level of No. 1784 was 80% that of No. 1774 in the second phase (Figure 7B). Similarly, the plasma LDL-C level of No. 1784 was 20 mg/dL higher than that of No. 1774 (Figure 6B). In the first phase of the response, although the difference in the LDL-C levels of both macaques was 50 mg/dL, it could be considered that the difference was 20 mg/dL in the second phase of the response due to the lower *LDLR* mRNA levels. It is possible that the efflux mechanism of intracellular CH may have been damaged in some way in these three macaques. For example, the intracellular concentration of CH may have been elevated more than in the other macaques, which then regulated the expression of *LDLR* mRNA and resulted in the higher plasma LDL-C and Total-C levels. A mutation in the ATP-binding cassette subfamily A member 1 (*ABCA1*) at positions 15:34700163 was annotated as *p*. (*Cys285Tyr*) in Ref Seq Mmul Ver. 8.0.1 and was found in both heterozygous SMIs; however, these positions were in introns 6–7 in Ref seq Mmul Ver.10. Thus, it appears that this mutation does not affect the efflux of CH.

At 5 weeks after terminating CH administration, the mRNA expression levels and LDL-C and Total-C levels in all three of the macaques recovered to the levels observed under the normal diet.

4 | DISCUSSION

Familial hypercholesterolemia caused by the *LDLR* mutation *p*. (*Cys82Tyr*) (rs8792544448, NP_000518.1 (*Homo Sapiens*)) was identified in rhesus macaques that originated in India. The plasma levels of LDL-C and Total-C in the heterozygous *LDLR p*. (*Cys82Tyr*) mutants were 50 mg/dL higher than those in normal animals. The average LDLR activity was 71.5% in the heterozygous animals and 42% in the homozygous animals. Cys82 bound to Cys95 forms one of three S-S bonds in the LDL receptor type A (LA2) repeat in LDLR.[50] Although steric hindrance of the Ca ion-binding region has been attributed to cleavage of S-S bonds due to the missense mutation, the mutation *p*. (*Cys82Tyr*) is in the LA2 repeat of the LDLR protein. Since the centre of the binding position of LDL is the LA4 and LA5 repeats,[51,52] this substitution was upstream from the centre resulting in the LDLR activity decreasing by only approximately 30% in the heterozygotes. In the homozygote, LDLR activity was reduced by 60%.

The hemizygous mutation of *MBTPS2* from G to A (*p*. (*Val241Ile*) in S2P) decreased the expression of *LDLR* mRNA and this mutation was sex-linked inheritance. The mRNA levels of *LDLR* of the A (*p. Ile241*) mutation was 80% that of the G mutation (*p. Val241*) after 3 weeks of 0.3% CH administration, resulting in plasma LDL-C and Total-C levels being 50 mg/dL higher than in the levels of the individual with G mutation (*p. Val241*). S2P has eight transmembrane segments (TMS), and TMS 4 to TMS 6 are conserved from bacteria to humans.[53] The *p*. (*Val241Ile*) mutation is in the fifth TMS of S2P,[54] which has the same sequence as the transmembrane helix/motif 2 (TMH2).[55] Although the mutation is 70 amino acid residues downstream from the zinc-bound active centre HEIGH (171–175)[56] and Asp467, TMH2 is a substrate binding site containing the substrate binding motif GpxxN/S/G and a hydrophobic region.[55] Therefore, it is possible that the difference in hydrophobicity caused by the *p*. (*Val241Ile*) mutation altered the binding capacity of the substrate, that is, SREBP cleaved by S1P,[57] and reduced binding activity by 20%. The reduction in the activity of S2P affected the reduction of *LDLR* mRNA levels. A mutation in the same position has been reported in humans, but the amino acid was substituted by methionine (rs148389641) and not isoleucine, and no clinical effect was reported.

The results clearly showed that the degree of the increase of LDL-C and Total-C attributable to CH in the diet of macaques differs between individuals according to their genetic background. Indeed, similar findings have been reported in humans. For example, the United States Department of Agriculture and United States Department of Health and Human Services announced that limiting the amount of CH in food is not necessary in 2015 because people's reactions to CH differ among individuals.[58]

Since the CH levels in the two SHIs in this study (i.e. No. 1784 and No. 1834), and others, recovered to ordinal levels after 5 weeks on a CH-free diet, these macaques can be used in future studies.

On the CH-free diet, all animals that were heterozygous for *p*. (*Cys82Tyr*) in *LDLR* had LDL-C levels that were 50 mg/dL higher than normal levels; however, the LDL-C/HDL-C and Total-C/HDL-C levels were lower than the risk level, then breeders will be able to maintain the animals more easily.

Rhesus macaques with mutations of *p*. (*Cys82Ttyr*) in *LDLR* and *p*. (*Val241Ile*) in *MBTPS2* will be well suited for use as model animals because the genetic background has been studied extensively, reproducibility in physiological responses to CH administration was demonstrated clearly using experiments involving the administration of CH, and macaques have a similar metabolism to humans. As a result, it is expected that future studies will focus on the development of medicines that promote the transcription of *LDLR*.

5 | CONCLUSION

We identified seven and one rhesus macaques with heterozygous and homozygous *p*. (*Cys82Tyr*) mutations in *LDLR*, respectively. The

activity of LDLR was 72% in the heterozygotes and 42% in the homozygote. Of these macaques, two animals were also hemizygous for the *p.* (*Val241Ile*) mutation in *MBTPS2*. The mRNA expression level of *LDLR* was reduced to 80% by this hemizygous mutation. Under 0.1% CH administration, the two genomic mutations increased plasma LDL-C levels by 100 mg/dL, increasing levels to above the risk level for developing cardiovascular disease.

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CONFLICT OF INTEREST STATEMENT

The authors do not have any conflicts of interest to declare regarding the publication of this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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