

Potential application of proprotein convertase subtilisin/kexin type 9 (PCSK9) gene to clinical settings

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List of abbreviations

ANOVA	Analysis of variance
ApoB	Apolipoprotein B
BMI	Body mass index
CVD	Cardiovascular disease
ELISA	Enzyme-linked immunosorbent assay
FH	Familial hypercholesterolemia
GOF	Gain-of-function
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
ICER	Incremental cost-effectiveness ratio
LAMP	Loop-mediated isothermal amplification
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptor
LLT	Lipid-lowering therapy
LOF	Loss-of-function
MCA	Melting curve analysis
OxPL	Oxidized phospholipid
OxLDL	Oxidized low-density lipoprotein
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/kexin type 9
ROS	Reactive oxygen species
ROX	X-Rhodamine
SNV	Single nucleotide variant
SQP	Short quenching probe
SREBP2	Sterol regulatory element-binding protein 2
TAMRA	Carboxytetramethylrhodamine fluorophore
VLDL	Very low-density lipoprotein
γ GT	Gamma-glutamyl transpeptidase

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Overviews

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a serine protease that belongs to the proprotein convertase family[1,2]. PCSK9 is newly discovered as a causative gene for hypercholesterolemia in genome-wide association studies[3] and is known to be a key molecule in low-density lipoprotein (LDL) regulation. The proprotein convertase family plays a role in protein maturation by digesting proteins[4]. PCSK9 can self-digest its proprotein region, resulting in a mature form of PCSK9 by proprotein-region reassembly. Blood LDL is mainly internalized by the LDL receptor (LDLR) in the liver and endothelial cells[5]. PCSK9 binds to LDLR and accelerates clathrin-mediated degradation by capturing LDLR in lysosomes instead of continuing with the LDLR recycling pathway through endosomes[6] (Figure 1). Under low pH conditions in the endosome or lysosome, the LDLR–PCSK9 bond is stronger than that in the blood[7,8]. This change in binding mode is a switch for accelerating clathrin-mediated degradation. Thus, LDLR density in cells is decreased by PCSK9, resulting in elevated LDL cholesterol (LDL-C) levels in the blood.

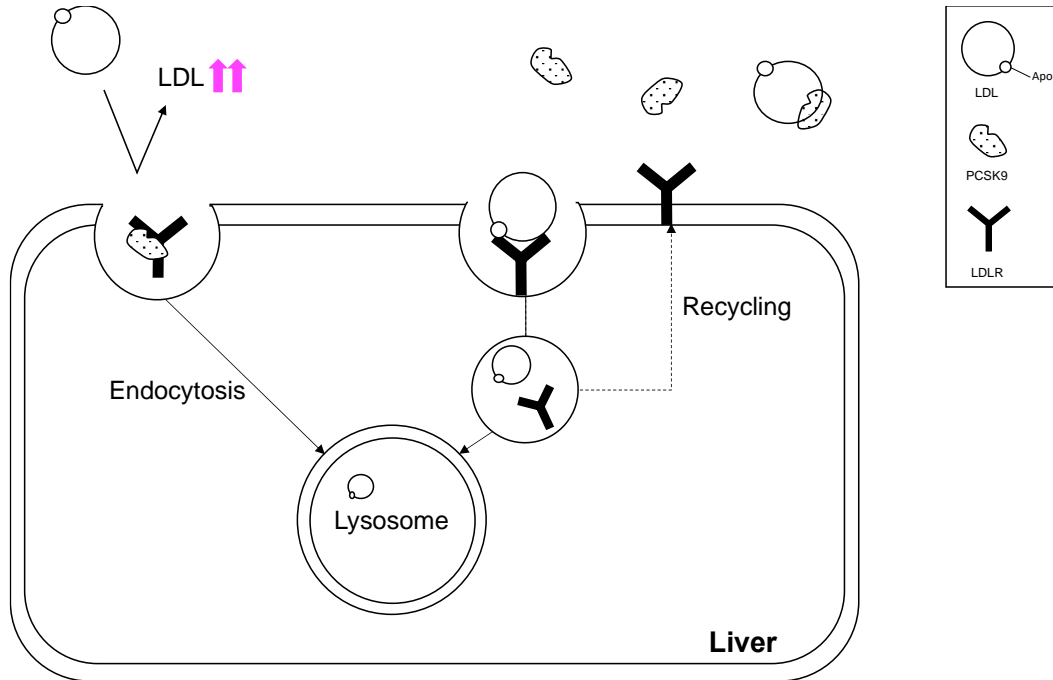


Figure 1. LDL-LDLR metabolic pathway.

LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; ApoB, apolipoprotein B; PCSK9, proprotein circulating convertase subtilisin/kexin type 9.

Familial hypercholesterolemia (FH) is an autosomal dominant genetic disease characterized by high levels of LDL-C in the blood, resulting in an increased risk of cardiovascular disease (CVD)[9], and the most frequent causative gene is *LDLR*[10]. The dysfunction of LDLR is induced by missense variants that are influenced by its various functions (i.e., LDL-bound, membrane-bound, and secretory)[11]. *APOB* is a causative gene that encodes apolipoprotein B (apoB), a component of LDL, which is a recognition component of LDLR, but p.R3527Q, a major causative variant, is found at very low frequency in Asian countries[12,13]. Recently, *PCSK9* was classified as another major causative gene of FH. In Japan, causative variants are comparatively common compared to those in Europe[12–15]. However, the diagnostic rate of FH in Japan is <1%, and an

overlooked FH population has been reported in a retrospective study[16,17].

Genetic variants of *PCSK9* alter its function[18]. These variants were categorized into two types based on their effects on LDL-C levels in the blood (Figure 2). Gain-of-function (GOF) variants accelerate LDLR degradation by increasing its secretion or affinity to LDLR, resulting in high levels of LDL-C in the blood[12,19–21]. In contrast, loss-of-function (LOF) variants weaken LDLR degradation by decreasing its secretion or affinity, resulting in low levels of LDL-C in the blood[22,23]. These variants alter the risk of CVD in relation to LDL-C levels. GOF variants show high levels of LDL-C (<12.9 mmol/L in homozygote untreated patients)[20], indicating a high risk of CVD[3,24]. LOF variants show low levels of LDL-C (<0.4 mmol/L), indicating a low risk for CVD without serious adverse events[25]. Hence, GOF variants are important for defining FH[19]. The most severe variant of *PCSK9* is p.D374Y[20]. p.D374Y shows strong affinity for LDLR because an important binding site is located[26,27]. In addition, the multimerization of PCSK9 is accelerated, leading to an increased LDLR degradation[28]. Another severe variant, p.S127R, also contributes to the high affinity for LDLR[19]. In addition, p.S127R increases apoB synthesis[29]. In Japan, p.E32K is the major causative variant, accounting for approximately 5% of FH cases, and is the most common variant of FH, including *LDLR*, *APOB* and *PCSK9*[12]. p.E32K is located in a positively charged domain that is an important binding site for protein maturation[30,31]. p.E32K shows an elevation in PCSK9 levels in the blood, leading to a moderate elevation of LDL-C (<7.6 mmol/L). Thus, the association between *PCSK9* variants and LDL regulation is well-understood.

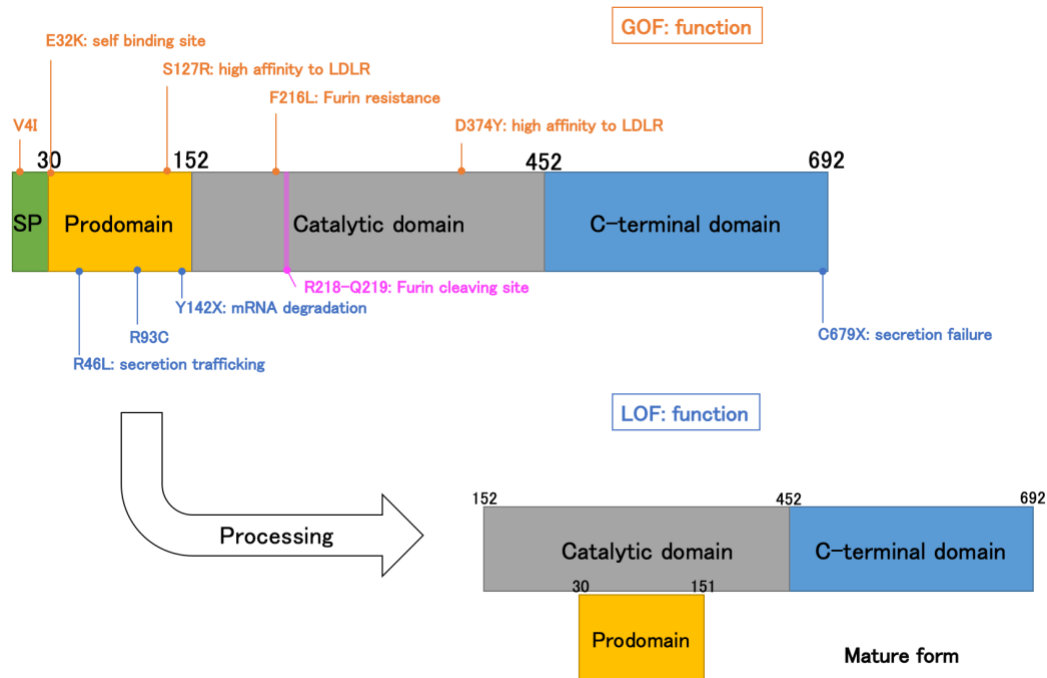


Figure 2. Secondary structure of PCSK9.

PCSK9, proprotein convertase subtilisin/kexin type 9; GOF, gain-of-function; LOF, loss-of-function; LDLR, low-density lipoprotein receptor; SP, signal peptide.

In contrast, the detection of mature and functional PCSK9 in the blood is difficult to define as a clinical marker. While structural forms of PCSK9 molecules in the circulation remain unknown, PCSK9 is mainly assembled with lipoproteins as the mature form. Non-functional PCSK9 is also reported in about 20% of all PCSK9 molecules in blood[32,33]. Mature PCSK9 is rendered non-functional by the protease furin[32]. The prodomain is processed in the blood (Figure 2), and its binding activity to LDLR decreases to <50% than that of mature PCSK9[33]. Furin-cleaved PCSK9 can be detected using a well-established enzyme-linked immunosorbent assay (ELISA). However, no significant difference was noted in circulating furin-cleaved PCSK9 levels between patients with FH and healthy controls[33]. PCSK9 binds to several lipoproteins in the blood[2]. The prodomain possesses

a binding region for apoB; thus, apoB-containing lipoproteins (i.e., chylomicron and very low-density lipoprotein [VLDL]) can bind to PCSK9. Recently, PCSK9 was reported to bind to high-density lipoprotein (HDL) in spite of the absence of apoB[34]. Lipoprotein-binding PCSK9 is considered to have a protective effect on mature PCSK9 owing to the inhibition of furin access[35]. However, the binding effect of lipoproteins to PCSK9 is debatable because of the opposite effect of LDL uptake under *in vivo* and physiological conditions[35,36]. Another obstacle is the lack of measurement assays for mature or functional PCSK9 as a clinical marker. All analyses were conducted using in-house assays or size exclusion chromatography[35,37]. Thus, the clinical significance of mature or functional PCSK9 is unknown and established assays are not ready to be used to resolve this mechanism.

The function of PCSK9 is also important for drug administration. Two approved drugs for lipid-lowering therapy (LLT) that inhibit PCSK9 are antibodies and RNA silencing[38–40]. The PCSK9 antibody was examined first. Large clinical trials have shown a reduction of up to 80% in PCSK9 levels in the blood[39,41]. Long-term administration of the PCSK9 antibody every two weeks maintains this low PCSK9 level with/without other LLT. RNA silencing of PCSK9 resulted in a similar or greater reduction in PCSK9 and LDL-C levels in the blood compared to the PCSK9 antibody[40]. The administration term was set every six months. However, these clinical studies did not consider the effect of genetic variants on PCSK9 reduction. GOF variants usually increase the levels of PCSK9 in the blood[13]; thus, genetic testing for *PCSK9* is important for the use of these drugs.

PCSK9 variants are good clinical markers of LDL-C. However, it remains unknown whether PCSK9 plays different roles in lipoprotein metabolism. Three studies were conducted to identify novel aspects of the GOF variants of *PCSK9* from basic to clinical

diagnosis. In Study I, we investigated the association between oxidative stress and *PCSK9* variants to identify new biological functions. In Study II, the response to LLT was investigated based on the correlation between blood PCSK9 and LDL-C levels in the *PCSK9* variants. In Study III, we developed a rapid genotyping method for *PCSK9* variants in clinical settings.

Study I – Oxidative stress and PCSK9: association between γ GT and *PCSK9* variants

Aims

The increased risk of CVD due to PCSK9 is related to LDL-C level. However, the GOF variants of *PCSK9* show a different low risk for CVD compared to other monogenic variants (i.e., *LDLR* and *APOB*) with similar levels of LDL-C[24,42,43]. High blood levels of PCSK9 were considered an independent risk factor for CVD because of their statistical significance after adjusting for other lipoprotein profiles (total cholesterol, triglycerides, HDL-C, and LDL-C)[44]. These opposite looking results indicate alternative functions of PCSK9 in CVD.

Gamma-glutamyl transpeptidase (γ GT) is a pro-oxidant and a risk marker for CVD[45,46]. High γ GT activity in the blood is associated with an increased risk of CVD[46]. γ GT is expressed on cell membranes when stimulated by the oxidation of DNA, proteins, and lipids[47–49], and its function is to induce the production of glutathione, an antioxidant, in cells[50]. γ GT is typically present in inflamed sites in response to oxidative stress[51].

Both γ GT and PCSK9 are expressed in hepatocytes as markers of CVD risk[45,52]. The association between γ GT, PCSK9, and its variants remains unknown. We aimed to examine the association between γ GT activity and the GOF variants in patients with hyper-LDL-cholesterolemia, an at-risk state for CVD.

Materials and Methods

Study population

This cross-sectional study enrolled 114 patients (mean age: 59 years, 38 males) with hyper-LDL-cholesterolemia (>5.17 mmol/L considered as an at-risk level of high LDL-C levels for CVD)[53]. Patients with moderate-to-severe liver and gallbladder disorders were excluded from this study. This study was approved by the Ethics Review Committee of Jichi Medical University (No. 22-004).

Biochemical analysis

Serum triglyceride, total cholesterol, HDL cholesterol (HDL-C) levels, and γ GT activities were measured using enzymatic methods. LDL-C levels were calculated using the Friedewald equation, because the triglyceride values of all patients were <4.5 mmol/L[54].

Genetic analysis

The major GOF variants of *PCSK9*, p.V4I and p.E32K, were detected by *PCSK9* genotyping using 2 ng of genomic DNA via a StepOnePlus real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA)[55,56]. Briefly, genomic DNA was amplified under isothermal conditions (64°C for 30 min), and variants were detected by quenching a short DNA probe with carboxytetramethylrhodamine fluorophore (TAMRA).

Statistical analysis

Student's t-test, chi-square test, or Fisher's exact test were used to analyze the differences in measured variables between patients with or without the variants. A multivariate-adjusted linear regression analysis was performed to compare the two groups. The triglyceride and γ GT values were log-transformed in the analyses because of their skewed distributions.

Statistical significance was set at $p < 0.05$. All statistical analyses were performed using R statistical package (version 3.3.0) (<https://www.R-project.org/>).

Results

The characteristics of the study participants are summarized in Table 1. A heterozygous p.E32K variant was observed in 12 patients (mean age: 55 years, three males). The frequency of the p.E32K allele was 5.2%. The variant was in Hardy–Weinberg equilibrium. γ GT activity in patients with p.E32K was significantly lower than that in patients without the variant ($p = 0.02$). No differences were noted in the other profiles (i.e., age, sex, alcohol consumption, smoking, statin use, LDL-C, HDL-C, triglycerides, and body mass index [BMI]) between the two variant-based groups. The difference in γ GT activity between the groups remained significant in multivariate analysis ($p = 0.03$).

Table 1. Characterization of population categorized by p.E32K alleles in hyper-LDL-cholesterolemia

Variables	p.E32K+, $n = 12$	p.E32K-, $n = 104$	p value	p value (adjusted)
Age, years	55 \pm 14	59 \pm 11	0.29	0.79
Male, n (%)	3 (25)	35 (34)	0.75	0.57
Alcohol, n (%)	5 (42)	39 (38)	0.76	0.51
Smoking, n (%)	3 (25)	35 (34)	0.75	0.66
Statin use, n (%)	1 (8)	20 (19)	0.69	0.65
BMI	24.4 \pm 3.5	24.3 \pm 3.9	0.88	0.37
Triglycerides, mmol/L	1.31 (1.06-1.45)	1.47 (1.07-2.02)	0.06	0.97
HDL-C, mmol/L	1.68 \pm 0.41	1.44 \pm 0.01	0.08	0.05
LDL-C, mmol/L	5.55 \pm 0.53	5.67 \pm 0.53	0.46	0.50
γ GT, IU/L	21 (16-28)	30 (18-49)	0.02*	0.03*

Mean \pm standard deviation; median (interquartile range); BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; γ GT,

gamma-glutamyl transferase; p.E32K+, patients with the gain-of-function p.E32K allele ; p.E32K-, patients without the gain-of-function p.E32K allele. * Significance level: $p < 0.05$.

Discussion

The GOF variant of *PCSK9*, p.E32K, is associated with the low activity of γ GT with hyper-LDL-cholesterolemia. Although, in general, γ GT is elevated by bile duct obstruction, alcohol or drugs[45], γ GT is considered an oxidative stress marker because of a necessary molecules for a generation of glutathione, an antioxidative molecules[50]. Thus, we focused on aspects of oxidative stress in this study. Both γ GT activity and *PCSK9* variants are associated with a risk of CVD[51,57,58]. This finding suggests the existence of a pathway that regulates oxidative stress via the γ GT–PCSK9 linkage (Figure 3).

Although the exact reason for this finding remains unknown, we can offer possible explanations. First, excessive LDL uptake in the liver leads to oxidative stress in hepatocytes, which promotes inflammation and oxidation (Figure 3, left panel)[59,60]. PCSK9 can reduce the oxidative stress burden by degrading LDLR in the liver, as LDL uptake which lead to an increase of oxidative stress does not occur during LDLR degradation. Because γ GT is released into the blood if oxidative stress occurs in hepatocytes, decreased LDL uptake by the GOF variant of *PCSK9* may produce lower γ GT activity in the blood than the non-GOF variant. Second, PCSK9 binds to other lipoprotein receptors[61]. Excessive reactive oxygen species (ROS) (e.g., oxidative phospholipids [oxPL] or triglycerides) have been reported to accumulate in PCSK9-null endothelial cells upon PCSK9 knockout via the scavenger receptor, CD36 (Figure 3, right panel)[60,62,63]. This oxidative stress induces macrophage cell formation, which is the first step in atherosclerotic

plaque development[64]. As the GOF variant of *PCSK9* enhances PCSK9 function, fully functionalized PCSK9 can suppress the accumulation of oxidative stress, where less oxidative stress may lower γ GT activity in the blood. The mechanisms underlying these findings need to be clarified in future studies. In particular, CD36 does not possess the major binding motif (i.e., EGF-like domains) to PCSK9 contrary to other lipoprotein receptors, and we can classify the function of the LDL receptor for PCSK9[60,61].

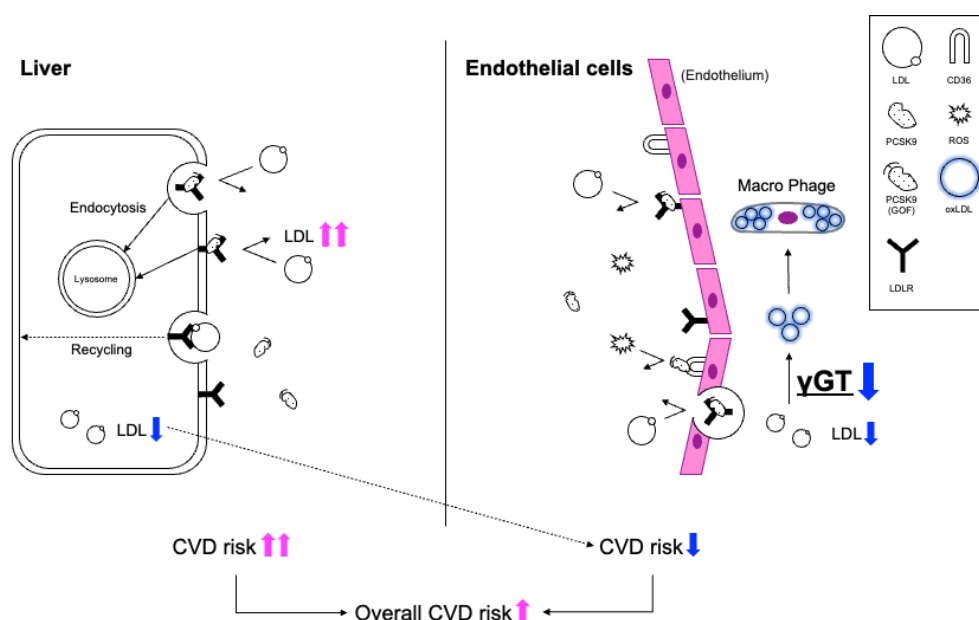


Figure 3. Different role for PCSK9 by site-specific location.

In the liver, PCSK9 shows high levels of LDL-C in the blood by inhibiting LDL uptake, resulting in an increased risk of CVD. In contrast, in endothelial cells in the early onset of plaque, PCSK9 works similarly to LDL uptake inhibition, but the effect may be opposite to the risk of CVD by inhibiting the supply of oxidative stress. γ GT, expressed by the elevation of oxidative stress, was also decreased by the inhibition of LDLR and CD36, a scavenger receptor, by PCSK9. The reduction in oxidative stress may also be observed in the liver

(dotted arrow). LDL, low-density lipoprotein; PCSK9, proprotein circulating convertase subtilisin/kexin type 9; GOF, gain-of-function; LDLR, low-density lipoprotein receptor; ROS, reactive oxygen species; oxLDL, oxidized low-density lipoprotein; CVD, cardiovascular disease; γ GT, gamma-glutamyl transpeptidase.

This inverse association may explain previous epidemiological observations. GOF variants of *PCSK9*, including p.E32K, have been shown to have a comparatively low risk for CVD, as compared with other genes (i.e., *LDLR* and *APOB*) causing hypercholesterolemia[43]. This observation may be explained by the idea that the GOF variants of *PCSK9* potentially reduce oxidative stress, as expressed by γ GT, more than other genes for increasing LDL, which can reduce the risk of CVD.

The detection of *PCSK9* variants could help to stratify the association between PCSK9 and CVD. PCSK9 is present in inflamed arterial sites[52]. In a mouse model, PCSK9 deficiency induced heart failure with a reduced ejection fraction[65]. Low levels of arterial PCSK9 contribute to a low risk of abdominal aortic aneurysm[66]. Therefore, it is important to identify arterial PCSK9 as a CVD marker. However, blood PCSK9 levels do not reflect arterial PCSK9 levels[65]. Arterial PCSK9, not blood PCSK9, is important for detecting CVD. Thus, the detection of *PCSK9* variants may be a good indicator to understand the mechanism of its localized PCSK9 function in CVD.

This study had several limitations. First, the sample size was relatively small particularly that of patients with GOF variants of *PCSK9*. Larger-number studies (e.g., genome-wide association studies), which include multi-confounders, are needed for sophisticating the evidence. Second, although there are several GOF variants of *PCSK9*,

p.E32K was selected as the major variant that is frequently found in the general population.

Third, we focused on only γ GT as a oxidative stress marker, but other oxidative stress markers (e.g., protein modification or lipid peroxide) need to be examined. These issues should be addressed in future research.

In conclusion, low γ GT activity was observed in patients with the GOF variant of *PCSK9* among those with hyper-LDL-cholesterolemia. Elucidating the mechanism of their association with oxidative stress-related pathways may help understand the development of CVD by *PCSK9* variants.

Study II – PCSK9 and LDL-C: influence of *PCSK9* variants in LDL-C under LLT

Aims

Statins, also known as hydroxymethylglutaryl-CoA reductase inhibitors, are the first-choice drugs for LLT in hypercholesterolemia[67,68]. Statins inhibit cholesterol biosynthesis in cells, resulting in a lack of intra-and extracellular cholesterol[69]. This physiological change stimulates LDLR expression via sterol regulatory element-binding protein 2 (SREBP2) to recover cholesterol in cells[70,71]. SREBP2 also binds to the SRE motif located in *PCSK9*. Both *PCSK9* and LDLR expression were upregulated. Patients administered statins showed an up to two-fold increase in *PCSK9* compared to before administration[72]. Under this *PCSK9* elevated condition, LDLR was still present on the cell surface[73]. GOF *PCSK9* showed up to ten-fold higher affinity, leading to faster degradation of LDLR[27]. This feature of GOF *PCSK9* may provide new insights into the response to statins compared to other hypercholesterolemias.

Materials and Methods

Study population

This cross-sectional study enrolled 70 patients (mean age: 53 years, 44 males) with FH who had undergone LLT. The patients were treated with statins at the maximum dose (40 mg/day atorvastatin, 4 mg/day pitavastatin, and 20 mg/day rosuvastatin) depending on the situation of the respective patients. Patients who received statins and Niemann–Pick C1–like 1 inhibitor (10 mg/day ezetimibe) or colestimide (4 mg/day) at the maximum dose were included. FH was defined based on clinical diagnostic criteria[16,67]. Patients with hepatic

and renal dysfunctions were excluded from the study. This study was approved by the Ethics Review Committee of Jichi Medical University (Nos. 22–003 and 22–007).

Biochemical analysis

The serum total cholesterol and HDL-C levels were measured using enzymatic methods. Serum triglyceride levels were measured using the total glycerol and enzymatic methods[74,75]. Serum LDL-C levels were calculated using the Friedewald equation (in the present study, the triglyceride levels of all patients were <4.5 mmol/L)[54]. Serum PCSK9 levels were measured using a PCSK9 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA).

Genetic analysis

PCSK9, *LDLR*, and *APOB* variants were examined by next-generation sequencing (NGS) with 50 ng of genomic DNA using NextSeq 500 (Illumina, San Diego, CA, USA). Genomic DNA was extracted from 200 µL of whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Library preparation was performed using TruSight One Sequencing Panel (Illumina). Sequencing was performed using a NextSeq 500/550 Mid Output Kit, 300 Cycles (Illumina). Variant data were obtained using ANNOVAR[76], and the analyzed variants of amino acid substitutions or splice regions were interpreted using the ClinVar database[77].

Statistical analysis

One-way analysis of variance (ANOVA) and Fisher's exact test were used to analyze the

differences in the measured variables among the groups. Pearson's correlation test was used to analyze the correlation between PCSK9 and LDL-C levels in each group. Comparison tests using Fisher's z-transformation were performed for obtaining correlation coefficients between the groups with *PCSK9* variant, *LDLR* variant, and variant-negative patients. Triglyceride values were log-transformed in the analyses because of their skewed distributions. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using R statistical package (version 3.3.0; <https://www.R-project.org/>).

Results

The characteristics of the study participants are summarized in Table 2. Two heterozygous GOF variants of *PCSK9*, p.V4I ($n = 1$) and p.E32K ($n = 6$), were observed in seven patients (mean age: 55 years, one male). These two variants have been reported to be common and have been reported to induce high LDL-C levels[12,21,24,78]. Heterozygous variants of *LDLR* were observed in 17 patients (mean age: 43 years, 13 males). Variants of *APOB* were not observed in the present study, and patients without these monogenic variants were defined as the variant-negative group (mean age, 56 years; 30 males). As shown in Table 2, the PCSK9 and LDL-C levels were similar between the groups. LDL-C levels did not reach the control levels (i.e., 2.59 mmol/L) from the Japanese Atherosclerosis Society[67]. HDL-C levels tended to be low and triglyceride levels tended to be high in patients with *PCSK9* variants, but the levels were not significantly different among the groups. The two variants, p.V4I and p.E32K, showed similar LDL-C (mean: 4.28 and 4.01 mmol/L, respectively) and PCSK9 levels (mean: 411 and 330 ng/mL, respectively). The prevalence of drugs used in LLT was not significantly different between groups. As shown in Figure 4, the PCSK9 and

LDL-C levels in patients with *PCSK9* variants were significantly positively correlated ($r = 0.79$, $p = 0.04$). In contrast, *PCSK9* and LDL-C levels were significantly negatively correlated in variant-negative patients ($r = -0.37$, $p = 0.01$) and insignificantly but negatively correlated in patients with *LDLR* variants ($r = -0.39$, $p = 0.12$). In addition, the correlation coefficient was significantly different between patients with *PCSK9* and *LDLR* variants ($p = 0.01$) as well as between patients with *PCSK9* variants and variant-negative patients ($p < 0.01$).

Table 2. Characterization of population that underwent LLT categorized by genetic variants

Variables	<i>PCSK9</i> +, $n = 7$	<i>LDLR</i> +, $n = 17$	Variant-negative, $n = 46$	p value
T-Chol, mmol/L	6.14 ± 0.93	6.60 ± 1.51	5.94 ± 1.17	0.17
Triglycerides, mmol/L	2.15 (2.04–2.24)	1.15 (0.86–1.51)	1.30 (1.01–1.68)	0.08
HDL-C, mmol/L	1.33 ± 0.34	1.50 ± 0.31	1.74 ± 0.53	0.05
LDL-C, mmol/L	4.06 ± 0.78	4.13 ± 1.16	3.55 ± 0.97	0.10
<i>PCSK9</i> , ng/mL	342 ± 150	385 ± 126	330 ± 84	0.17
Lipid-lowering therapy				0.08
Statins, n (%)	6 (86%)	15 (88%)	45 (98%)	-
Coestimide, n (%)	1 (14%)	0 (0%)	0 (0%)	-
Statins + ezetimibe, n (%)	0 (0%)	2 (12%)	1 (2%)	-

Mean \pm standard deviation or median (interquartile range). Triglyceride values are displayed as medians (interquartile range) because of skewed distribution. LLT, lipid-lowering therapy; T-Chol, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; *LDLR*, low-density lipoprotein receptor; *PCSK9*, proprotein circulating convertase subtilisin/kexin type 9; *PCSK9*+, patients with *PCSK9* variants; *LDLR*+, patients with *LDLR* variants. Significance level: $p < 0.05$.

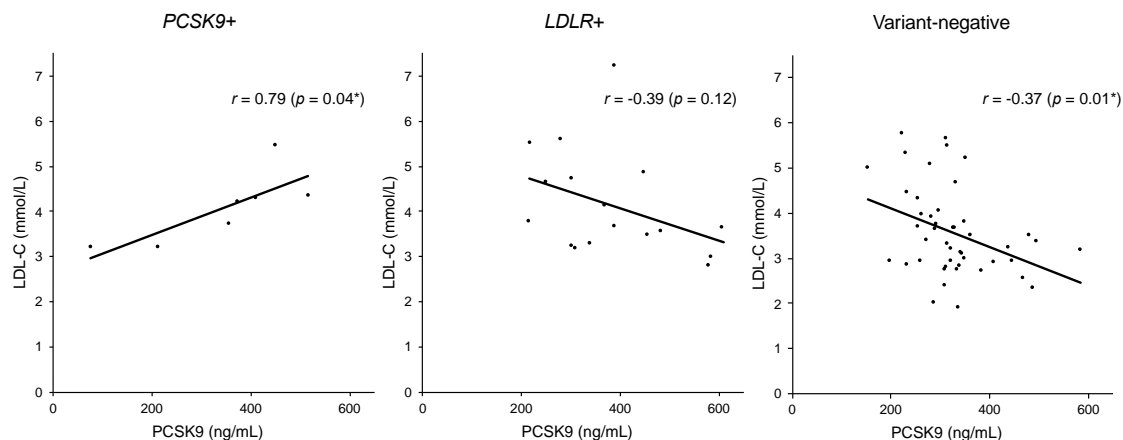


Figure 4. Correlations between PCSK9 and LDL-C levels by gene variant.

LDL-C, low-density lipoprotein cholesterol; PCSK9, proprotein circulating convertase subtilisin/kexin type 9; LDLR, low-density lipoprotein receptor; *PCSK9+*, patients with *PCSK9* variants; *LDLR+*, patients with *LDLR* variants; r values and p -values analyzed by Pearson's correlation tests. Significance level: (correlation coefficient) $p < 0.05$.

Discussion

In Study II, a significant positive correlation was observed between PCSK9 and LDL-C levels in patients with FH who underwent LLT. In contrast, no positive correlation was observed in patients with *LDLR* variants or with variant-negative patients. This is the first study to demonstrate that the correlation between PCSK9 and LDL-C levels could be modulated by *PCSK9* variants “under LLT” (Figure 5). This implies *PCSK9* variants may be considered as genetic markers for assessing the response of LLT to FH.

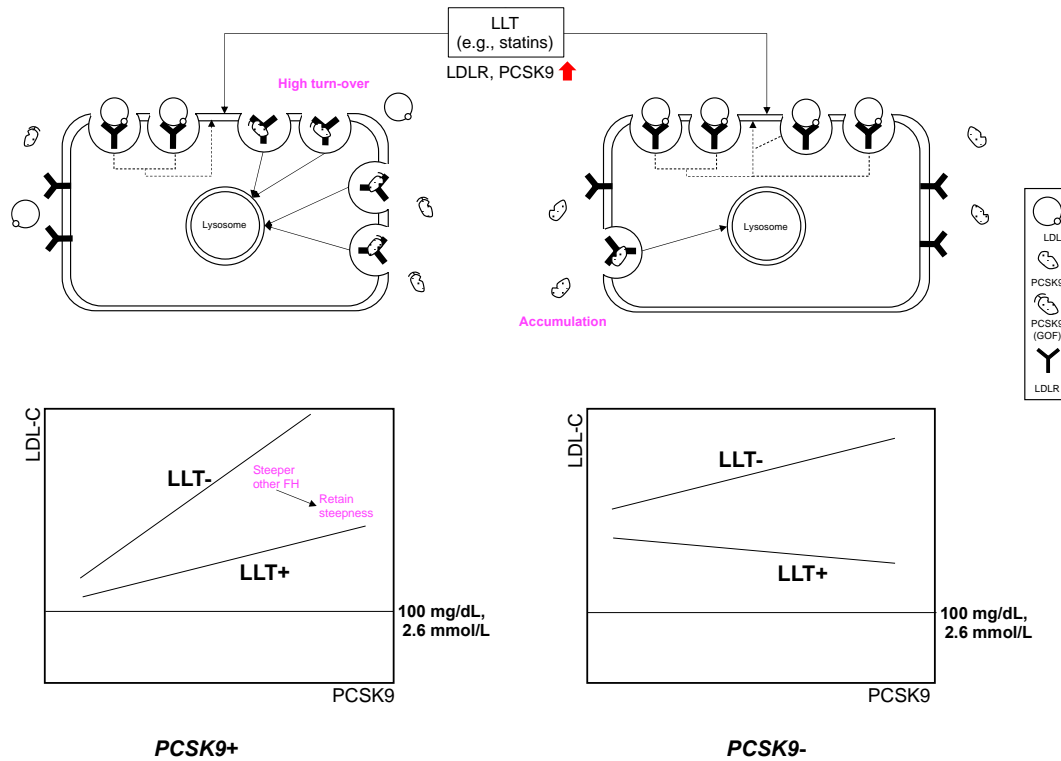


Figure 5. Different feedback by cholesterol depletion in patients with *PCSK9* variants.

Under LLT, SREBP2 feeds back LDLR over-expression upon the lack of cholesterol in the liver, resulting in an increase of LDLR. Simultaneously, blood PCSK9 is elevated by SREBP2; thus, an inverse correlation with levels of LDL-C in the blood may be observed against blood PCSK9 in the group without the GOF variants of *PCSK9* (lower right panel).

In contrast, in patients with GOF variants of *PCSK9*, GOF PCSK9 enhances the turnover of LDLR degradation; thus, the regulation between PCSK9 and LDL-C in the blood is stronger than that without GOF variants of *PCSK9*, leading to a steep regression curve without LLT.

Under LLT, the steepness of the curve is lesser than that without LLT. However, the positive correlation persists (lower left panel) even after LDLR and PCSK9 overexpression.

LLT, lipid-lowering therapy; SREBP2, sterol regulatory element-binding protein 2; LDL, low-density lipoprotein; PCSK9, proprotein convertase subtilisin/kexin type 9;

GOF, gain-of-function; LDLR, low-density lipoprotein receptor; LLT+, patients under LLT; LLT-, patients without LLT; *PCSK9*+, patients with GOF variants of *PCSK9*. *PCSK9*-, patients without GOF variants of *PCSK9*.

A mild negative correlation was observed between PCSK9 and LDL-C levels in patients without the *PCSK9* variants. Although a mild negative correlation was similarly observed in previous studies on patients with hypercholesterolemia who underwent LLT, *PCSK9* variants were not examined in these studies[79–81]. Some possible explanations for these mild correlations were considered. In balanced condition without LLT, the mild positive correlation was observed between PCSK9 and LDL-C levels[82]. LLT, such as statin therapies, induce upregulation of *LDLR* expression for the internalization of LDL-C to be used in cells by SREBP2[83]. Simultaneously, PCSK9 expression was upregulated to regulate excess LDL uptake via LDLR[73]. As a result, statins reduced LDL-C levels and increased PCSK9 levels in the opposite direction in the blood[18,72,84]; LLT weakened the positive correlation observed in individuals “without LLT.” This mild negative correlation is also thought to be due to the different responses to LLT among individuals[79,80].

Furthermore, adopting various lifestyle modifications, including diet and exercise, as an additional treatment for hypercholesterolemia may partly weaken this correlation in patients who undergo LLT[85–87]. The notable finding of the present study is that the positive correlation between PCSK9 and LDL-C levels in FH patients with *PCSK9* variants was retained even underwent LLT, similar to their positive correlation in “without LLT”[82,88]. *PCSK9* variants observed in the present study were of the GOF type[12,21,78], which promotes the rapid turnover and binding prevalence of LDLR (the turnover accelerates the

degradation of LDLR by its high affinity with LDLR) compared with non-*PCSK9* variants[8,27,29], resulting in a failure of LDL uptake in hepatocytes. Specifically, this process which is a regulation of LDL-C by PCSK9 can retain even under LLT. In the present study setting, the positive correlation between PCSK9 and LDL-C levels during LLT may only appear in patients with *PCSK9* variants.

A positive correlation between PCSK9 and LDL-C levels is a condition of CVD risk[12,88], and again, in the present study, such a correlation was seen in patients with *PCSK9* variants even “underwent LLT.” p.E32K has been reported to have a strong effect on this correlation because the regression curve between PCSK9 and LDL-C was observed to be steeper than that of the controls without LLT[12]. In estimating a slope value, LDL regulation still remains under LLT at the same level as the healthy controls or patients with *LDLR* variants without LLT[12,82]. p.E32K is located at the processing region and related to the maturation of PCSK9 to obtain adequate binding for LDL degradation[30]. Although the function of p.V4I, which is located at the signal peptide region, was not clearly defined, p.V4I is located in the processing region as well as p.E32K[21,78]. The possibility of having the same function as that of p.E32K was considered. PCSK9 inhibitors (i.e., antibody or RNA silencing) can be used as candidate drugs for severe hypercholesterolemia and show good long-term reduction of LDL-C[38,40]. However, the PCSK9 inhibitors are more expensive than other drugs[89]. In this study, GOF PCSK9 still controlled LDL-C levels under LLT. Thus, the addition of PCSK9 inhibitors to the first choice drug, statins, is a suitable treatment, particularly for *PCSK9* variants.

The present study had some limitations. First, the sample size was relatively small, particularly in the GOF variants of *PCSK9*. We will plan to analyze larger number of

samples (e.g., using genome-wide association study). Second, the cross-sectional study may not have fully determined causality. Third, although lifestyle factors are known to modify PCSK9 and LDL-C levels[85–87], they were not examined in the present study. Further detailed analyses are required to corroborate the results of this study.

In the present study, blood PCSK9 and LDL-C levels were positively correlated in patients with GOF variants of *PCSK9* that underwent LLT. LDL-C-PCSK9 correlation is a potential CVD condition, and variant detection may be useful for additional treatment with statins, such as PCSK9 inhibitors, in patients with *PCSK9* variants.

Study III – Novel genotyping of *PCSK9* variants: new detection systems

Aims

In the two previous studies, the clinical advantage of the detection of the GOF variant of *PCSK9* was demonstrated. A new genetic test method is needed to establish its suitability for clinical settings. The new method uses the LAMP reaction as the backbone of the method and a short quenching probe (SQP) for variant detection without the use of an exclusive thermal cycler.

Melting curve analysis (MCA) is a major detection method for single nucleotide variants (SNV) in clinical settings where speed is needed[90,91]. Although SQP are suitable for SNV detection owing to a significant decay of binding energy compared with a perfectly matched allele[92,93], SQP cannot use MCA because a complementary strand competes to bind a target sequence[94]. Therefore, SQP cannot be used in conventional polymerase chain reaction (PCR)-based methods for SNV detection.

The LAMP method can overcome this problem owing to its two unique features (Figure 6). First, the LAMP amplicon possesses free single-stranded regions as loop structures[55,95]. These loop structures allow the binding of SQP without conflict with a complementary strand. Second, self-amplification produces a large number of amplicons that is 10-times higher than that produced by PCR[55]; thus, SQP can detect a target region via the loop structure with several-fold molecules. However, no remarkable method for SQP detection has yet been established.

In this study, we established genotyping for *PCSK9* using the LAMP method and SQP. Next, the newly established genotyping, SQP genotyping, was validated in clinical

samples from FH patients and compared with conventional genetic analyses. Finally, SQP genotyping was optimized in clinical settings to shorten turnaround time and procedures.

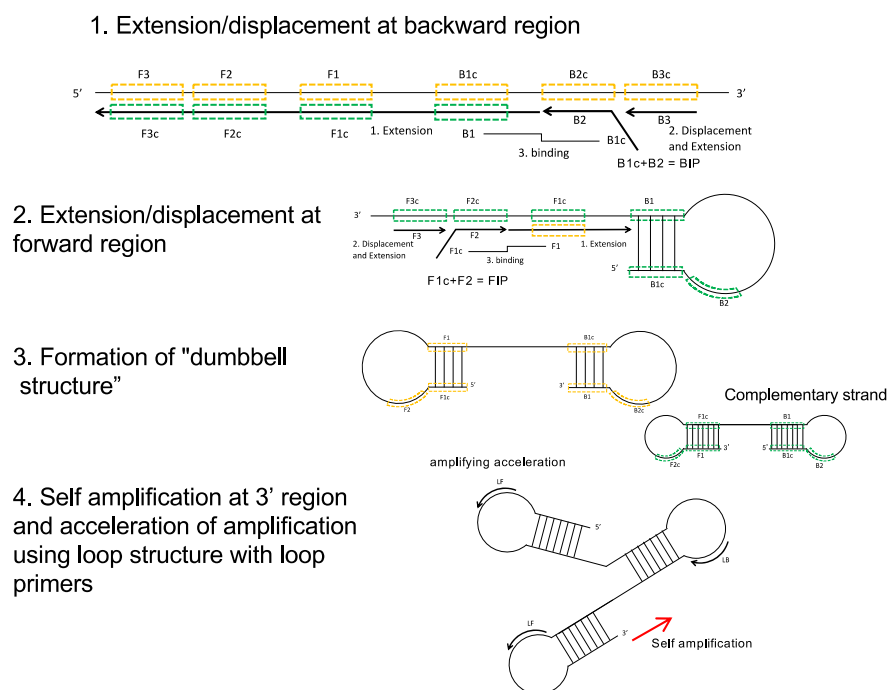


Figure 6. Schematic diagram of the LAMP reaction.

1. The primer BIP (B1c + B2) binds to the B2c site and extends the complementary strand. After extension, primer B3 also extended a complementary strand from the external B3c site, while displacing the BIP-extended complementary strand. 2. Using the amplicon from step 1 as a template, the FIP primer (F1c + F2) binds to the F2c site in a similar fashion and extends a complementary strand, while primer F3 also extends a complementary strand that displaces the FIP-extended complementary strand. 3. A “dumbbell structure” is formed from the amplicon of step 2. 4. Using this “dumbbell structure” as a minimum unit, self-amplification occurs and amplicons of various lengths (<50 kb) are synthesized. The primers LF and LB targeted the loop structures to accelerate the reactions. LAMP, loop-mediated

amplification.

Materials and Methods

Development of a genotyping assay

To design this method, the two most common *PCSK9* variants p.V4I and p.E32K[12,21] were selected. We first designed the LAMP primers F3, FIP, B3, BIP, LF, and LB. Four SQPs were designed: variant and wild-type for p.V4I and p.E32K labeled TAMRA. LAMP primers were designed to locate the SQP at the loop regions[95]. To reduce nonspecific detection, a blocking oligo was added for p.V4I detection[96]. All oligonucleotides were designed using NCBI RefSeq (NG_009061.1)[97]. The concentrations and sequences of the primers, SQP, and blocking oligos are shown in Table 3, and the locations of the SQPs are shown in Figure 7 and 8.

Table 3. List of oligonucleotides used for SQP genotyping

p.V4I, primer	Sequence (5'- 3')	Conc. used in LAMP (mmol/L)	Conc. used in PCR (mmol/L)	p.E32, primer	Sequence (5'- 3')	Conc. used in LAMP (mmol/L)	Conc. used in PCR (mmol/L)
F3 V4I	CAGTCCTC CCCACCG C	0.02	0.04	F3 E32K	GGCCTCT AGGTCT CCTCG	0.02	0.04
B3 V4I	TCCTCCTC GGAACGC AAG	0.02	0.04	B3 E32K	CTCGGA ACGCAA GGCTAG	0.02	0.04
FIP V4I	CGGCCAC CAGGACC GCGCCTCT AGGTCTCC TCGC	0.16	-	FIP E32K	CCACCA GGACCG CCTGGA CCAGGA CAGCAA CCTCTCC	0.16	-
BIP V4I	TGCCACTG CTGCTGCT GCCTAGC ACCAGCT CCTCGTA	0.16	-	BIP E32K	GCTGCC ACTGCT GCTGCTC CAGCTC CTCGTA	0.16	-

					GTCGC		
LF V4I	GGCCAGG	0.08	-	LF	CTGACG	0.08	-
	GGAGAGG			E32K	GTGCCC		
	TTG				ATGAG		
LB V4I	GTGCGCA	0.08	-	LB	GCTGCT	0.08	-
	GGAGGAC			E32K	GCTGCTC		
	GA				CT		
SQP for wild-type V4I	TGGAGCT	0.04	0.04	SQP for wild-type	CCTCGTC	0.04	0.04
	GAC-[TAMRA]			E32K	CTC-[TAMRA]		
SQP for variant V4I	GGCACCA	0.04	0.04	SQP for variant	CTCGTCC	0.04	0.04
	TC-[TAMRA]			E32K	TTC-[TAMRA]		
BO for wild-type V4I	TGGAGCT	2	2	-	-	-	-
	GAT						
BO for variant V4I	GGCACCG	0.4	0.4	-	-	-	-
	TC						

LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction; Conc., concentration; LF, forward loop; LB, backward loop; SQP, short quenching probe; BO, blocking oligonucleotide.

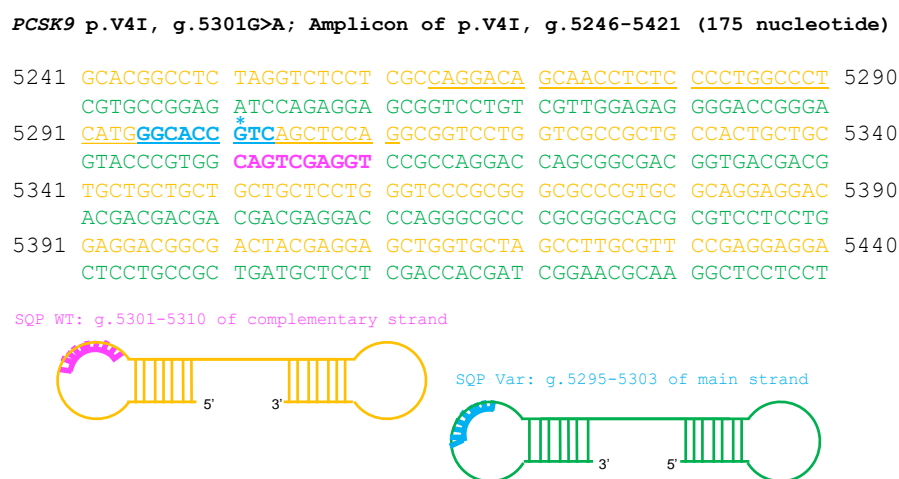


Figure 7. Schematic diagram of the loop region to detect p.V4I alleles.

Upper panel: orange, sense strand of RefSeq (NG_009061.1) from g.5241 to g.5440; green, complementary strand; magenta (bold), SQP site to detect wild-type (g.5301 to g.5310); blue

(bold), SQP site to detect variants (g.5295 to g.5303); *, SNV; underline, amplicon loop region to detect SNV. Lower panel: Scheme of each LAMP amplicon loop region used to detect SNV. Magenta (bold), the SQP site used to detect the wild-type. Blue (bold), SQP site to detect variants. LAMP, loop-mediated amplification; PCSK9, proprotein convertase subtilisin/kexin type 9; SQP, short quenching probe; SNV, single nucleotide variant.



Figure 8. Schematic diagram of the loop region to detect p.E32K alleles.

Upper panel: orange, sense strand of RefSeq (NG_009061.1) from g.5241 to g.5440; green, complementary strand; violet (bold), SQP site to detect wild-type (g.5385 to g.5394); red (bold), SQP site to detect variants (g.5384 to g.5393); *, SNV; underline, amplicon loop region to detect SNV. Lower panel: Scheme for each LAMP amplicon loop region. Violet (bold), SQP site to detect wild-type; red (bold), SQP site to detect variants. LAMP, loop-mediated amplification; PCSK9, proprotein convertase subtilisin/kexin type 9; SQP, short quenching probe; SNV, single nucleotide variant.

After heating the template DNA (plasmid or genomic DNA) at 95 °C for 5 min, a mixture of all the reaction components was added to the LAMP reactions. Each reaction mixture (25 µL) contained 20 units of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA, USA), F3, B3, FIP, BIP, LF, LB, SQP, or blocking oligos (see Table 3 for the final concentrations used in the reaction), 1 × X-Rhodamine (ROX) reference dye (Takara Bio, Shiga, Japan), 1.2 fg of plasmid DNA or 2 ng of genomic DNA, and an optimized buffer (1.4 mmol/L of each deoxynucleoside triphosphate, 0.5% of Tween 20, 8 mmol/L of MgSO₄, 30 mmol/L of KCl, 20 mmol/L of Tricine, pH 8.6), as previously described[98]. LAMP reactions were performed using the StepOnePlus real-time PCR system (Thermo Fisher Scientific) at 64 °C for 30 min. MCA was conducted between 25 °C-75 °C at a ramp speed of +0.15 °C/s. Quenching (i.e., SNV detection) was detected every second using a universal filter installed in the StepOnePlus Real-Time PCR system (excitation/emission = 535 nm/585 nm). SQP genotyping was validated using positive and no-template controls. Plasmid DNA with the targeting sequences for p.V4I and p.E32K (g.5172 to g.5462) cloned into the pTAKN-2 vector (Eurofins Genomics, Tokyo, Japan) was used as the positive control for the variant alleles. The genomic DNA of wild-type alleles, confirmed using NGS, was used as a positive control. Distilled water was added instead of the DNA samples in the no-template controls.

Comparison of amplification method

To compare the sensitivity of LAMP with that of conventional PCR, the same amount of template was also amplified in PCR reactions using the PrimeSTAR HS Premix (Takara

Bio) and oligonucleotide mixtures for PCR at the concentrations specified in Table 3, along with 1× ROX reference dye and SQP. The thermal cycling conditions for PCR were as follows: 98 °C hot start for 30 s, followed by 40 cycles at 95 °C for 5 s and 68 °C for 1 min. MCA was conducted under the same conditions as SQP genotyping with the LAMP reaction.

The data from both amplifications were normalized to the ROX reference dye and the derivative value (i.e., delta fluorescence per delta temperature: dF/dT) was calculated using normalized fluorescence data. Detection thresholds were defined from each SQP mean \pm 3 standard deviations in non-detectable homozygotes. Homozygous wild-type or variant alleles were defined when SQP was detected for either the wild-type or variant allele, and heterozygous alleles were defined when both SQP were detected.

Sensitivity and quantification of SQP genotyping

The limits of sensitivity and quantification for each SQP genotyping were determined using a mixed template with variant and wild-type alleles. A total of 10^4 copies/reaction of plasmid or genomic DNA (equal to 33 ng/reaction of genomic DNA) were used as the background calibrator. The amount of template added was between 10^0 and 10^4 copies/reaction (0.01%–100% mutation rate). A mutation rate of 100% was not included in the background calibrators. SQP genotyping was performed as previously described for the LAMP reaction.

Validation of the assay for patient samples

This study enrolled unrelated 36 patients with FH (mean age: 50 years, 16 males) based on the Japanese FH criteria[16,67]. The study protocol was approved by the Ethics Review

Committee of Jichi Medical University (No. 22-003). The serum lipid levels were measured using enzymatic methods[99]. Serum PCSK9 levels were measured using a commercial ELISA kit (R&D Systems). All data were obtained from the patients who underwent LLT. Student's t-test and the chi-square or Fisher's exact test were used to analyze the differences in measured variables between patients with and without *PCSK9* variants. Total genomic DNA was extracted from the blood using the QIAamp DNA Mini Kit (Qiagen), following the manufacturer's instructions. Target variant sequencing was performed using the genomic DNA from the NextSeq 500 sequencing system (Illumina). Library preparation was performed using TruSight One sequencing panel (Illumina). Sequencing was performed using 2×150 bp paired-end chemistry. Enrichment was performed using the BWA Enrichment software (version 2.1.0) powered by BaseSpace Onsite (Illumina). Annotation was performed using wANNOVAR[76], and non-synonymous variants were identified in $\leq 1\%$ of the East Asian population in the Exome Aggregation Consortium[100]. The variants were defined using the ClinVar database[77] and from previous reports. Using NGS, two heterozygous variants, p.V4I and p.E32K, were identified in seven patients. Two of the seven patients were double-heterozygous (patient 1, *PCSK9* p.V4I and the *LDLR* p. C160R [NG_009060.1 from RefSeq]; patient 2, *PCSK9* p.E32K and *LDLR* p.K811X). Genomic DNA samples from all patients were analyzed using SQP genotyping for comparison with NGS.

Adaptation to clinical use

To adapt the high-throughput screening assay, MCA, a complex procedure, must be removed. Generally, MCA requires precise temperature control[101], which limits the use of

exclusive devices instead of a high-resolution definition of melting temperature. This is an obstacle preventing rapid detection by genetic testing[102,103]; thus, we performed an endpoint assay. After the LAMP reaction, the amplicons were heated at 95 °C for 15 s at a ramp rate of 3.0 °C/s and cooled to 25 °C to facilitate the binding of SQPs to the loop. The turnaround time for this step was 15 min. Binding was then analyzed and relative fluorescence was calculated using the no-template control. To validate the endpoint assay, the same patient samples were used for MCA.

Results

Assay development

SQP genotyping results comparing the two different amplification methods, LAMP vs. PCR, are shown in Figure 9. Using LAMP, two variants, p.V4I and p.E32K, were clearly distinguished. However, these variants were not detected using PCR. The melting temperatures of the SQPs were less than 40 °C, which was more than 40 °C lower than the melting temperature of the amplicons obtained by the LAMP reaction (data not shown). The turnaround time of the whole procedure was approximately 3 h, including genomic DNA extraction (<1 h) and SQP genotyping (30 min for LAMP reaction and 2 h for MCA).

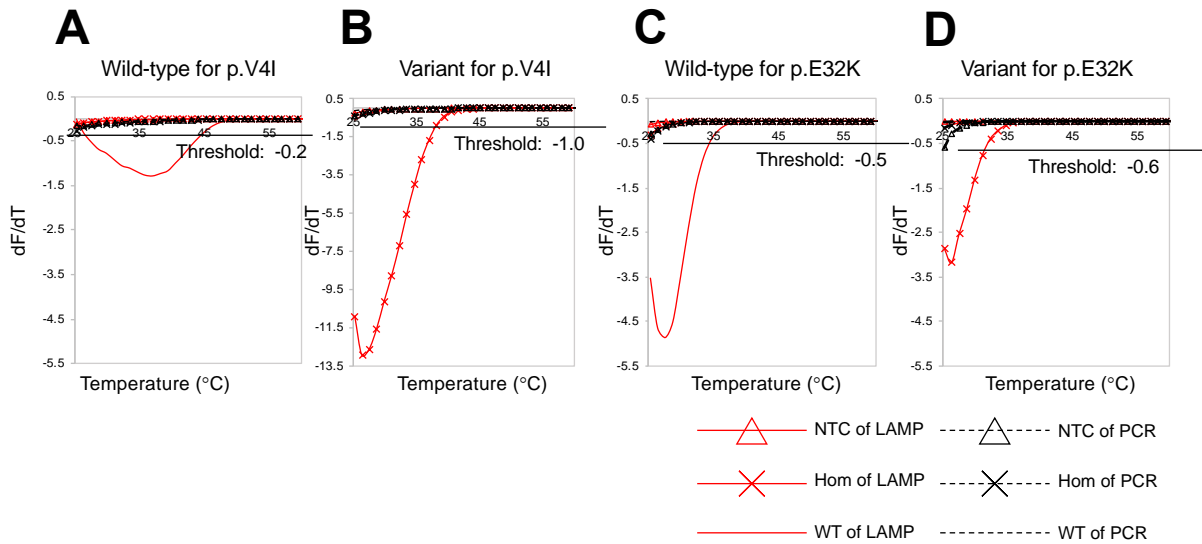


Figure 9. SQP genotyping for *PCSK9* variants using different amplification methods.

Detection of the (A) wild-type p.V4I allele, (B) variant p.V4I allele, (C) wild-type p.E32K allele, and (D) variant p.E32K allele. LAMP, loop-mediated amplification; PCR, polymerase chain reaction; NTC, no-template control; Hom, positive control for homozygous variant alleles; WT, positive control for wild-type alleles; SQP, short quenching probe; black line, detection threshold.

The limits of sensitivity and quantification are shown in Figure 10A–10D. The limits were different for each allele (Table 4). Wild-type detection of the p.E32K allele was detected and quantified at 0.01%, which was the ideal result. Picking up the wild-type detection of the p.V4I allele, we conducted further analyses to determine the importance of loop structures. The LAMP amplicon for p.E32K was used instead of the LAMP amplicon for the p.V4I allele, whose end position of the loop structure was four nucleotides slid forward (Figure 10E, lower panel). The limit of sensitivity was 0.01% for the model (wild-type detection of p.V4I). This sensitivity significantly decreased when SQPs were not

designed from the loop structure of the LAMP amplicon until the detection was diminished (Figure 10E, upper panel). The combined data highlights the advantages of using the LAMP–SQP method for genotyping.

Table 4. Limit of sensitivity and quantification of SNV using SQP genotyping

Variable	p.V4I WT	p.V4I variant	p.E32K WT	p.E32K variant
Sensitivity, %	0.01	0.01	0.01	>10
LOQ, %	1	>10	0.01	>10

Mutation rate = target SNV/background calibrator. WT, wild-type; SQP, short quenching probe; SNV, single nucleotide variant; LOQ, limit of quantification.

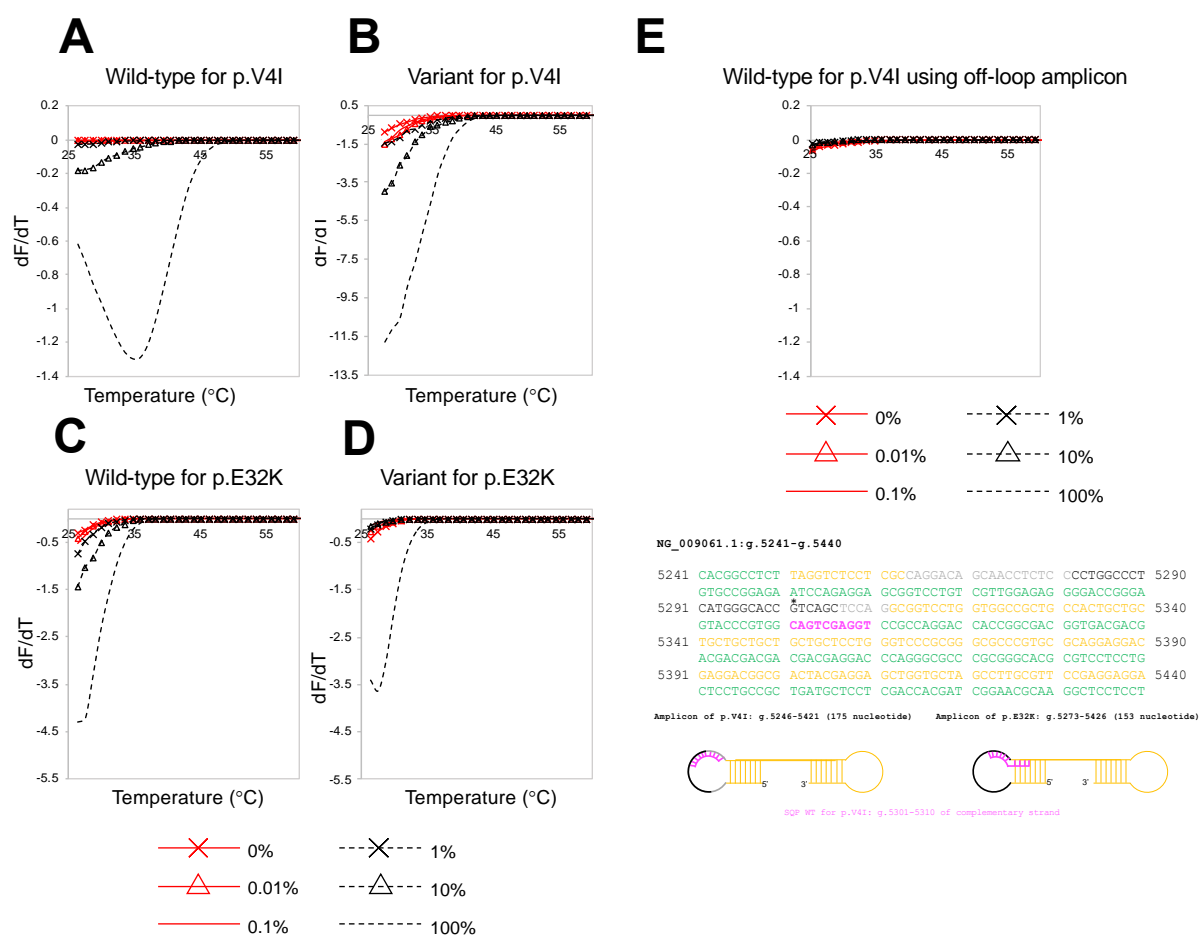


Figure 10. Limit of detection of SQP genotyping.

Detection of the (A) wild-type p.V4I allele, (B) variant p.V4I allele, (C) wild-type p.E32K allele, and (D) variant p.E32K allele. (E) Upper panel: difference in SQP detection with a loop structure. (A) and (E) only differ in the structure of the amplicons. Lower panel: schematic diagram of SQP position to detect the wild-type p.V4I allele at each amplicon. The amplicon of the p.E32K detection slides four nucleotides forward in the end of loop region from g.5311 to g.5306, resulting in a conflict between the SQP and the complementary strand of the target SNV. Magenta (bold), SQP site to detect wild-type p.V4I (g.5301 to g.5310); grey, amplicon loop region for p.V4I detection; black, amplicon loop region for p.E32K detection; *, SNV; SQP, short quenching probe; SNV, single nucleotide variant.

Patient studies

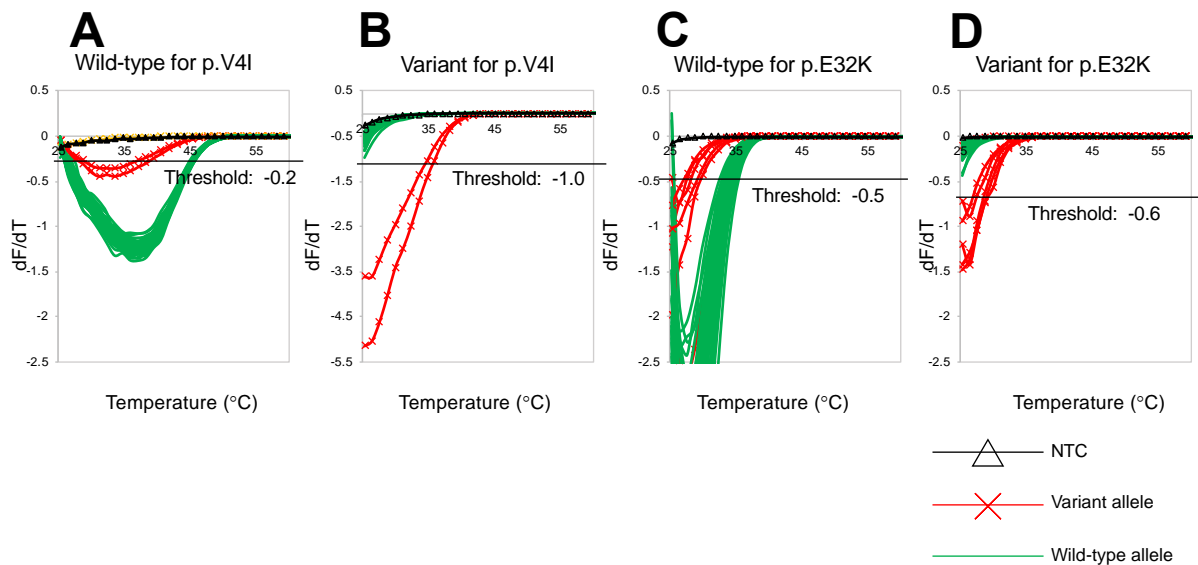
The characteristics of the patients with FH are shown in Table 5. Seven patients with *PCSK9* variants and 29 patients without *PCSK9* variants were categorized using NGS. All lipid and *PCSK9* levels were similar among the categorized patients. In SQP genotyping, seven patients with *PCSK9* variants and 29 patients without *PCSK9* variants could be clearly distinguished (Figure 11). The sensitivity and specificity for the detection of targeted-*PCSK9* variants were both 100%, among all patients (Table 6). The allele distribution in each patient matched that obtained by conventional sequencing.

Table 5. Characterization of SQP-genotyped population categorized by *PCSK9* variants

Variable	<i>PCSK9</i> +, <i>n</i> = 7	<i>PCSK9</i> -, <i>n</i> = 29	<i>p</i> value
Age, years	48 ± 17	47 ± 16	0.69
Male, <i>n</i> (%)	1 (14)	11 (38)	0.38
TG, mmol/L	2.22 (1.81-2.26)	1.20 (0.86-1.51)	0.04*
HDL-C, mmol/L	1.39 ± 0.31	1.56 ± 0.50	0.40
LDL-C, mmol/L	4.76 ± 1.19	4.03 ± 1.33	0.19
<i>PCSK9</i> , ng/mL	454 ± 227	358 ± 102	0.10

Mean ± standard deviation; median (interquartile range). TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; *PCSK9*, proprotein circulating convertase subtilisin/kexin type 9; *PCSK9*-, patients without the gain-of-function *PCSK9* variant; *PCSK9*+, patients with the gain-of-function *PCSK9* variant.

Significance level: *p* < 0.05.

**Figure 11. SQP genotyping for *PCSK9* variants in clinical samples.**

Detection of the (A) wild-type p.V4I allele, (B) variant p.V4I allele, (C) wild-type p.E32K allele, and (D) variant p.E32K allele. Black line, detection threshold; NTC, no-template

control.

Table 6. Comparison of specificity and sensitivity between SQP genotyping and NGS in clinical samples

	NGS positive	NGS negative	Total
SQP positive	7	0	7
SQP negative	0	29	29

SQP, short quenching probe (genotyping); NGS, next-generation sequencing

Adaptation to clinical use

The endpoint assay yielded the same results as NGS and MCA (Table 7). Although the turnaround time for the MCA was 2.5 h for detection, the analysis time was shortened to 15 min for the endpoint assay. Furthermore, only two temperatures were set (64 °C for amplification and 95 °C for SQP dissociation) and cooled to room temperature (0–25 °C) for the analysis.

Table 7. Endpoint SQP genotyping for *PCSK9* variants

	p.E32K variant	p.E32K wild-type	NGS	p.V4I variant	p.V4I wild-type	NGS
ID1	106%	68%	-	116%	8%	-
ID2	114%	63%	-	65%	64%	+
ID3	113%	68%	-	125%	38%	-
ID4	114%	65%	-	73%	61%	-
ID5	102%	68%	-	107%	47%	-
ID6	116%	63%	-	125%	60%	-
ID7	110%	68%	-	124%	72%	-
ID8	112%	64%	-	124%	26%	-
ID9	98%	83%	+	134%	52%	-

ID10	105%	66%	-	114%	56%	-
ID11	116%	64%	-	150%	42%	-
ID12	114%	70%	-	124%	53%	-
ID13	90%	81%	+	119%	42%	-
ID14	93%	76%	+	130%	87%	-
ID15	111%	67%	-	114%	46%	-
ID16	110%	63%	-	130%	35%	-
ID17	109%	66%	-	119%	42%	-
ID18	116%	68%	-	122%	64%	-
ID19	111%	62%	-	125%	57%	-
ID20	116%	67%	-	123%	44%	-
ID21	125%	62%	-	124%	60%	-
ID22	116%	69%	-	127%	54%	-
ID23	112%	67%	-	124%	65%	-
ID24	113%	63%	-	115%	88%	-
ID25	94%	63%	+	123%	49%	-
ID26	112%	64%	-	124%	41%	-
ID27	108%	59%	-	51%	71%	+
ID28	115%	64%	-	142%	60%	-
ID29	95%	62%	+	141%	86%	-
ID30	112%	70%	-	119%	59%	-
ID31	111%	64%	-	120%	63%	-
ID32	104%	52%	-	123%	50%	-
ID33	109%	56%	-	137%	51%	-
ID34	104%	56%	-	108%	57%	-
ID35	110%	66%	-	113%	46%	-
ID36	122%	63%	-	154%	43%	-
Threshold	<100%	<85%		<70%	<100%	

SQP, short quenching probe; PCSK9, proprotein convertase subtilisin/kexin type 9; NGS, next-generation sequencing; red highlights, detection under each threshold.

Discussion

This study demonstrated a novel genotyping method based on the binding of sequence-specific SQP to the loop regions of LAMP amplicons for the detection of *PCSK9* variants.

The detection mechanism is illustrated in Figure 12. Our SQP genotyping method can detect a difference of even a single nucleotide substitution based on fluorescence quenching, and hence allows rapid (<1.5 h) and specific detection of SNV.

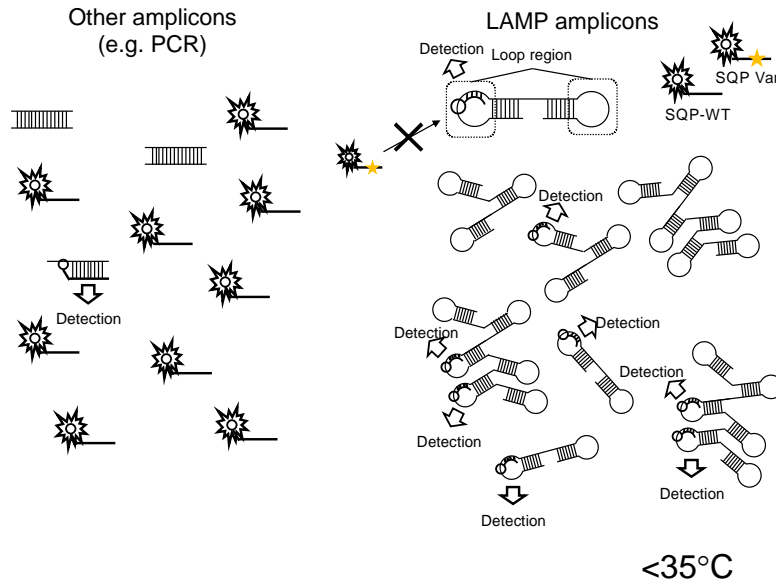


Figure 12. Schematic diagram of SQP genotyping.

At low temperatures (<35°C), amplicons derived from conventional amplification methods (e.g., PCR) are double-stranded and difficult to bind to SQP because there are no single-stranded binding sites (left panel). In contrast, LAMP amplicons possess loop structures with single-stranded binding sites for SQP (right panel). SQP-labeled fluorescence is quenched when double-stranded that is the detection of SNV. SQP, short quenching probe; PCR, polymerase chain reaction; LAMP, loop-mediated amplification; SNV, single nucleotide variant.

As shown in Figure 13, conventional genetic screening methods, including NGS and Sanger sequencing, require several days to achieve diagnosis, mostly because of the time required for sample preparation[104,105]. In contrast, the turnaround time of our SQP genotyping method can be shortened to within one and half hour including sample preparation, detection, and analysis. Our SQP genotyping method does not require the identification of variants and any post-calculation steps, such as MCA, because the specificity to each allele can obtain a threshold of sufficient fluorescence intensity for identification. This method can be used in clinical laboratories that do not have an exclusive thermal cycler, such as a canonical plate reader for other clinical tests (i.e., ELISA or minimal inhibitory concentration plates), because precise temperature control is not required. The LAMP method using SQP genotyping could also be applied to crude samples[106]. This feature of the LAMP method makes it cost-effective (one-tenth of the cost per sample compared with other amplification methods)[107]. A one-step DNA extraction device that is specific to the LAMP method is available[108]. These features enable a higher throughput when conducting genetic screening in a large number of samples.

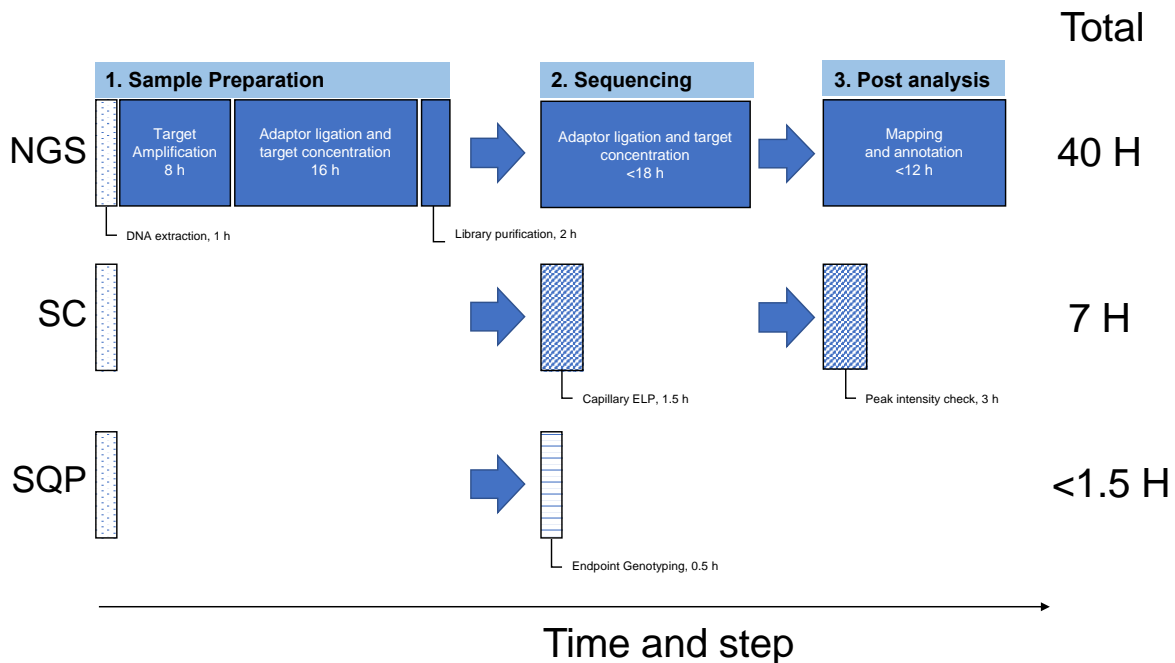


Figure 13. Turnaround time between conventional assays and SQP genotyping.

Two conventional variant definitions, NGS and SC, require additional analysis steps and time for each: sample preparation, sequencing, and analysis. In the case of NGS and SC, costs and turnaround time vary widely depending on the number of samples. Turnaround time is estimated per 96 samples. NGS, next-generation sequencing; SC, Sanger sequencing; SQP, short quenching probe genotyping; ELP, electrophoresis.

In this study, two pathogenic variants in exon 1, p.V4I and p.E32K, which are pathogenic variants of FH in Japan, were detected in patients with FH[12,21,109]. p.E32K is a hotspot of FH in Japan[12]. The frequency of the p.E32K and p.V4I variants was higher in the Asian population (0.013–0.2%) than in the global population (0.000–0.001%)[77]. Other GOF variants of *PCSK9* are comparatively rare in Japanese FH patients[19,20]. GOF variants of *PCSK9* differ depending on regional or ethnic factors; thus, the method needs to

be customized. In the genetic screening of patients with FH in Asia and South America[13,15,110], GOF variants of *PCSK9* were not detected. Our SQP genotyping is feasible in countries where the precise frequency of the variants is still unknown, such as in Asia, South America, and Africa, or in developing countries where establishing a valid healthcare system for screening is difficult[111].

In conclusion, the results of the present study revealed that SQP genotyping provided sufficient sensitivity and specificity for diagnosing FH based on the identification of *PCSK9* variants. SQP genotyping provides a solution for large-scale genetic screening owing to its speed and ease of use. Further studies on various genes are required to determine the clinical utility of genotyping *PCSK9* variants in FH patients.

Perspectives

Across these studies, new functions of GOF variants of *PCSK9* were revealed, and the importance of detecting GOF variants of *PCSK9* was indicated. Study I indicated that *PCSK9* controlled oxidative stress in cells, and GOF variants of *PCSK9* weakened oxidative stress in cells by inhibiting the uptake of ROS. In Study II, GOF *PCSK9* showed a different response to LLT, retaining the positive correlation between LDL-C and *PCSK9*. This correlation indicates a high risk of CVD. In Study III, a clinically acceptable genotyping assay, SQP genotyping, was developed that is high-throughput and user friendly. Recently, several functional types of *PCSK9* have been observed in both blood and endothelial cells. The functional types of *PCSK9* have not been fully categorized by measuring *PCSK9* levels in the blood[52,66,112]. Detection of *PCSK9* variants may provide a solution for assessing these functions. Furthermore, SQP genotyping can be used to detect the variants in clinical settings.

An increase in *PCSK9* levels in the blood is a concrete risk factor for CVD, in relation to an increase in LDL-C levels. The GOF variants of *PCSK9* universally raise the levels of LDL-C; thus, the risk increases more than that in healthy controls. In hypercholesterolemia, an at-risk state for CVD, *PCSK9* may play an alternative role in the early stage of plaque formation. *PCSK9* inhibits the uptake of ROS (i.e., LDL-C, triglyceride, and oxPL) in endothelial cells[52,60,82]. In this case, the GOF variants of *PCSK9* may show an opposite effect (i.e., a protective effect) on CVD by protecting against exposure to oxidative stress. The effects of gene variants are consistent regardless of other factors of CVD such as diet or daily deviation of biochemical tests[113]. The *PCSK9* variant may be an important marker for assessing CVD, especially in high-risk groups (Figure 14A1).

PCSK9 genotyping can be used as personalized medicine, especially for companion diagnosis with drugs. Major LLT such as statins inhibit cholesterol biosynthesis. In Study II, monotherapy did not reach the control levels of LDL-C, 2.59 mmol/L[67]. In addition, the regulation of *PCSK9* to LDL-C was retained only in patients with GOF variants, which is a good target for additional therapy. Recently, two *PCSK9*-related drugs, one of which is an antibody and the other involves RNA silencing, have been approved. Patients with GOF variants can easily experience the effects of the drug owing to a direct reduction in *PCSK9* levels. The *PCSK9* inhibitors are expensive compared to traditional drugs[114], and genetic tests are good for assessing drug decisions as companion diagnostics (Figure 14A2).

As a clinical test, cascade screening is cost-effective for FH considering the incremental cost-effectiveness ratio (ICER) threshold (e.g., \$20,000–30,000 in the UK and \$50,000–150,000 in the US) (Figure 14B)[111]. Developed healthcare systems, such as the assignment of genetic experts, patient adherence, and highly accurate genetic tests, are needed for its effectiveness. In a simulation, the cost of genetic testing was the highest for cascade screening at \$250[115]. Countries with high diagnostic rates have well-designed genetic screening systems[116]. However, the diagnostic rate in Japan is estimated to be less than 1 % [16]. SQP genotyping allows for cascade screening in countries without developed healthcare systems owing to ease of use. Universal screening in children was also tried in a few countries[116,117]. From the ICER threshold, genetic tests for neonates looks cost-effective (\$3,800) in a pilot study in Australia[116]. In the future, we need to focus on cohorts with larger number of patients.

In conclusion, the detection of *PCSK9* variants can be applied to personalized medicine (e.g., CVD risk assessment and companion diagnosis) or genetic screening of FH.

Optimizing near-patient and high-throughput, SQP genotyping is one solution for these diagnostic applications.

***PCSK9* genotyping**

A. Personalized medicine

1. CVD risk assessment in at-risk population
 - GOF variant Risk reduction

2. Decision of treatment strategy
 - Combination therapy
 - Companion diagnosis for PCSK9 inhibitor
 - Antibody Recommendation for patient with GOF variant
 - RNA silencing

B. Genetic screening

- | | |
|---------------------|--|
| Cascade screening | Countries with developing HS |
| Universal screening | Newborn screening with high-throughput |
-

Figure 14. Clinical applications of *PCSK9* genotyping.

PCSK9, proprotein convertase subtilisin/kexin type 9; CVD, cardiovascular disease; GOF, gain-of-function; LOF, loss-of-function; HS, health-care system; LDL, low-density lipoprotein.

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Major publications related to fulfilment of PhD course

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Conflict of interests

M.H. worked at Eiken Chemical Co. Ltd. and received research funding from Eiken Chemical Co., Ltd.

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