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Clinical whole exome sequencing in severe hypertriglyceridemia

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ABSTRACT

Background: Little data exist regarding the clinical application of whole exome sequencing (WES) for the molecular diagnosis of severe hypertriglyceridemia (HTG).

Methods: WES was performed for 28 probands exhibiting severe HTG (≥1000 mg/dl) without any transient causes. We evaluated recessive and dominant inheritance models in known monogenic HTG genes, followed by disease-network gene prioritization and copy number variation (CNV) analyses to identify causative variants and a novel genetic mechanism for severe HTG.

Results: We identified possible causative variants for severe HTG, including three novel variants, in nine probands (32%). In the recessive inheritance model, we identified two homozygous subjects with lipoprotein lipase (LPL) deficiency and one subject harboring compound heterozygous variants in both *LPL* and *APOA5* genes (hyperchylomicronemia). In the dominant inheritance model, we identified probands harboring deleterious heterozygous variants in *LPL*, glucokinase regulatory protein, and solute carrier family 25 member 40 genes, possibly associated with this extreme HTG phenotype. However, gene prioritization and CNV analyses did not validate the novel genes associated with severe HTG.

Conclusions: In 28 probands with severe HTG, we identified potential causative variants within nine genes associated with rare Mendelian dyslipidemias. Clinical WES may be feasible for such extreme cases, potentially leading to appropriate therapies.

1. Introduction

Severe hypertriglyceridemia (HTG) is characterized by serum triglyceride (TG) levels ≥1000 mg/dl [1] and can originate from primary or secondary causes [2]. In an earlier study, we have shown that the condition resulted from secondary causes in 74% of our subjects with HTG, with 36.9% of the cases transiently induced by a variety of environmental factors [3]. In contrast, primary severe HTG derives to some extent from genetic etiology. To date, genes, such as apolipoprotein A-V (*APOA5*), apolipoprotein C-II (*APOC2*), apolipoprotein E (*APOE*), glucokinase regulator (*GCKR*), lipase I (*LIPI*), lipoprotein lipase (*LPL*), and solute carrier family 25, member 40 (*SLC25A40*), have been reported to be associated with monogenic HTG [4–7].

Plasma triglyceride (TG) level has been shown to be one of the residual risk factors for coronary artery disease (CAD) in this era of statin use [8]. In addition, a recent Mendelian randomization study has suggested that elevated plasma TG is a causal factor of CAD, rather than merely a biomarker, and should be targeted for CAD treatment and prevention [9]. Thus, identifying novel molecules associated with plasma TG could contribute to the discovery of novel pharmacological targets to combat CAD alongside statins. However, till date, few studies have investigated the genetic causes for severe HTG using a comprehensive genetic approach. An additional challenge is the occasional difficulty with gathering a sufficient number of a patient's family members to check the co-segregation of the putative variants in a clinical setting [10]. In this study, therefore, we investigated the usefulness of proband-first whole exome sequencing for identifying the genetic causes of primary severe HTG; this could potentially uncover the genetic etiology throughout the coding region of the human genome [11–13]. In addition, we evaluated the detail phenotypic characteristics

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Table 1
Causative variants identified by the autosomal recessive inheritance model.

Family #	Gene	Mutation status	Chr.	Position (hg19)	rsID	Ref.	Alt.	NA change	AA change	HGMD	ExAC MAF (East Asians)
8	*APOA5*	Compound Hetero	11	116,661,394	rs201229911	G	C	c.551C > G	p.Thr184Ser	Listed[a]	0.21%
	LPL		8	19,811,751	rs118204061	T	C	c.662T > C	p.Ile221Thr	Listed[a]	0
18	*LPL*	Homo	8	19,816,833	rs118204071	G	A	c.1081G > A	p.Ala361Thr	Listed	n.a.
20	*LPL*	Homo	8	19,813,384	rs118204077	C	T	c.808C > T	p.Arg270Cys	Listed	0

AA change, amino acid change; Alt, alternate allele; APOA5, apolipoprotein A5; CADD, Combined Annotation Dependent Depletion; Chr., chromosome; ExAC, The Exome Aggregation Consortium; Hetero, heterozygous; Homo, homozygous; HGMD, Human Gene Mutation Database; HTG, hypertriglyceridemia; MAF, minor allele frequency; n.a., not available; NA change, nuclear acid change; LPL, lipoprotein lipase; and Ref, reference allele.
[a] Reported individually as recessive diseases.

Table 2
Possible causative variants identified by the autosomal dominant inheritance model.

Family #	Gene	Mutation status	Chr.	Position (hg19)	rsID	Ref.	Alt.	NA change	AA change	HGMD	ExAC MAF (East Asians)	Meta SVM	Meta LR	CADD C-score
14	*GCKR*	Hetero	2	27,721,143	rs146175795	G	A	c.307G > A	p.Val103Met	Listed	0.95%	D	D	23.0
21	*GCKR*	Hetero	2	27,721,143	rs146175795	G	A	c.307G > A	p.Val103Met	Listed	0.95%	D	D	23.0
22	*GCKR*	Hetero	2	27,720,198	rs183618032	G	A	c.148G > A	p.Val50Ile	Not listed[a]	0.012%	D	D	23.0
25	*SLC25A40*	Hetero	7	87,479,214	rs200954020	C	T	c.314G > A	p.Ser105Asn	Not listed[a]	0.37%	D	D	34
27	*LPL*	Hetero	8	19,796,977	NA	T	G	c.26T > G	p.Leu9Arg	Not listed[a]	NA	T	D	22.3
28	*LPL*	Hetero	8	19,813,438	rs1800011	G	A	c.862G > A	p.Ala288Thr	Listed	0.058%	D	D	33

Abbreviations: CREB3L3, cyclic AMP-responsive element-binding protein 3-like protein 3; and GCKR, glucokinase regulatory protein.
[a] Novel variants associated with hypertriglyceridemia.

of severe HTG probands by causative genes.

2. Methods

2.1. Study population

The study included a total of 28 probands who fulfilled the criterion for severe HTG (*i.e.*, HTG ≥ 1000 mg/dl) without any transient secondary cause defined as previously described [3]. Written informed consent was obtained from all of the participants.

2.2. Clinical evaluations

The presence of diabetes was defined as previously described by the Japan Diabetes Society [14] or by the use of diabetes medication. Body mass index (BMI) was defined as body weight in kilograms divided by the square of height measured in meters. CAD was defined by the presence of angina pectoris, myocardial infarction, or severe stenotic region(s) in the coronary arteries identified by either an angiogram or computed tomography. Ultrasound examination of the liver was performed by echocardiographic experts to detect fatty liver. Subjects with both liver brightness and hepatorenal echo contrast were considered to have fatty liver in this study. Alcohol intake was assessed by a medical interview. A history of pancreatitis was assessed by reviewing medical records.

2.3. Biochemical analysis

Blood samples were drawn for assays following overnight fasting. Serum levels of total cholesterol (TC), TG, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were determined enzymatically (Qualigent, Sekisui Medical, Tokyo, Japan) using automated instrumentation based on assays previously described [15]. LPL mass in postheparin plasma was measured according to previously reported methods; briefly, postheparin plasma was obtained 15 min after an injection of 30 U/kg heparin, and LPL mass in the postheparin plasma was measured by a sandwich enzyme immunoassay [16]. The *APOE* phenotype was separated by isoelectric focusing and detected by western blot with *APOE* polyclonal antibodies (phenotyping apoE IEF system, JOKOH, Tokyo, Japan). We also classified the study patients' hyperlipidemia according to Fredrickson's classification, based on the appearance of the serum lipid profile [17]. Ultracentrifugation and agarose gel electrophoresis were performed as previously described [8].

2.4. Whole exome sequencing

Genomic DNA for each subject was isolated from peripheral white blood cells using a standard DNA extraction protocol. DNA was pooled, selected for size, ligated to sequencing adapters, and amplified to enrich for targets, which were sequenced using the Kapa DNA Library Preparation kit or the Illumina Nextera sample preparation kit. Exome capture was performed with the Nextera Rapid Capture Exome kit with a bait/target size of 38 Mb (Illumina Inc.). Exome-enriched products were sequenced using the Illumina HiSeq2000. The target coverage for each subject was at least 20-fold in ≥90% of targeted exons. Subjects with < 90% of targeted exon coverage with at least 20-fold reads were not included in the subsequent analysis.

2.5. Bioinformatics

Paired-end reads for the samples were aligned using the Burrows–Wheeler Aligner on the human reference genome build GRCh37.75 using quality score calibration, soft clipping, and adapter trimming. Following the exclusion of PCR duplicate reads using Picard, insertion–deletions and single nucleotide polymorphisms (SNPs) were called using the Genome Analysis Toolkit [18] (GATK, version 3.4). Variants (single nucleotide variants [SNVs] or indels) were filtered on the basis of the Phred-scaled genotype quality score. *Re*-alignment was performed and the calling algorithm merged with the output of the GATK Haplotype Caller. The GATK Variant Quality Score Recalibration (VQSR) tool was used to update the quality score of each variant. All samples were annotated using SnpEff [19] version 4.1 to classify variants (missense, nonsense, splice site, synonymous, intronic, or stop gain/loss). The frequency filter adopted allele frequency estimates from the Exome Aggregation Consortium, East Asian cohort database [20].

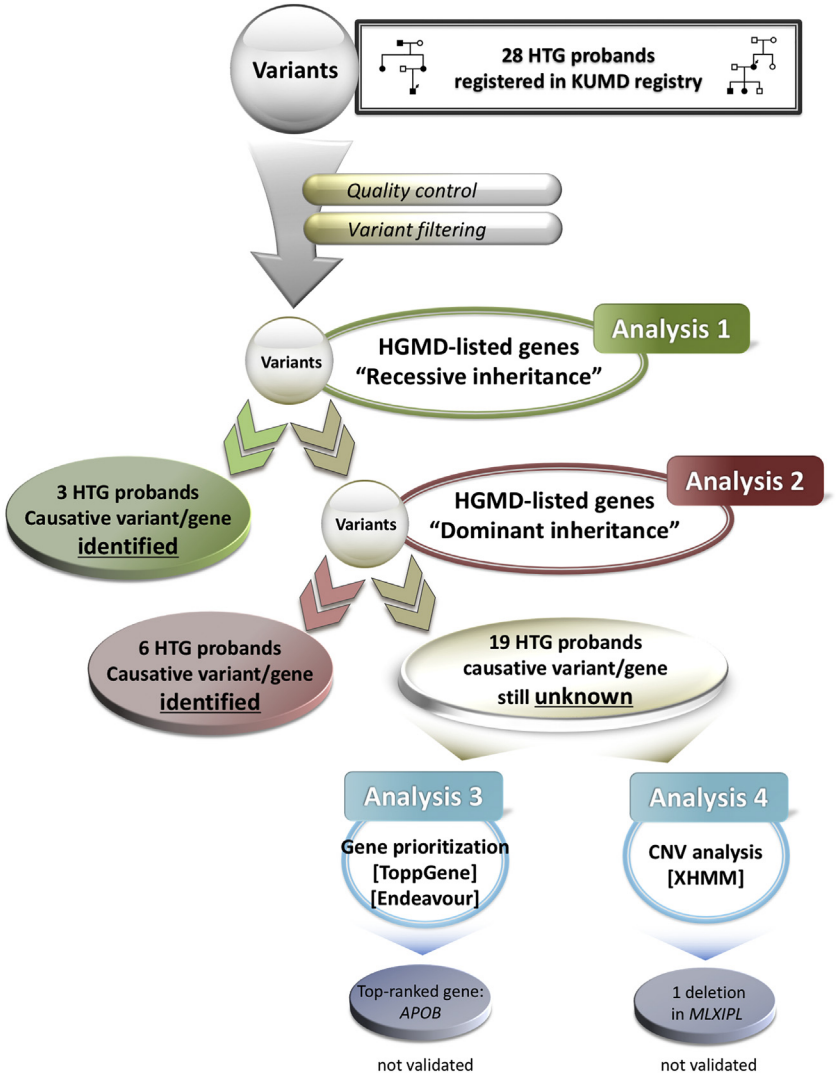

Fig. 1. Scheme of this study.
Step 1 involved application of the standard filters, including minor allele frequency < 1%, nonsynonymous variants, and deleterious variants predicted by the *in silico* annotation tool. Step 2 involved four analyses, including investigations among Human Gene Mutation Database-listed genes, gene prioritization analyses, and a copy number variation analysis. After applying these filters, follow-up studies were conducted to determine the causality of variants as well as the pattern of inheritance.

Initially, three independent filters were applied as primary filtering: (1) Variant Quality Score Recalibration (VQSR) filter PASS variants, (2) minor allele frequency (MAF) < 1% in the East Asian population; and (3) missense, premature stop, frameshift, and canonical splice site variants predicted by SnpEff. In addition, we checked for deleterious variants in genes that have been shown to be associated with monogenic HTG by the human gene mutation database [21] (HGMD® professional, version 2016.1) (Supplemental Table 1), firstly assuming an autosomal recessive inheritance model (homozygous or compound heterozygous), as Analysis 1, and then checking an autosomal dominant inheritance model (a single heterozygous variant) in those genes, as Analysis 2. In order to validate the deleteriousness of novel heterozygous missense variants, we applied the following stringent pathogenicity criteria: (1) Combined Annotation Dependent Depletion (CADD, version 1.3) scaled C-score [22] > 20; and (2) indicated to be "damaging" in a database of human non-synonymous SNVs and their functional predictions and annotations [23] (dbNSFP, version 2.9), MetaSVM, or MetaLR. The CADD C-score is an integrated damaging score calculated by 63 software annotations for each variant, and the MetaSVM / MetaLR are support vector machine- (SVM) or logistic regression- (LR) based ensemble scores that incorporate 10 software annotations with a 1000 Genomes Project allele frequency for maximizing sensitivity and specificity to classify causative missense variants. In addition, the pathogenicity of each variant was investigated according to the American College of Medical Genetics and Genomics (ACMG) Standards and Guidelines [24].

2.6. Candidate gene prioritization analysis

In Analysis 3, candidate gene prioritization analysis was performed using two publicly available web-based software packages, ToppGene [25] (https://toppgene.cchmc.org) and Endeavour [26] (https://endeavor.esat.kuleuven.be/Default.aspx). We used 15 HGMD-listed monogenic HTG-causing genes (Supplemental Table 1) as a training gene set. To perform this gene-based analysis, we converted lists of the damaging variants of each proband into genes by the annotation and evaluated how many subjects shared each gene. Damaging variants were defined as those with a CADD C-score > 10. Ninety-nine genes of which four or more subjects shared the variants were used for this analysis. This gene prioritization was performed using all data source for both ToppGene and Endeavor. The gene priority rankings from each tool were combined and a final composite ranking was created.

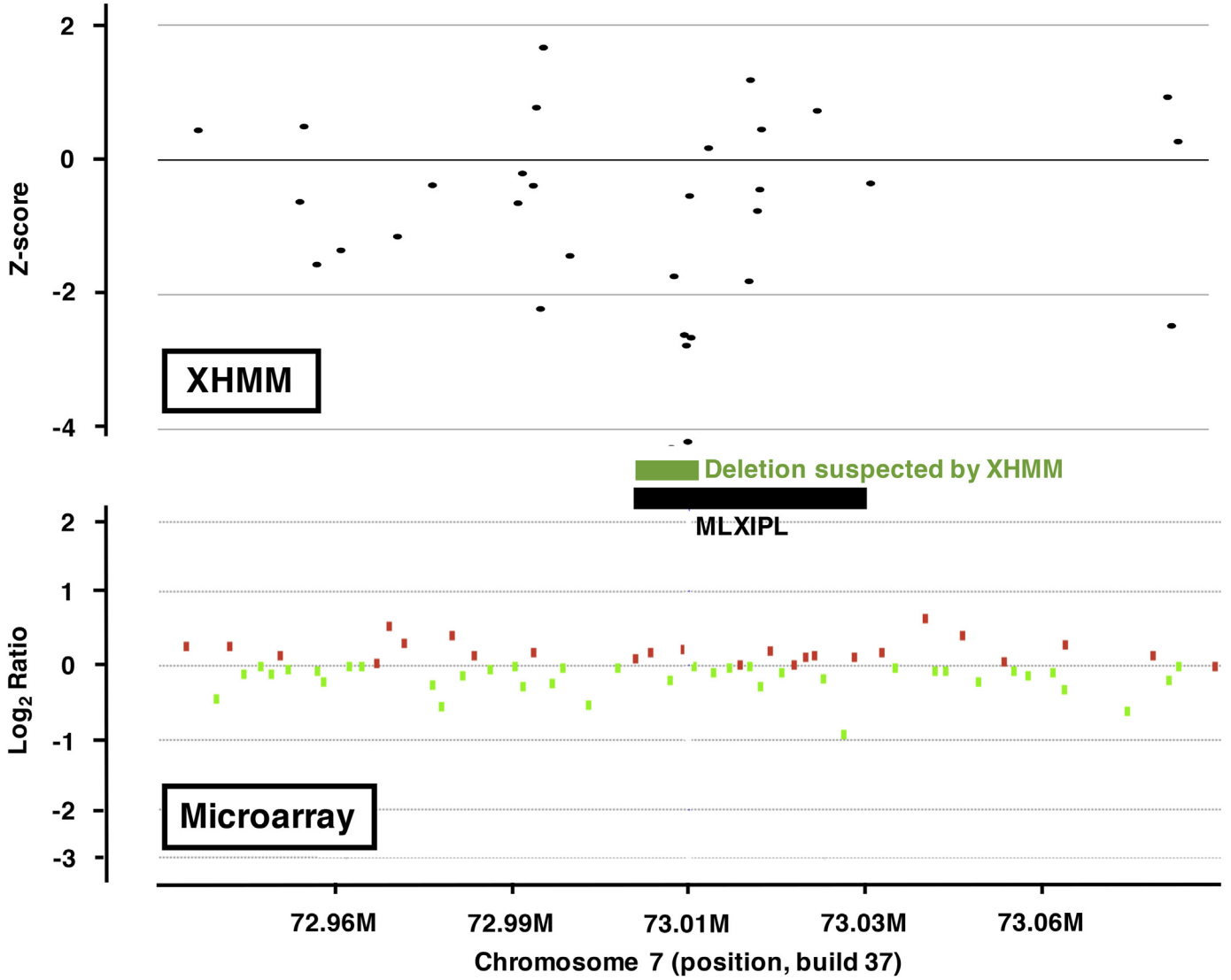

Fig. 2. Suspected deletion point in the *MLXIPL* gene by XHMM and the microarray validation.
XHMM software predicted that this proband (HTG-1) may have had a deletion point in the *MLXIPL* gene (upper scatter plot), but the microarray data was unable to validate the deletion at this point (lower scatter plot). In the XHMM result, each dot represents a normalized *Z*-score calculated from the depth of coverage in each exon and adjusted by the first principal component. In the microarray result, each dot indicates the $\log_2$ ratio of each probe. Red plots indicate $\log_2$ ratio > 0, and green plots indicate $\log_2$ ratio < 0.

2.7. Copy number variation analysis

We assessed copy number variation (CNV) using the eXome Hidden Markov Model (XHMM) as Analysis 4. The detailed CNV analysis pipeline and commands have previously been described [21]. To adjust the statistics by principal components, we recruited 75 additional subjects as controls from Kanazawa University Mendelian Disease Registry, and so a total of 113 whole exome sequenced subjects were incorporated in the CNV analysis. We evaluated CNV within 15 HGMD-listed genes associated with HTG, as well as genes previously reported to be primarily associated with HTG by genome-wide association studies [27]. (Supplemental Table 2.)

2.8. Array comparative genomic hybridization

Array Comparative Genomic Hybridization (CGH) was performed using SurePrint G3 Human CGH microarrays 1 × 1 M (Agilent Technologies) following the manufacturer's instructions. Briefly, 1 μg of genomic DNA corresponding to either a human reference control (Promega) or test samples were fragmented by heating at 95 °C for 10 min. Fragmented DNA was labeled with Cy3 (reference DNA) and Cy5 (test samples) fluorescent dUTP, using the SureTag Complete Labeling Kit (Agilent Technologies). Purification columns (Agilent Technologies) were used to remove any unincorporated nucleotides and dyes. The labeled samples along with human Cot-1 DNA were added together and hybridized on the array slides. Hybridization of labeled DNA to SurePrint G3 Human CGH Arrays (4 × 180 K) (Agilent Technologies) was performed in a hybridization oven at 65 °C at 20 rpm for 24 h. The slide was scanned at 3-μm resolution on an Agilent Microarray Scanner System (Agilent Technologies). Agilent CytoGenomics software (Agilent Technologies) was used to visualize, detect, and analyze chromosomal patterns within the microarray profiles.

2.9. Follow-up study

As far as was possible, we assessed the genotype and phenotype of the probands' family members in order to validate and support the causalities of identified variants and the pattern of inheritance [28].

Table 3
Baseline and phenotypic characteristics of hypertriglyceridemia probands.

Family #	Age (year)	Sex	BMI (kg/m^2)	TC (mg/dl)	TG (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	LPL mass (ng/ml)	APOA1 (mg/dl)	APOA2 (mg/dl)	APOB (mg/dl)	APOC2 (mg/dl)	APOC3 (mg/dl)	APOE (mg/dl)	Alcohol	DM	AST (IU/L)	ALT (IU/L)	γ-GTP (IU/L)	Fredrickson	CAD	Fatty liver	Pancreatitis	APOE IEF
1	79	M	24.8	280	1672	27	28	28	129	30.9	108	20.6	44	14.7	−	+	41	35	239	2b	−	−	−	3/3
2	60	M	23.4	358	2738	22	28	NA	78	22.5	66	21.9	42.9	20.1	−	−	36	17	21	5	−	−	+	3/3
3	51	M	25.0	336	1956	40	114	48	135	29.5	139	12.6	21.7	12.7	+	+	38	25	231	2b	−	−	+	3/3
4	36	F	26.1	457	1971	22	NA	20	86	13.6	89	16	22.3	12.9	+	−	15	21	25	2b	−	−	+	3/3
5	50	M	30.1	306	1775	26	NA	128	108	26.8	131	27.7	41.8	16	+	+	37	45	216	2b	+	+	−	4/3
6	50	F	24.0	351	1006	35	74	234	124	30.5	142	13.3	28.3	17.9	−	−	20	28	55	4	−	−	−	4/3
7	33	F	15.2	317	1354	13	NA	41	92	11.8	82	12.2	20.7	11.4	−	−	16	30	81	4	−	+	−	4/3
8	25	M	29.0	208	1292	31	42	124	116	27.1	81	10.4	25.1	14.3	+	−	20	36	208	5	−	−	−	4/3
9	35	M	24.0	571	1885	32	NA	176	116	21.8	161	46.1	77.;9	31.1	+	+	14	20	87	3	−	+	−	NA
10	41	M	29.0	334	1975	29	71	300	120	26.1	100	26.1	35.2	14.6	+	−	50	78	43	5	−	+	−	3/3
11	66	M	NA	340	1184	31	NA	NA	NA	NA	NA	NA	NA	NA	−	−	25	40	40	5	−	−	−	3/3
12	47	M	23.6	332	2359	40	18	215	159	38.2	122	25.8	73	27	+	−	25	32	203	5	−	−	−	4/3
13	33	M	38.3	456	3807	35	66	NA	163	37.4	144	23.4	45.8	32.1	+	+	35	64	76	5	−	+	−	3/3
14	44	M	26.7	331	1253	49	NA	NA	154	57.7	151	18.8	47.8	18	+	+	29	44	313	4	−	+	−	3/2
15	60	M	NA	184	1032	51	32	NA	153	34	89	15.2	44.6	12	+	+	118	93	493	4	−	−	−	3/3
16	52	F	25.6	316	1353	33	61	NA	155	57.2	157	34.9	41.8	20.1	−	+	49	52	49	4	−	−	−	3/3
17	59	M	19.9	300	1315	4	33	NA	108	5.3	96	7.9	19.8	13.3	+	−	210	310	600	3	−	−	−	NA
18	63	M	23.2	354	1736	26	56	28	109	22.5	81	19	40	22.5	−	−	22	18	23	5	−	−	+	3/2
19	60	M	22.7	248	1276	44	NA	234	158	34.5	109	7.6	19.3	7.3	+	−	21	11	74	5	−	−	−	3/2
20	45	F	24.5	476	4781	20	27	37	103	13.5	89	15.3	54.3	34.5	−	+	10	13	33	5	−	−	+	3/3
21	34	F	30.8	1159	4257	29	NA	25	166	28.5	288	31.2	129	85.2	−	+	30	15	10	4	−	−	−	3/3
22	26	F	NA	462	1662	52	NA	44	261	36.1	178	20.4	52.3	24.3	+	+	16	8	16	4	−	−	+	3/2
23	65	F	23.6	274	2152	37	NA	324	NA	NA	NA	NA	NA	NA	−	−	34	35	28	4	−	+	−	4/3
24	2	F	17.9	194	1349	21	NA	28	NA	NA	NA	NA	NA	NA	−	−	20	8	8	2b	−	−	−	NA
25	5	F	NA	506	2694	20	NA	17	NA	NA	NA	NA	NA	NA	−	−	NA	NA	NA	2b	−	−	+	3/3
26	43	M	24.5	276	1141	36	53	37	143	29.6	105	15.8	31.7	12.1	+	+	24	24	36	2b	−	−	−	3/3
27	40	M	29.1	558	2790	18	51	95	94	22.2	110	36.9	60.2	19.8	−	+	16	46	30	5	−	−	−	3/3
28	40	M	24.8	664	2936	21	4	99	105	NA	105	NA	NA	31.8	−	−	42	57	180	2b	−	+	−	4/3

Abbreviations:; APOA1, apolipoprotein A1; APOA2, apolipoprotein A2; APOB, apolipoprotein B; APOC2, apolipoprotein C2; APOC3, apolipoprotein C3; APOE, apolipoprotein E; AST, aspartate transaminase; ALT, alanine transaminase; BMI, body mass index; CAD, coronary artery disease; F, female; γ-GTP, gamma-glutamyl transpeptidase; HDL-C, high-density lipoprotein cholesterol; IEF, isoelectric focusing; LDL-C, low-density lipoprotein cholesterol; LPL, lipoprotein lipase; NA, not available; M, male; TC, total cholesterol; TG, triglyceride.

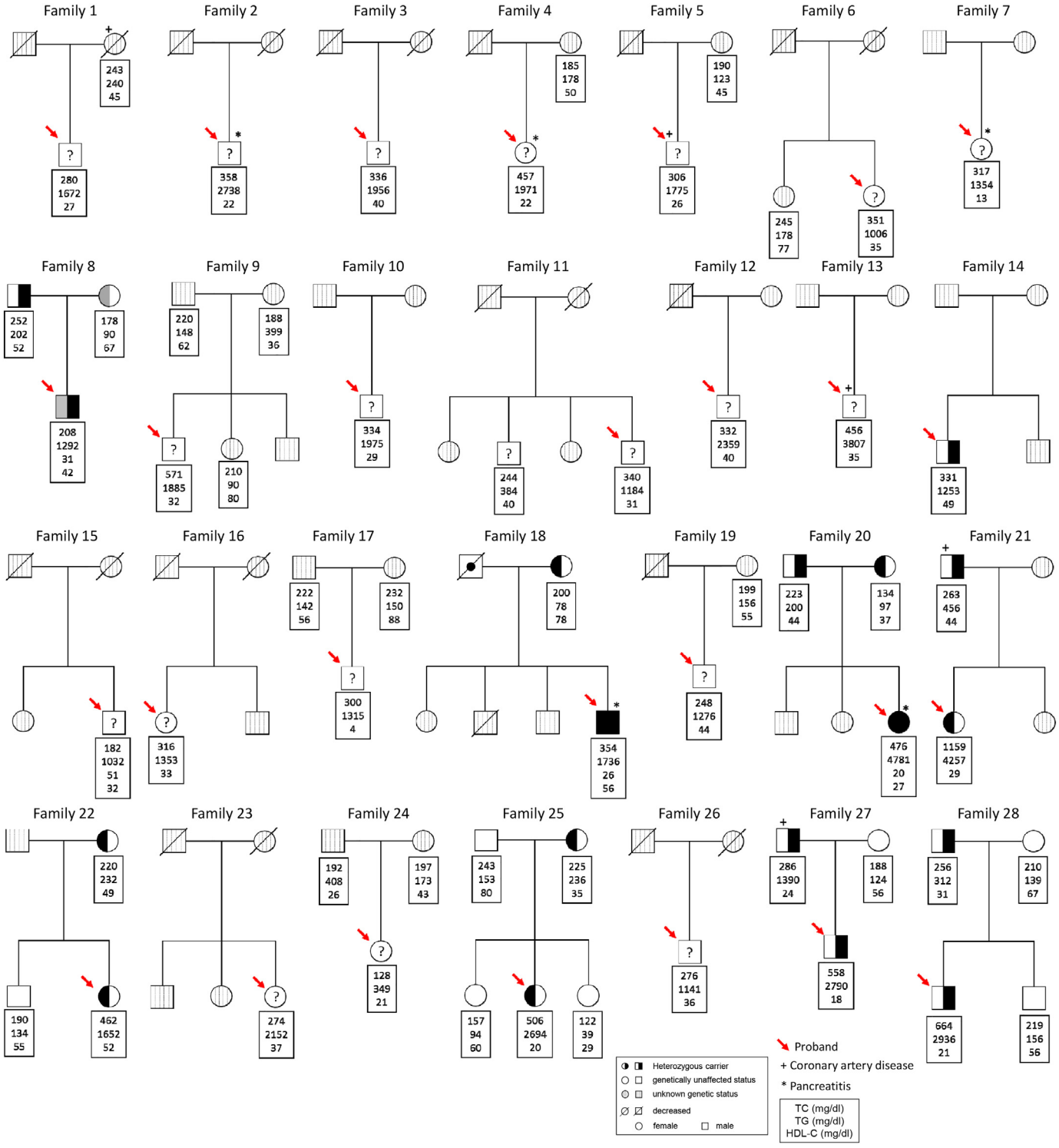

Fig. 3. Pedigrees of study subjects.
Squares indicate male subjects, and circles indicate female subjects. Borderlines indicate subjects with unknown genetic/phenotypic status. Total cholesterol level (mg/dl), triglyceride level (mg/dl), and high-density glycoprotein cholesterol level (mg/dl) are displayed below each individual identifier. Whole exome-sequenced subjects are indicated with red arrows; subjects with a history of coronary artery disease are indicated with "+," and subjects with a history of pancreatitis are indicated with *: In Family 8, the proband's father exhibited an *APOA5* gene variant, and his mother exhibited a *LPL* gene variant.

2.10. Statistical analysis

Categorical variables are expressed as percentages. Continuous variables with a normal distribution are presented as the mean ± SD. For values not distributed normally, the median and interquartile range (IQR) were reported. The levels of TG, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transpeptidase (γ-GTP) were log-transformed for the statistical analyses. *P*-values were calculated by using Student's *t*-test (for continuous variables) or Fisher's exact test (for categorical variables) in comparisons between subjects identified with causative variants and the remainder. R software (R Project for Statistical Computing, version 3.1.1) was used

Table 4
Phenotypic characteristics by causative genes.

Causative genes	HTG subjects identified causative variant (n = 9)					Causative variants		
	LPL		LPL + APOA5	GCKR	SLC25A40	Identified	Unidentified	P value
Zygosity	Homo	Hetero	Compound hetero	Hetero	Hetero	–	–	
No. of subjects	2	2	1	3	1	8	19	
Age	40 ± 0	54 ± 13	25	35 ± 9	5	36 ± 16	49 ± 17	0.07
Male gender	2 (100)	1 (50)	1	1 (33)	0	5 (63)	13 (68)	0.54
Body mass index	27.0 ± 3.0	23.9 ± 0.92	29.0	28.8 ± 2.9	NA	26.9 ± 2.8	24.6 ± 5.0	0.17
Alcohol intake	0 (0)	0 (0)	1	2 (67)	0	3 (38)	11 (58)	0.42
Coronary artery disease	0 (0)	0 (0)	0	0	0	0	1 (5)	1.00
Diabetes Mellitus	1 (50)	1 (50)	0	3 (100)	0	5 (63)	8 (42)	0.69
Fatty liver	0 (0)	0 (0)	0	1 (33)	0	2 (25)	6 (32)	1.00
Pancreatitis	2 (100)	2(100)	0	1 (33)	1	4 (50)	3 (16)	0.17
Lipid profile								
TC	611 ± 75	415 ± 86	208	650 ± 450	506	524 ± 270	328 ± 91	0.07
TG	2860 ± 100	3260 ± 2200	1292	2390 ± 1600	2694	2600 ± 1300	1750 ± 690	0.08
HDL-C	20 ± 2	23 ± 4	31	43 ± 13	20	30 ± 13	30 ± 11	0.86
LDL-C	28 ± 33	42 ± 20	42	NA	NA	36 ± 21	53 ± 28	0.22
Lipoproteins								
ApoAI	100 ± 8	106 ± 4	116	190 ± 60	NA	138 ± 56	127 ± 27	0.59
ApoAII	22	18 ± 6	27	41 ± 15	NA	30 ± 14	28 ± 12	0.81
ApoB	110 ± 4	85 ± 6	81	210 ± 73	NA	135 ± 71	115 ± 28	0.46
ApoC2	37	17 ± 3	10	23 ± 7	NA	22 ± 9	20 ± 10	0.77
ApoC3	60	47 ± 10	25	76 ± 46	NA	58 ± 33	38 ± 18	0.17
ApoE	26 ± 8	29 ± 8	14	43 ± 37	NA	31 ± 23	17 ± 7	0.13
LPL mass	97 ± 3	33 ± 6	124	35 ± 13	17	59 ± 41	139 ± 110	0.03
Hepatobiliary enzymes								
AST	29 ± 20	16 ± 8	20	25 ± 8	NA	23 ± 10	44 ± 46	0.06
ALT	52 ± 8	16 ± 4	36	22 ± 19	NA	30 ± 18	51 ± 67	0.28
γ-GTP	105 ± 100	28 ± 7	208	113 ± 170	NA	102 ± 120	137 ± 160	0.46

Abbreviations as Tables 1–3.
P values are from comparisons between probands with identified causative variants and the remainder.

for the analyses.

2.11. Ethical considerations

This study was approved by the Ethics Committee of Kanazawa University. All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent for genetic analyses were obtained from all the subjects included in the study.

3. Results

3.1. Exome sequencing and bioinformatics analysis

The number of aligned variants in the eight subjects that passed the standard quality control was 150,491. Of those, 37,522 were missense, premature stop, canonical splice sites, and frameshift variants. Variants with MAF > 1% in East Asians were then excluded and the remaining 12,406 variants were used for further analyses. Filtering according to the 15 genes shown to be associated with monogenic forms of HTG (Supplemental Table 1), we identified homozygous or compound heterozygous causative variants in three HTG probands (Table 1, Analysis 1). We then detected possible causative heterozygous variants, including three novel ones, in six of the HTG probands (Table 2, Analysis 2). All these identified variants were also considered as pathogenic or likely pathogenic according to the ACMG guideline (see Fig. 1).

For the remaining 19 probands, we applied the gene prioritization analysis (Analysis 3) and found the *APOB* gene to be the top-ranked gene (Supplemental Table 3). Among the four proband harboring *APOB* variants (Supplemental Table 4), two probands (HTG-6 and 23) had the same variant (NM_000384.2:c.2863C > T), but the allele frequency of this variant was 5.2% in the Japanese population according to the HapMap-JPT database [29], which indicated that this variant was less likely to be causative. The other two probands (HTG-2 and 16) each had a unique variant (NM_000384.2:c.8111C > T and NM_000384.2:c.7193A > T); however, both variants were assessed as "tolerated" by both MetaSVM and MetaLR, suggesting that there was not enough evidence to indicate that these variants could cause such a extreme situation. In addition, in one proband (HTG-1) we found a CNV in the *MLXIPL* gene; this was reported to be associated with TG by genome-wide association studies and was shown by the XHMM method (Analysis 4) to have the potential to cause monogenic severe HTG. However, a microarray analysis failed to validate the CNV (Fig. 2).

Overall, we identified potential causative genes (or variants) in nine (32%) of the 28 probands with severe HTG, using four bioinformatics methods.

3.2. Characteristics of the study subjects

Clinical characteristics of the study subjects are shown in Table 3 and Fig. 3. Detailed results using ultracentrifugation and agarose gel electrophoresis are shown in Supplemental Fig. 1–28. Median TC, TG, and HDL-C levels were 335 mg/dl [IQR: 295–458], 1756 mg/dl [IQR: 1288–2443], and 30 mg/dl [IQR:22–36], respectively.

We evaluated phenotypic characteristics of subjects by causative genes (Table 4). Subjects with identified causative variants tended to have higher plasma TC and TG levels, and lower LPL mass and AST, compared to those without such identified variants.

3.3. Familial hyperchylomicronemia

We identified two subjects with *LPL* deficiency (HTG-18 and 20) who had homozygous variants in *LPL* gene

(NM_000237.2:c.1081G > A, and NM_000237.2:c.808C > T) and who exhibited a significant reduction of plasma *LPL* mass (28 ng/ml and 37 ng/ml, respectively). We also detected two subjects (HTG-27 and 28) with heterozygous variants in the *LPL* gene (NM_000237.2:c.26T > G, and NM_000237.2:c.862G > A), who exhibited a mild-to-moderate reduction of *LPL* mass (95 mg/ml and 97 ng/ml, respectively). The follow-up study revealed that dominant as well as recessive forms of inheritance were observed for the variants in *LPL* gene in terms of HTG phenotype. Furthermore, we identified one subject (HTG-8) who had compound heterozygous variants in both the *LPL* gene (NM_000237.2:c.662T > C) and the *APOA5* gene (NM_052968.4:c.551C > G); this subject's *LPL* mass level was mildly reduced (124 ng/ml). Both variants have been shown to be associated with familial hyperchylomicronemia [23]. Interestingly, both individuals harboring homozygous variants in the *LPL* gene had histories of recurrent pancreatitis, whereas none of the others had a history of this condition.

3.4. Types 2b and 4 hyperlipoproteinemia

We also identified three other subjects (HTG-14, 21, and 22) who harbored deleterious variants in the *GCKR* gene (NM_001486.3:c.307G > A, and NM_001486.3:c.148G > A;), which were possibly associated with this extreme phenotype. Two of these three subjects without any apparent relationship exhibited the identical variant. The follow-up study suggested that both of those variants could cause the dominant form of the HTG phenotype. We also identified one subject (HTG-25) who exhibited type V hyperlipoproteinemia, potentially caused by a missense variant in the *SLC25A40* gene (NM_018843.3:c.314G > A); this subject's mother carried the same variant and also exhibited the HTG phenotype.

4. Discussion

In this study, we investigated the usefulness of Clinical WES for detecting the genetic causes of severe HTG. We successfully identified causative variants in nine (32%) of the 28 probands with severe HTG; these were within known genes to cause rare monogenic hypertriglyceridemia. We applied further bioinformatics methods such as gene prioritization and CNV analyses; however, we were unable to validate any novel genes associated with HTG. In terms of phenotypic characteristics, the subjects with identified causative variants tended to have higher plasma TC and TG levels compared to the other subjects.

Our use of clinical WES enabled us to detect causative variants in 32% of the severe HTG probands in our study. These results were consistent with previous investigations using a targeted gene strategy for extreme cases of HTG [30,31] and other Mendelian diseases [12] with pedigrees. Although we should be cautious about using bioinformatics damaging scores to identify causative variants instead of investigating co-segregation, we were able to detect reasonable causative variants by applying stringent thresholds by high sensitivity and specificity *in silico* damaging predictions (MetaLR MetaSVM) as well as detailed phenotype evaluation of the probands. These findings indicate that DNA testing could be an efficient strategy for revealing a molecular diagnosis of HTG, even for probands with extreme phenotypes such as severe HTG; this could help to guide individualized therapeutic strategies.

Three probands exhibited a recessive inheritance model in the *LPL* and *APOA5* genes, and six showed a dominant model in *GCKR*, *LPL*, and *SLC25A40*. Interestingly, the proband with *APOA5 / LPL* compound heterozygote variants was a novel case jointly associated with increasing plasma TG. Regarding variants in the *LPL* gene, there were two homozygous probands (*LPL* deficiency) and two heterozygous probands, which demonstrated the large phenotypic variation in both lipid profile and *LPL* mass. A number of recent studies have described similar situations where a single variant caused a mild phenotype of a disorder previously known as “recessive,” leading to a paradigm shift for the recognition of traditional Mendelian disorders through comprehensive genetic analyses.

We tried to identify novel molecules associated with plasma TG level by several bioinformatics methods; however, we were unable to validate any novel genes. The *APOB* gene was the top-ranked gene according to the gene prioritization analysis and had a strong correlation with previously known TG-associated genes. Recent Mendelian randomization trials have suggested that apolipoprotein B-containing lipoproteins that include TG are causal factors for coronary artery disease [9]. This suggests that the *APOB* gene could be a reasonable novel therapeutic target for HTG, but in this study, we were unable to validate the causality of *APOB* variants for plasma TG levels by checking the variant-level evaluation. Furthermore, we tried to evaluate the CNVs that were associated with TG, but we could not validate the true CNV signal from the XHMM software by the microarray test. Our results may indicate the difficulty of exploring novel genes associated with extreme phenotypes, even when using promising bioinformatics methods that have been previously reported. In addition, we may consider another etiology of increasing TG by different approaches such as polygenic scores, epigenetics, or whole-genome sequencing.

Regarding phenotypic characteristics, we found that the probands identified with causative variants tended to exhibit higher plasma TC and TG compared to the others. However, there was insufficient power to prove the statistically significant relationships. However, this could indicate that higher plasma TG may be derived from a certain type of genetic variants in subjects with primary HTG. In addition, HTG subjects with hyperchylomicronemia, caused for example by the *LPL* gene, are prone to pancreatitis as well as CAD [32,33]. Thus, identifying causative variants for HTG subjects has the potential to be an effective method to evaluate future life-threatening events, especially pancreatitis and CAD, in the current era of precision medicine [34].

This study had some limitations. First, the novel variants in *GCKR*, *LPL*, and *SLC25A40* identified in the present study were not functionally assessed. However, the combination of *in silico* prediction and the investigation of relatives strongly suggested causality. Second, although some of the dominant forms of co-segregation were confirmed by follow-up studies for relatives of the probands, the relatives harboring the same variants did not always exhibit “severe” HTG, leaving the possibility that some other factors may simultaneously affect the severe phenotypes.

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Disclosures

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2018.10.041.

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