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Genetic Analysis in a Taiwanese Cohort of 750 Index Patients with Clinically Diagnosed Familial Hypercholesterolemia

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Aim: Familial hypercholesterolemia (FH) is underdiagnosed in most countries. The genetic heterogeneity of FH requires an algorithm to efficiently integrate genetic testing into clinical practice. We aimed to report the spectrum of genetic mutations from patients with clinically diagnosed FH in Taiwan.

Methods: Patients with LDL-C>190 mg/dL or those with probable or definite FH according to the Taiwan Lipid Guidelines underwent genetic testing. Samples from 750 index patients from the Taiwan FH registry were screened using custom-made mass spectrometry, followed by targeted next generation sequencing (NGS) and/or multiplex ligation-dependent probe amplification (MLPA) if found negative.

Results: The mean age of the patients was 52.4±15.1 years and 40.9% were male. Mutations were detected in 445 patients (59.3%). The distribution of mutations was as follows: *LDLR* (*n*=395), *APOB* (*n*=58), *PCSK9* (*n*=0), and *ABCG5* (*n*=3). The most common mutations were *APOB* c.10579 C>T (p.R3527W) (12.6%), *LDLR* c.986 G>A (p.C329Y) (11.5%), and *LDLR* c.1747 C>T (p.H583Y) (10.8%). *LDLR* c.1187-10 G>A (IVS 8-10) and *APOB* c.10580 G>A (p.R3527Q) were detected using targeted NGS in Taiwan for the first time. Four novel mutations were identified, including *LDLR* c.1060+2 T>C (IVS 7+2), *LDLR* c.1139 A>C (p.E380A), *LDLR* c.1322 T>C (p.A431T)+c.1867 A>G (p.I623V), and ABCG5 c.1337 G>A (p.R447Q).

Conclusion: *LDLR* and *APOB*, but not *PCSK9*, mutations were the major genetic causes of FH. Four novel mutations in *LDLR* or *ABCG5* were identified. This genetic screening method using mass spectrometry, targeted NGS, and MLPA analysis provided an efficient algorithm for genetic testing for clinically diagnosed FH in Taiwan.

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Key words: Cholesterol, Familial hypercholesterolemia, Gene mutation, Lipids and lipoprotein metabolism, Mass spectrometry

Introduction

Familial hypercholesterolemia (FH) is an autosomal-dominant hereditary lipid disorder that causes increased low-density lipoprotein cholesterol (LDL-C) levels and results in premature coronary artery disease and mortality[1, 2]. FH is known to be caused by mutations in three different genes. The most commonly mutated is the gene coding for low-density lipoprotein receptor (*LDLR*), resulting in defective synthesis, assembly, transport, and recycling of the LDLR. Mutations in apolipoprotein B (*APOB*), encoding the ligand of the LDLR, cause a phenotypically identical condition[3]. Mutations in a third gene, Proprotein Convertase Subtilisin Kexin type 9 (*PCSK9*), which degrades the LDLR, have

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recently been reported in about 1% of FH cases in Caucasians[4] and 8% in Japanese[5], but has not been reported in Taiwanese patients[6].

Identification of patients with FH can be achieved by clinical diagnosis, i.e., by examination of personal and family history. The criteria for the clinical diagnosis of FH have been established and reported by the 2017 Taiwan Lipid Guidelines, a modification of the Dutch Lipid Clinic Network Score (DLCNS) for FH in Taiwan[7]. The diagnosis of FH is dependent on the total scores and can be definite (when the score is more than 8), probable (6-8), or possible (3-5).

The detection rates of FH vary widely across countries, but it is generally underdiagnosed in most countries[4, 8]. In Taiwan, the detection rate of FH has improved from less than 1% in 2013 [4] to 3.8% in 2019 [8]. The Taiwan FH registry, a national, multi-center, observational registry supported by the Taiwan Society of Lipids and Atherosclerosis, has been dedicated in the active recruitment and management of FH individuals since 2016 and has enrolled more than a thousand FH individuals, which may have contributed to the improved detection rate of FH in Taiwan. The identification and early treatment of affected individuals is desirable, and a DNA-based genetic diagnosis provides confirmation of the clinical diagnosis and enables early patient management.

We have developed a custom-made mass spectrometry-based genotyping assay that can simultaneously detect 68 known FH mutations in Taiwan. The initial result for the validation of this assay has been reported in 2017, which showed that the assay sensitivity and specificity were 92.5% and 100%, respectively[9]. Considering its low cost, rapid turnaround time, and flexibility, it has been used as the first line genotyping assay for FH in Taiwan. If the result of this assay returns negative, a more comprehensive targeted next generation sequencing (NGS) of a panel of hypercholesterolemia-related genes, including *LDLR*, *APOB*, *PCSK9*, *LDLRAP1*, *ABCG5*, and *ABCG8*, was performed to detect any novel variations not included in the mutation panels of the mass spectrometry assay. If the targeted NGS result was still negative, multiplex ligation-dependent probe amplification (MLPA) analysis was performed to detect *LDLR* large gene rearrangements, which could not be detected using current NGS technology[10]. This unique algorithm was used for the genetic diagnosis of patients enrolled in the Taiwan FH registry with definite or probable FH **(Fig. 1)**. In the present study, we aimed to report the spectrum of mutations from patients with severe hypercholesterolemia enrolled in the national registry of FH in Taiwan.

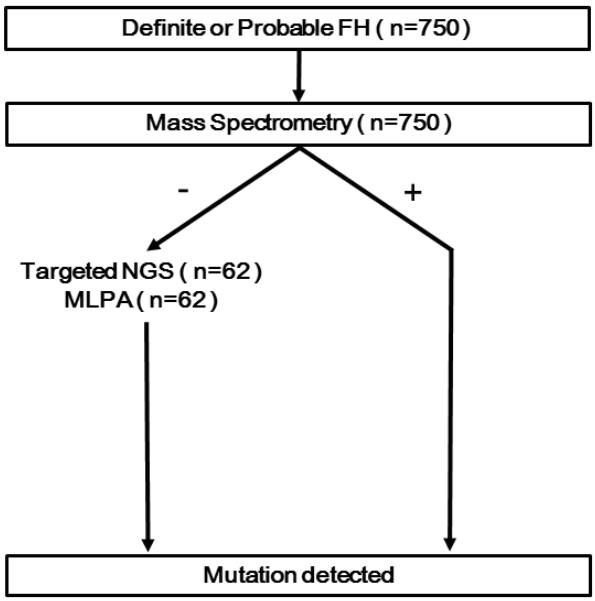


Fig. 1. The algorithm for the genetic diagnosis of patients with definite or probable familial hypercholesterolemia (FH) enrolled in this study

All patients were initially screened by a custom-made mass spectrometry, which can simultaneously detect 68 known FH mutations in Taiwan. If its result was negative and the LDL-C level was >250 mg/dL, a more comprehensive targeted next generation sequencing (NGS), was performed to detect any novel variations not included in the mutation panels of mass spectrometry. If the targeted NGS was still negative, multiplex ligation-dependent probe amplification (MLPA) analysis was necessary to detect *LDLR* large gene rearrangements.

Materials and Methods

Study Subjects

Subjects with severe hypercholesterolemia were referred from the participating hospitals of the Taiwan FH registry. We followed the criteria for the diagnosis of FH according to the 2017 Taiwan Lipids Guideline[7]. Index patients, those who have LDL-C> 190 mg/dL or fit the criteria of probable or definite FH according to the guideline, could be enrolled for genetic testing. Subjects with evidence of secondary hypercholesterolemia, e.g., hypothyroidism, nephrotic syndrome, or diabetes, were excluded. The protocol was approved by the Institutional Review Board of the Taipei Veterans General Hospital and each participating hospital. Informed consent was obtained from each patient.

DNA Extraction

DNA was isolated from 10 mL of venous blood

collected from subjects in the clinic. Genomic DNA was extracted from peripheral leukocytes using a QIAamp DNA Blood Minikit (Qiagen, Hilden, Germany). The yield and purity of each DNA sample was assessed using UV spectrophotometry.

MassARRAY-Based Mutation Detection

The FH mutations to be studied were selected according to the known mutation frequencies from our previous study[9]. Sequences covering the selected alterations were taken from the databases of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/ index.html).

The entire multiplex reaction process was performed according to the manufacturer's instructions, including PCR amplification, shrimp alkaline phosphatase treatment, and primer extension reaction using the iPLEX Gold assay (Agena Bioscience, San Diego, California), which have been described in detail before[9]. Briefly, genomic DNA was amplified via multiplex PCR (PCR Accessory and Enzyme Kit; Agena Bioscience) and unincorporated dNTPs were deactivated. The single-base extension reaction, consisting of the iPLEX enzyme, terminator mix, and extension primer mix, were subject to thermocycling conditions (iPLEX Gold Kit; Agena Bioscience). After desalting, the PCR products were spotted on SpectroCHIP II arrays using a MassARRAY nanodispenser and analyzed using the MassARRAY platform. Mass signals for the different alleles were captured with high accuracy by MALDI-TOF MS, and Typer v4.0 (Agena Bioscience) was used to process the raw data obtained from the assays.

Targeted Next Generation Sequencing for FH-related Genes

If the mass spectrometry genotyping assay failed to detect causative mutations, the high throughput genome sequencer Illumina MiSeq was used to comprehensively determine the DNA sequence of all six well-known hypercholesterolemia-related genes (*LDLR*, *APOB*, *PSCK9*, *LDLRAP1*, *ABCG5*, and *ABCG8*). The target DNA sequences of the candidate regions: coding exons of *LDLR* (exon 1-18), *APOB* (exon 2-29), *PSCK9* (exon 1-12), *LDLRAP1* (exon 2-9), *ABCG5* (exon 1-13), and *ABCG8* (exon 1-13) were enriched using amplicon-based methods. To cover these target regions, 189 amplicons were designed, amplified, purified, and quantified before library construction. PCR primer designs were run through BLAST and necessary modifications were made to avoid the effect of known SNPs and pseudogene amplification. Each PCR reaction at a volume of 25 μL contained 300 μM each of the corresponding primers, 200 ng genomic DNA, and RealStart DNA Polymerase Premix (Yeastern Biotech Co., Ltd., New Taipei City, Taiwan). The PCR conditions were as follows: 94℃ for 15 min, followed by 18 cycles of 94℃ (30s), 60℃ (3 min), and 72℃ (1 min), and a final extension of 72℃ for 7 min. The final pooled amplicons were used to prepare the DNA library for the Illumina sequencer by performing end-repairing, addition of A-overhangs, adaptor ligation, and size selection (150 – 250 bp). Library preparation was carried out using an Illumina TruSeq Nano DNA Library Prep Kit, and the resulting library was pooled equally for sequencing (Illumina MiSeq sequencer, 2×250bp). The raw output of each individual run was approximately 30 Mb, and the average depth of the target regions was >1000×. The sequence of each read was trimmed based on the quality score (Q30). Reads were aligned to the human hg19 reference genome using BWA-MEM (http://bio-bwa.sourceforge.net/) while GATK Unified Genotyper (GATKLite version 2.3–9) was used for calling variants. After variant calling, we used the Illumina VariantStudio 3.0 to annotate the identified variants for the subsequent statistical analyses.

MLPA Analysis

The SALSA P062-D2 LDLR MLPA kit was obtained from MRC-Holland (Amsterdam, the Netherlands). The SALSA MLPA Probemix P062-D2 LDLR contained 33 MLPA probes. This included 20 probes for the *LDLR* gene, one flanking probe upstream of the *LDLR* gene, and 12 reference probes that detect autosomal chromosomal locations. Reactions were carried out in 200-μL tubes with a PTC-225 thermocycler (MJ Research). Genomic DNA (50 – 250 ng) from each subject was diluted in 5 μL of distillation-distillation H2O (ddH_2O) and denatured at 98℃ for 5 min. MLPA buffer and probe mix (1.5 μL each) were then added and the mixture was heated to anneal to the target genomic DNA. Annealed probes were ligated and followed by inactivation at 98℃ for 5 min. The ligation reaction mixture was aliquoted for multiplex amplification using a pair of common primers, one of which was labeled with the fluorescent dye FAM (5-carboxyfluorescein). The addition of Taq polymerase to the reaction was followed by thermocycling. Two microliters of the reaction solution were used for fragment analysis on the 3730xl capillary sequencer (Applied Biosystems, Foster City, California), with LIZ-600size standards (Applied Biosystems). The procedure was performed according to the manufacturer's instructions.

Data analysis of the MLPA was performed using

Table 1. Clinical characteristics of the probands

	Characteristics (n = 750)
Age at recruitment (years)	52.4 ± 15.1
Sex (% male)	40.9
BMI (kg/m^2)	24.8 ± 4.1
TC (mg/dl)	325.2 ± 66.6
LDL-C (mg/dl)	237.9 ± 56.4
HDL-C (mg/dl)	52.7 ± 18.4
TG (mg/dl)	146.3 ± 87
Fasting glucose (mg/dl)	118.4 ± 31.4
Medication at recruitment (%)	
Statins	61%
Ezetmibe	24%
Family history of premature CVD (%)	38.4%
Tendon xanthomas (%)	22.1%

BMI, body mass index; CVD, cardiovascular disease; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol; TG, triglyceride.

Coffalyser.Net software (www.mrcholland.com.). Electropherograms of fragments from the MLPA analysis of *LDLR* from normal subjects showed a profile composed of 33 peaks (range, 136–490 nt). In addition, the multiplex contained nine control fragments, generating an amplification product between 64 and 105 nt. Successful ligation was indicated by a peak (representing the D-fragment, 92 nt) with a size comparable to that of the other chromosome-specific probes in the multiplex. The relative areas under the curve (AUCs) for the peaks in each sample were determined. The relative peak AUC for each probe was calculated using 4-7 adjacent peaks as internal controls. The fraction of each peak was then divided by the median peak fraction of the corresponding fragment from 15 normal control samples. In 15 normal individuals, these calculations gave values close to 1.0, which corresponded to the normalized mean peak area and standard deviations for an individual with two copies of the target sequence. Copy number results >1.3 or <0.70 were flagged. Calculations were performed on samples processed within an assay run.

Variant Classification for Mutations

Variants were analyzed for pathogenicity according to polyphen-2, SIFT, or the recommendations of the American College of Medical Genetics (ACMG). A variant was considered as pathogenic mutation based on the following criteria: (1) in silico prediction (polyphen-2 or SIFT); (2) if it was registered as pathogenic in Clinvar, or HGMD, or meet the criteria of ACMG guideline[11], otherwise, it was classified as variants of unknown significance.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences software (version 20.0, SPSS Inc., Chicago, Illinois). All data were expressed as mean ± standard deviation or frequency (percentage).

Results

Baseline Characteristics of the Patients

From January 2017 to June 2020, a total of 750 patients were referred from hospitals participating in the Taiwan FH registry. The mean age of the patients was 52.4 ± 15.1 years, and 40.9% of them were male. The highest recorded level of LDL-C in the patients before treatment was 237.9 ± 56.4 mg/dL; 61% of them received statins and 24% of them received ezetimibe. A family history of premature cardiovascular disease (CVD) was present in 38.4% of the patients while tendon xanthoma was present in 22.1%. The baseline characteristics of these patients are presented in **Table 1**.

Genetic Diagnosis of the Patients

All 750 patients were initially screened with a custom-made mass spectrometry technique, and genetic mutations were detected in 415 patients. A total of 62 cases with negative mass spectrometry results and LDL-C levels >250 mg/dL were further analyzed with targeted NGS and MLPA. The combination of NGS and MLPA had identified additional 30 mutations, including 25 patients with positive NGS results and 5 patients with positive MLPA. Therefore, genetic mutations were detected in

445 patients (59.3%), including 443 patients with FH mutations. Overall, the distribution of FH mutations was: *LDLR* (n=395), *APOB* (n=58), and *PCSK9* (n=0). In addition, there were 2 cases of sitosterolemia detected by *ABCG5* mutation **(Table 2)**.

The three most common genetic mutations were *APOB* c.10579 C>T (p.R3527W), found in 56 cases (12.6%); *LDLR* c.986 G>A (p.C329Y), found in 51 cases (11.5%); and *LDLR* c.1747 C>T (p.H583Y), found in 48 cases (10.8%) **(Table 2)**. Private mutations, those mutations with a low frequency (less than 1%), were present in 74 cases (23.7%). All private mutations are listed in **Table 2**.

The linked *LDLR* c.1291 G>A (p.A431T)+c.1867 A>G (p.I623V) were novel mutations and were present in two cases (0.4%) in our series. These mutations were classified as pathogenic mutation (p.A431T) and likely pathogenic mutation (p.I623V) by ACMG criteria. One index patient, a 4-year-old boy, presented with xanthoma over both ankles since birth. Three *LDLR* mutations found in this patient were detected simultaneously by mass spectrometry. Based on the Sanger sequencing analysis of DNA collected from his family, we confirmed that *LDLR* c.1291 G>A (p.A431T) and *LDLR* c.1867 A>G (p.I623V) were in one allele and *LDLR* c.1747 C>T (p.H583Y) was in the other allele **(Fig. 2)**.

Genetic Mutations Diagnosed by NGS

A total of 62 cases with negative mass spectrometry results and LDL-C levels >250 mg/dL were further analyzed with targeted NGS. An additional 25 patients with positive genetic mutations were identified with this method **(Table 2)**. The *LDLR* c.1187-10 G>A (G>A at IVS 8-10) is a known *LDLR* pathogenic mutation and has been reported before, however it was first identified in our series by targeted NGS **(Table 2)**. The *LDLR* c.1060+2 T>C (T>C at IVS 7+2) is a novel intronic mutation of *LDLR*, and was classified as a pathogenic mutation by ACMG criteria. The proband was a 32-year-old male with an untreated total cholesterol level of 362 mg/dL and LDL-C level of 294 mg/dL. His mother, a 57-year-old with a positive mutation, had an untreated total cholesterol level of 382 mg/dL and LDL-C level of 263 mg/dL. His 55-year-old aunt, with a negative mutation, had total cholesterol of 202 mg/dL and an LDL-C level of 103 mg/dL. Therefore, the *LDLR* c.1060+2 T>C mutations co-segregated well with LDL-C levels, at least in this small family study **(Fig. 3)**.

Two *LDLR* variants identified using targeted NGS showed uncertain pathogenicity as estimated by polyphen-2 and SIFT in silico analyses **(Table 2)**. Both *LDLR* c.811 G>A (p.V271I) and *LDLR* c.1139 A>C (p.E380A) were predicted as benign based on in silico analysis. They were classified as likely benign (p.V271I) and likely pathogenic mutation (p.E380A) by ACMG criteria. However, the *LDLR* c.1139 A>C (p.E380A) did not co-segregate well with the phenotype of hypercholesterolemia. A 77-year-old female proband has an LDL-C level of 199 mg/dL and her daughter's LDL-C level was 193 mg/dL. However, her granddaughter with positive mutation had an LDL-C level of 108 mg/dL, which did not support the pathogenicity of *LDLR* c.1139 A>C (p.E380A) **(Fig. 4)**.

The *APOB* c.10580 G>A (p.R3527Q) mutation was first detected in two probands (0.4%) using targeted NGS in our series **(Table 2)**. Although both were referred from the same lipid clinic in Tainan city, they did not have clear relationship with each other.

Genetic Mutations Diagnosed by MLPA

Genetic analysis using MLPA was necessary if both mass spectrometry and targeted NGS examinations were negative for *LDLR* mutations. Additional 5 *LDLR* large insertions/deletions were detected through MLPA analysis, including three exon deletions and two duplications. There probands were one male and four females. Xanthoma was present in two and premature CVD in three of these probands. The untreated LDL-C levels ranged from 261 to 588 mg/dL. Each of these deletion/duplication mutations was rare and detected in only a single proband **(Table 2 and Table 3)**.

Homozygous FH in Taiwan

There were 14 cases of homozygous FH detected in this series. Thirteen of these were diagnosed by mass spectrometry only. One proband was compound heterozygous with the *LDLR*-R257W+D589N / IVS 4+2 T>C mutation, with a linked R257W+D589N mutation detected by mass spectrometry and IVS 4+2 T>C detected by targeted NGS. We detected five simple homozygous mutations, including one *APOB* c.10579 C>T (p.R3527W) homozygote, seven compound heterozygotes of *LDLR*, and two double heterozygotes (*LDLR* and *APOB*). The linked R257W+D589N mutation was present in three compound heterozygous patients (patients No. 2, No. 4, and No. 9), since it was a frequent mutation and was present in 23 probands (5.2%) in this series **(Table 2)**. Patient No. 9 had two linked double mutations: *LDLR* c.769 C>T (p.R257W)+c.1765 C>A (p.D589N) in one allele and *LDLR* c.1322 T>C (p.A431T)+c.1867 A>G (p.I623V) in another allele. The clinical characteristics and genetic information of these

Table 2. Frequencies of all detected mutations in this cohort

Gene	Mutation	Designation	Region affected	Frequency (*n*, %)	Polyphen-2	SIFT	ACMG	dbSNP	Methodology*
APOB	NM_000384.2: c.10579 C>T	R3527W	Exon 26	56 (12.6%)	Probably damaging (score: 1.000)	Damaging (score: 0.056)	Pathogenic	rs144467873	1
LDLR	NM_000527.4: c.986 G>A	C329Y	Exon 07	51 (11.5%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Pathogenic	rs761954844	1
LDLR	NM_000527.4: c.1747 C>T	H583Y	Exon 12	48 (10.8%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Pathogenic	rs730882109	1
LDLR	NM_000527.4: c.268 G>A	D90N	Exon 03	26 (5.8%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Pathogenic	rs749038326	1
LDLR	NM_000527.4: c.1432 G>A	G478R	Exon 10	26 (5.8%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Likely Pathogenic	rs144614838	1
LDLR	NM_000527.4: c.769 C>T+ c.1765 G>A	R257W+D589N	Exon 05+12	23 (5.2%)	Probably damaging (score: 0.993) + Probably damaging (score: 0.998)	Damaging (score: 0.006) + Tolerated (score=0.083)	Likely Pathogenic + Uncertain Significance	rs200990725 + rs201971888	1
LDLR	NM_000527.4: c.2054 C>T	P685L	Exon 14	15 (3.4%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Pathogenic	rs28942084	1
LDLR	NM_000527.4: c.1241 T>G	L414R	Exon 09	13 (2.9%)	Probably damaging (score: 0.957)	Damaging (score: 0)	Pathogenic	rs748554592	1
LDLR	NM_000527.4: c.1246 C>T	R416W	Exon 09	11 (2.5%)	Probably damaging (score: 0.998)	Damaging (score: 0)	Pathogenic	rs570942190	1
LDLR	NM_000527.4: c.1953,1954 del [TA]	M652Fs	Exon 13	11 (2.5%)	-	-	Pathogenic	rs875989935	1
LDLR	NM_000527.4: c.1322 T>C	I441T	Exon 09	10 (2.2%)	Probably damaging (score: 0.996)	Damaging (score: 0.001)	Pathogenic	rs879254862	1
LDLR	NM_000527.4: c.1016 T>G	L393L	Exon 07	9 (2.0%)	-	-	Pathogenic	-	1
LDLR	NM_000527.4: c.1867 A>G	I623V	Exon 13	7 (1.8%)	Benign (score: 0)	Tolerated (score=0.902)	Likely Pathogenic	rs555292896	1
LDLR	NM_000527.4: c.1174 Ins[T]	C392Fs	Exon 08	6 (1.6%)	-	-	Pathogenic	rs879254813	1
LDLR	NM_000527.4: c.2389 G>A	V797M	Exon 16	6 (1.3%)	Benign (score: 0.449)	Tolerated (score=0.056)	Pathogenic	-	1
LDLR	NM_000527.4: c.510 del [C]	P171Fs	Exon 04	5 (1.1%)	-	-	Pathogenic	-	1
LDLR	NM_000527.4: c.1448 G>A	W483X	Exon 10	5 (1.1%)	-	-	Pathogenic	rs875989921	1
LDLR	NM_000527.4: c.1474 G>A	D492N	Exon 10	5 (1.1%)	Probably damaging (score: 1.000)	Damaging (score: 0.024)	Pathogenic	rs373646964	1
LDLR	NM_000527.4: c.1879 G>A	A627T	Exon 13	5 (1.1%)	Possibly damaging (score: 0.847)	Damaging (score: 0.001)	Pathogenic	rs879255066	1
LDLR	NM_000527.4: c.599 T>G	F200C	Exon 04	4 (0.9%)	Possibly damaging (score: 0.796)	Tolerated (score=0.1)	Likely Pathogenic	rs879254586	1
LDLR	NM_000527.4: c.1186+2 T>G	IVS8+2 T>G	Intron 08	4 (0.9%)	-	-	Pathogenic	rs779921498	2
LDLR	NM_000527.4: c.1268 T>C	I423T	Exon 09	4 (0.9%)	Possibly damaging (score: 0.845)	Damaging (score: 0.001)	Pathogenic	rs879254849	1
LDLR	NM_000527.4: c.1723 C>T	L575F	Exon 11	4 (0.9%)	Probably damaging (score: 1.000)	Damaging (score: 0.001)	Pathogenic	rs1205480064	1
LDLR	NM_000527.4: c.516 C>G	D172E	Exon 04	3 (0.7%)	Probably damaging (score: 0.997)	Damaging (score: 0.045)	Pathogenic	rs879254557	1
LDLR	NM_000527.4: c.694 +2 T>C	IVS4+2 T>C	Intron 04	3 (0.7%)	-	-	Pathogenic	rs200238879	2
LDLR	NM_000527.4: c.1016 T>C	L339P	Exon 07	3 (0.7%)	Probably damaging (score: 0.985)	Damaging (score: 0)	Pathogenic	-	1
LDLR	NM_000527.4: c.1291 G>A	A431T	Exon 09	3 (0.7%)	Probably damaging (score: 1.000)	Damaging (score: 0.001)	Pathogenic	rs28942079	1
LDLR	NM_000527.4: c.1691 A>G	N564S	Exon 11	3 (0.7%)	Probably damaging (score: 1.000)	Damaging (score: 0.039)	Pathogenic	rs758194385	1

(Cont. Table 2)

Gene	Mutation	Designation	Region affected	Frequency (*n*, %)	Polyphen-2	SIFT	ACMG	dbSNP	Methodology*	
LDLR	NM_000527.4: c.68-2 A>C	IVS2-2 A>C	Intron 01	2 (0.4%)	-	-	Likely Pathogenic	-	1	
LDLR	NM_000527.4: c.190 +4 A>T	IVS2+4 A>T	Intron 02	2 (0.4%)	-	-	Pathogenic	rs769446356	2	
LDLR	NM_000527.4: c.338 del [AGTTTC] ins T	E113Fs	Exon 04	2 (0.4%)	-	-	Pathogenic	-	1	
LDLR	NM_000527.4: c.940 +1 G>R	IVS6+1 G>A	Intron 06	2 (0.4%)	-	-	Pathogenic	rs879254729	1	
LDLR	NM_000527.4: c.1216 C>T	R406W	Exon 09	2 (0.4%)	Probably damaging (score: 1.000)	Tolerated (score=1)	Pathogenic	-	1	
LDLR	NM_000527.4: c.1291 G>A+ c.1867 A>G	A431T+I623V	Exon 09+13	2 (0.4%)	Probably damaging (score: 1.000) + Benign (score: 0.000)	Damaging (score: 0.001) + Tolerated (score=0.902)	Pathogenic + Likely Pathogenic	rs28942079 / rs555292896	1	Novel
LDLR	NM_000527.4: c.1420 C>Y	Q474X	Exon 10	2 (0.4%)	-	-	Pathogenic	rs201967266	1	
LDLR	NM_000527.4: c.1592 T>A	M531K	Exon 11	2 (0.4%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Likely Pathogenic	*--	1	
LDLR	NM_000527.4: c.1609 G>T	G537X	Exon 11	2 (0.4%)	-	-	Pathogenic	rs879254958	1	
LDLR	NM_000527.4: c.1618 G>A	A540T	Exon 11	2 (0.4%)	Probably damaging (score: 1.000)	Damaging (score: 0.002)	Pathogenic	-	1	
LDLR	NM_000527.4: c.1807 A>T	K603X	Exon 12	2 (0.4%)	-	-	Pathogenic	rs879255029	1	
APOB	NM_000384.2: c.10580 G>A	R3527Q	Exon 26	2 (0.4%)	Probably damaging (score: 1.000)	Damaging (score: 0.039)	Likely Pathogenic	rs5742904	2	
LDLR	NM_000527.4: c.64 del [G]	A22Fs	Exon 01	1 (0.2%)	-	-	Pathogenic	rs879254393	1	
LDLR	NM_000527.4: c.101 G>C	C34S	Exon 02	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Likely Pathogenic	rs879254406	1	
LDLR	NM_000527.4: c.253 C>T	Q85X	Exon 03	1 (0.2%)	-	-	Pathogenic	rs875989893	2	
LDLR	NM_000527.4: c.310 T>C	C104R	Exon 03	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Pathogenic	rs879254464	1	
LDLR	NM_000527.4: c.313 +1 G>A	IVS3+1 G>A	Intron 03	1 (0.2%)	-	-	Pathogenic	rs112029328	1	
LDLR	NM_000527.4: c.344 G>A	R115H	Exon 04	1 (0.2%)	Probably damaging (score: 1.000)	Tolerated (score=0.069)	Likely Pathogenic	rs201102461	1	
LDLR	NM_000527.4: c.536 A>C	E179A	Exon 04	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Pathogenic	-	1	
LDLR	NM_000527.4: c.562 del[T]	Y188Fs	Exon 04	1 (0.2%)	-	-	Pathogenic	-	1	
LDLR	NM_000527.4: c.590 G>A	C197Y	Exon 04	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Pathogenic	rs376459828	1	
LDLR	NM_000527.4: c.626 G>A	C209Y	Exon 04	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Pathogenic	rs879254600	1	
LDLR	NM_000527.4: c.664 T>C	C222R	Exon 04	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Pathogenic	rs577934998	2	
LDLR	NM_000527.4: c.681 C>A	D227E	Exon 04	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0.001)	Pathogenic	rs121908028	1	
LDLR	NM_000527.4: c.682 G>T	E228X	Exon 04	1 (0.2%)	-	-	Pathogenic	rs121908029	1	
LDLR	NM_000527.4: c.799 G>T	E267X	Exon 05	1 (0.2%)	-	-	Pathogenic	-	1	
LDLR	NM_000527.4: c.811 G>A	V271I	Exon 05	1 (0.2%)	Benign (score: 0.000)	Tolerated (score=0.253)	Likely Benign	rs749220643	2	

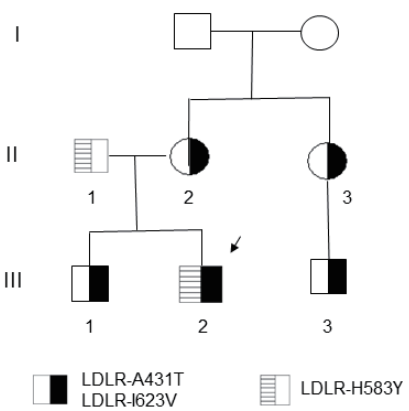
(Cont. Table 2)

Gene	Mutation	Designation	Region affected	Frequency (*n*, %)	Polyphen-2	SIFT	ACMG	dbSNP	Methodology*	
LDLR	NM_000527.4: c.817 +1 G>A	IVS5+1 G>A	Intron 05	1 (0.2%)	-	-	Pathogenic	rs879254685	1	
LDLR	NM_000527.4: c.828 C>A	C276X	Exon 06	1 (0.2%)	-	-	Likely Pathogenic	rs146651743	1	
LDLR	NM_000527.4: c.947 A>G	N316S	Exon 07	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0.002)	Pathogenic	rs730882094	1	
LDLR	NM_000527.4: c.1048 C>T	R350X	Exon 07	1 (0.2%)	-	-	Pathogenic	-	1	
LDLR	NM_000527.4: c.1054 T>A	C352S	Exon 07	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Pathogenic	rs879254769	1	
LDLR	NM_000527.4: c.1057 G>A	E353K	Exon 07	1 (0.2%)	Benign (score: 0.437)	Tolerated (score=0.187)	Likely Pathogenic	rs370471092	1	
LDLR	NM_000527.4: c.1060+2 T>C	IVS7+2 T>C	Intron 07	1 (0.2%)	-	-	Pathogenic	rs774069731	2	Novel
LDLR	NM_000527.4: c.1139 A>C	E380A	Exon 08	1 (0.2%)	Benign (score: 0.009)	Tolerated (score=0.411)	Likely Pathogenic	-	2	Novel
LDLR	NM_000527.4: c.1187-10 G>A	IVS8-10 G>A	Intron 08	1 (0.2%)	-	-	Likely Pathogenic	rs765696008	2	
LDLR	NM_000527.4: c.1195 G>A	A399T	Exon 09	1 (0.2%)	Probably damaging (score: 0.993)	Damaging (score: 0.006)	Pathogenic	rs730882099	1	
LDLR	NM_000527.4: c.1222 G>A	E408K	Exon 09	1 (0.2%)	Probably damaging (score: 0.995)	Damaging (score: 0.004)	Pathogenic	rs137943601	2	
LDLR	NM_000527.4: c.1247 G>T	R416L	Exon 09	1 (0.2%)	Benign (score: 0.144)	Damaging (score: 0)	Pathogenic	-	1	
LDLR	NM_000527.4: c.1285 G>T	V429L	Exon 09	1 (0.2%)	Benign (score: 0.307)	Damaging (score: 0.016)	Pathogenic	rs28942078	2	
LDLR	NM_000527.4: c.1384 G>A	V462I	Exon 10	1 (0.2%)	Benign (score: 0.005)	Tolerated (score=0.434)	Uncertain Significance	rs750363970	1	
LDLR	NM_000527.4: c.1552 A>G	K518E	Exon 10	1 (0.2%)	Possibly damaging (score: 0.5166)	Tolerated (score=1)	Likely Pathogenic	rs879254937	1	
LDLR	NM_000527.4: c.1597 T>C	W533R	Exon 11	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Pathogenic	rs879254951	1	
LDLR	NM_000527.4: c.1661 C>T	S554L	Exon 11	1 (0.2%)	Possibly damaging (score: 0.685)	Tolerated (score=0.167)	Uncertain Significance	rs879254976	1	
LDLR	NM_000527.4: c.1693 G>C	G565R	Exon 11	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Pathogenic	-	1	
LDLR	NM_000527.4: c.1706-1 G>A	IVS12-1 G>A	Intron 11	1 (0.2%)	-	-	Pathogenic	rs879254996	2	
LDLR	NM_000527.4: c.1721 G>A	R574H	Exon 12	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Pathogenic	rs777188764	2	
LDLR	NM_000527.4: c.1726 del [T]	Y576Fs	Exon 12	1 (0.2%)	-	-	Pathogenic	-	1	
LDLR	NM_000527.4: c.1783 C>T	R595W	Exon 12	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0)	Likely Pathogenic	rs373371572	1	
LDLR	NM_000527.4: c.1851-1862 del [AGTATTTTGGAC]	597-600delVFWT	Exon 13	1 (0.2%)	-	-	Likely Pathogenic	-	1	
LDLR	NM_000527.4: c.1988-1 G>C	IVS14-1 G>C	Intron 13	1 (0.2%)	-	-	Pathogenic	rs1555807335	1	
LDLR	NM_000527.4: c.2096 C>T	P699L	Exon 14	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0.001)	Likely Pathogenic	rs201573863	1	
LDLR	NM_000527.4: c.2099 A>G	D700G	Exon 14	1 (0.2%)	Probably damaging (score: 0.999)	Damaging (score: 0.003)	Likely Pathogenic	rs879255139	1	
LDLR	NM_000527.4: c.2140 G>T	E714X	Exon 14	1 (0.2%)	-	-	Pathogenic	rs869320652	2	
LDLR	NM_000527.4: c.2215 C>T	Q739X	Exon 15	1 (0.2%)	-	-	Likely Pathogenic	rs370018159	1	

(Cont. Table 2)

Gene	Mutation	Designation	Region affected	Frequency (*n*, %)	Polyphen-2	SIFT	ACMG	dbSNP	Methodology*	
LDLR	NM_000527.4: c.2446 A>T	K816X	Exon 17	1 (0.2%)	-	-	Pathogenic	rs879255213	1	
LDLR	NM_000527.4: c.817-?_?del	Exon 6-18 del	-	1 (0.2%)	-	-	Pathogenic	-	3	
LDLR	NM_000527.4: c.941-?_1186+?del	Exon 7-8 del	-	1 (0.2%)	-	-	Pathogenic	-	3	
LDLR	NM_000527.4: c.1187-?_2140+?del	Exon 9-14 del	-	1 (0.2%)	-	-	Pathogenic	-	3	
LDLR	NM_000527.4: c.1-?_940+?dup	Exon 1-6 dup	-	1 (0.2%)	-	-	Pathogenic	-	3	
LDLR	NM_000527.4: c.68-?_940+?dup	Exon 2-6 dup	-	1 (0.2%)	-	-	Pathogenic	-	3	
ABCG5	NM_022436.2: c.1166 G>A	R389H	Exon 09	1 (0.2%)	Probably damaging (score: 1.000)	Tolerated (score: 0.095)	Likely Pathogenic	rs119480069	2	
ABCG5	NM_022436.2: ABCG5 c.1336 C>T	R446X	Exon 10	1 (0.2%)	-	-	Pathogenic	rs199689137	2	
ABCG5	NM_022436.2: ABCG5 c.1337 G>A	R446Q	Exon 10	1 (0.2%)	Probably damaging (score: 1.000)	Damaging (score: 0.000)	Uncertain Significance	-	2	Novel

*Methodology used for genetic detection: 1: mass spectrometry, 2: next generation sequencing, 3: MLPA



Family member	Age	CVD	Tendon Xanthoma	TC (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	TG (mg/dL)
II-1	44	-	-	316	252	40	136
II-2	34	-	-	272	228	36	144
II-3	32	-	-	274	199	41	107
III-1	6	-	-	285	231	45	85
III-2	4	-	+	380	334	38	62
III-3	14	-	-	230	172	50	46

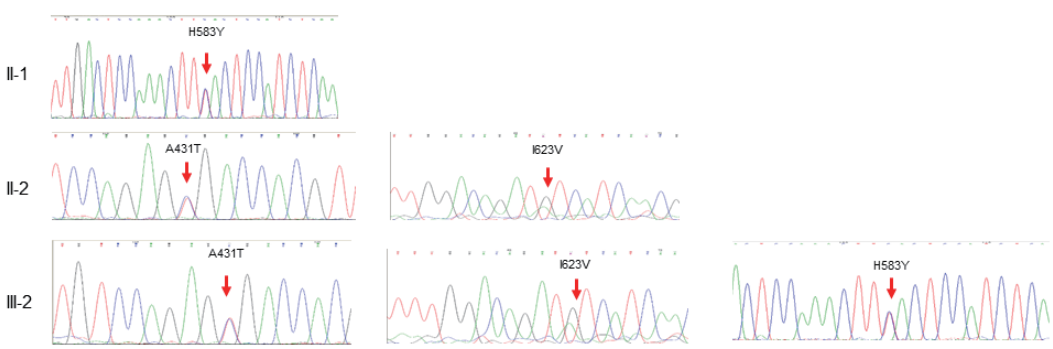
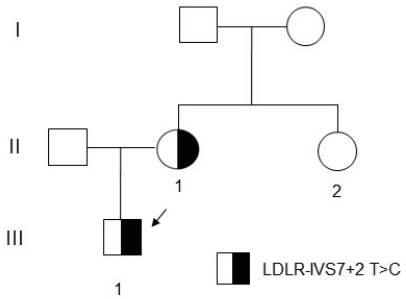


Fig. 2. Genetic diagnosis of a family with *LDLR* mutation

The index patient was a 4-year-old boy that presented with xanthoma over both ankles since birth. Genetic testing showed three *LDLR* mutations detected by mass spectrometry. Based on the Sanger sequencing analysis of DNA collected from his family, the *LDLR* c.1322 T>C (p.A431T) + *LDLR* c.1867 A>G (p.I623V) are in one allele and *LDLR* c.1747 C>T (p.H583Y) is in the other allele. CVD, cardiovascular disease; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol; TG, triglyceride.



Family member	Age	CVD	Tendon Xanthoma	TC (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	TG (mg/dL)
Ⅱ-1	57	-	-	382	263	39	334
Ⅱ-2	55	-	-	202	103	40	290
Ⅲ-1	32	+	-	362	294	45	59

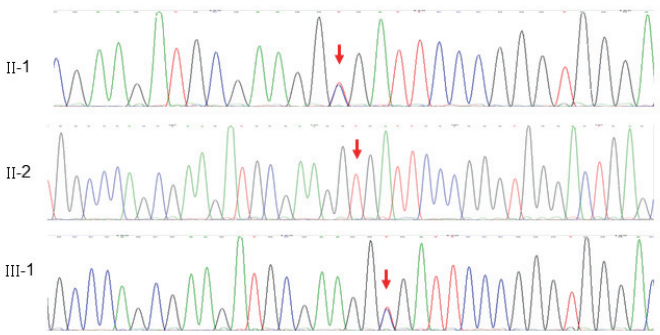
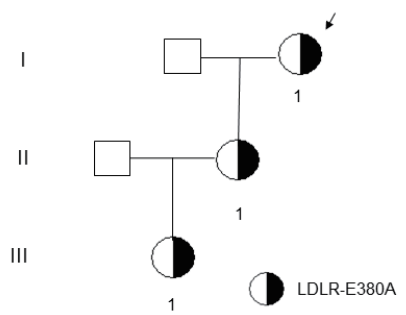


Fig. 3. Family pedigree of *LDLR* c.1060 + 2 T > C (T > C at IVS 7 + 2)

The index patient was genetically diagnosed as *LDLR* c.1060+2 T > C (T > C at IVS 7+2), which was a novel intronic mutation of *LDLR*. The *LDLR* c.1060 + 2 T > C mutations co-segregated well with LDL-C levels in this family study. CVD, cardiovascular disease; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol; TG, triglyceride.



Family member	Age	CVD	Tendon Xanthoma	TC (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	TG (mg/dL)
Ⅰ-1	77	-	-	309	199	60	246
Ⅱ-1	52	-	-	310	193	72	120
Ⅲ-3	25	-	-	198	108	54	254

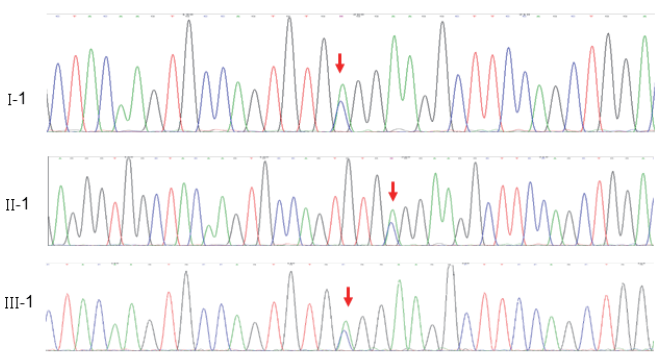


Fig. 4. Family pedigree of *LDLR* c.1139 A > C (p.E380A)

The index patient was genetically diagnosed as *LDLR* c.1139 A > C (p.E380A), which was novel and has not been reported before. However, this genotype did not co-segregate well with the phenotype of hypercholesterolemia in this family. CVD, cardiovascular disease; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol; TG, triglyceride.

Table 3. LDLR mutations identified by MLPA analysis

Age	Sex	LDL-C	Premature CVD	Xanthoma	cDNA (HGVS)		No. of proband
60	F	588	Y	Y	Exon 6-18 deletion	NM_000527.4 : c.817-?_?del	1
46	F	261	N	N	Exon 7-8 deletion	NM_000527.4 : c.941-?_1186+?del	1
42	F	282	N	N	Exon 9-14 deletion	NM_000527.4: c.1187-?_2140+?del	1
66	F	341	Y	N	Exon 1-6 duplication	NM_000527.4 : c.1-?_940+?dup	1
45	M	448	Y	Y	Exon 2-6 duplication	NM_000527.4 : c.68-?_940+?dup	1

CVD, cardiovascular disease; LDL-C, low-density lipoprotein-cholesterol

patients are presented in **Table 4**.

Sitosterolemia in Taiwan

In our NGS panel, we also included *ABCG5* and *ABCG8*, which could help us to identify sitosterolemia in Taiwan. In our study, two patients with clinical diagnosed FH were diagnosed to be sitosterolemia. Three *ABCG5* mutations, including *ABCG5* c.1166 G>A (p.R389H), *ABCG5* c.1336 C>T (p.R446X), and a novel *ABCG5* c.1337 G>A (p.R446Q), were detected using targeted NGS in two hypercholesterolemic probands. These three ABCG5 mutations were predicted as probably damaging by polyphen-2 analysis **(Table 2)**. They were classified as likely pathogenic mutation (p.R389H), pathogenic mutation (p.R446X), and uncertain significance (p.R446Q) by ACMG criteria. The first patient, a 2-year-old boy, presented with total cholesterol of 791 mg/dL, triglycerides of 197 mg/dL, HDL-C of 42.4 mg/dL, and LDL-C of 591 mg/dL. He was diagnosed as compound heterozygous with the mutations *ABCG5* c.1336 C>T (p.R446X) and *ABCG5* c.1337 G>A (p.R446Q). The second patient, a 5-year-old girl, presented with a total cholesterol of 514 mg/dL, triglycerides of 92 mg/dL, HDL-C of 57 mg/dL, and LDL-C of 445 mg/dL. Her genotype was also compound heterozygous, containing the *ABCG5* c.1166 G>A (p.R389H) and *ABCG5* c.1337 G>A (p.R446Q) mutations. Both the parents of these two probands had normal cholesterol levels; however, we have not checked their serum plant sterol concentrations.

Discussion

All patients with severe hypercholesterolemia, referred from each participating hospital of the Taiwan FH Registry, were initially screened by a custom-made mass spectrometry assay. If the result was negative, targeted NGS and MLPA analysis were performed. Genetic mutations were detected in 59.3% of the patients. These mutations were detected in *LDLR* (395 cases, 86%), *APOB* (58 cases, 13 %), but not in *PCSK9*. The three most common genetic mutations were *APOB* c.10579 C>T (p.R3527W) (12.6%),

Table 4. Clinical characteristics and mutations of probands with homozygous FH (*N*=14)

Patient No.	1	2	3	4	5	6	7
Age	34	64	48	45	48	63	65
Sex	F	F	M	M	F	F	M
TC (mg/dL)	583	442	377	517	420	448	550
LDL-C (mg/dL)	467	354	306	432	308	322	362
HDL-C (mg/dL)	57	32	40	37	40	57	93
TG (mg/dL)	88	111	229	241	150	162	81
Xanthoma	Y	N	N	Y	N	N	N
CVD	N	Y	N	N	Y	N	Y
Mutations	P685L	R257W+D589N IVS3+3 A>TA	H583Y	R257W+D589N IVS4+2 T>C	D90N *APOB*-R3527W	G478R F200C	D90N *APOB*-R3527W
FH classification	Simple	Compound	Simple	Compound	Double	Compound	Double
Genetic tests	MS	MS	MS	MS+NGS	MS	MS	MS

Patient No.	8	9	10	11	12	13	14
Age	46	31	32	25	45	74	50
Sex	M	M	M	M	M	M	F
TC (mg/dL)	313	474	285	322	369	416	352
LDL-C (mg/dL)	210	382	235	276	265	316	301
HDL-C (mg/dL)	45	49	39	27	35	54	26
TG (mg/dL)	112	53	137	71	348	319	145
Xanthoma	N	N	N	N	N	N	N
CVD	N	N	Y	Y	N	Y	N
Mutations	D90N I623V	R257W+D589N A431T+I623V	*APOB*-R3527W	IVS2+4 A>T	L339P I623V	R416W H583Y	IVS2+4 A>T
FH classification	Compound	Compound	Simple	Simple	Compound	Compound	Simple
Genetic tests	MS	MS	MS	MS	MS	MS	MS

CVD, cardiovascular disease; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; MS, mass spectrometry; NGS, next generation sequencing; TC, total cholesterol; TG, triglyceride.

LDLR c.986 G>A (p.C329Y) (11.5%), and *LDLR* c.1747 C>T (p.H583Y) (10.8%). *LDLR* c.1187-10 G>A (IVS 8-10) and *APOB* c.10580 G>A (p.R3527Q) mutations, which have not been reported in Taiwan, were detected by targeted NGS for the first time in our study. Four novel mutations, including *LDLR* c.1060+2 T>C (IVS 7+2), *LDLR* c.1139 A>C (p.E380A), *LDLR* c.1322 T>C (p.A431T)+c.1867 A>G (p.I623V), and *ABCG5* c.1337 G>A (p.R447Q) were identified by targeted NGS examination. In addition to FH, two patients with clinical diagnosed FH were diagnosed to be sitosterolemia by *ABCG5* mutation.

Numerous genetic mutations of *LDLR* have been reported[9, 10]. This genetic heterogeneity of *LDLR* was also present in this series. The pattern and frequency of major *LDLR* mutations were similar to those from a previous systematic review of FH from China, Hong Kong and Taiwan[12]. However, the mutation profiles are different from those in Japanese patients with heterozygous FH[5].

The *LDLR* c.1187-10 G>A (G>A at IVS 8-10) is an intronic mutation located at the poly-pyrimidine tract of intron 8 of *LDLR*. The *LDLR* c.1187-10 G>A mutation creates a splicing donor site which is eight nucleotides upstream the previous splicing donor site. This eight-nucleotide insertion in the 5′ region of exon 9 causes a frameshift and creation of premature stop codon in exon 9 of *LDLR*. This is a known *LDLR* pathogenic mutation and has been reported before[13], however it was first identified in our series by targeted NGS.

The linked *LDLR* c.1291 G>A (p.A431T)+c.1867 A>G (p.I623V) were novel and have not been reported before. Interestingly, a single *LDLR* c.1867 A>G (p.I623V) mutation was present in seven cases (1.8%) and the single *LDLR* c.1291 G>A (p.A431T) mutation was present in three cases (0.7%), both of which were not rare in our series **(Table 2)**. This combination of linked mutations suggested that the *LDLR* 431 and *LDLR* 623 position might be hotspots for new mutations or through DNA recombination.

Two novel *LDLR* mutations were identified by targeted NGS in this series. The *LDLR* c.1060+2 T>C (T>C at IVS 7+2) was an intronic mutation located at intron 7 of *LDLR*. It co-segregated well with high plasma LDL-C levels in an index case and his family. The *LDLR* c.1139 A>C (p.E380A) was a missense mutation at the coding region in exon 8 of *LDLR*. It was predicted as benign and was tolerated by polyphen-2 and SIFT analysis, respectively. Furthermore, it did not co-segregate well with high plasma LDL-C levels in the index family. Therefore, further genetic testing is necessary for this family.

The *APOB* c.10579 C>T (p.R3527W) was the most common mutation in Taiwan, the causative mutation in 56 (12.6%) patients in this cohort. This finding was similar to that from our previous study[14]. In a study conducted in Hong Kong, *APOB* c.10579 C>T was identified in 6 (6.3%) probands among 96 Chinese participants with clinical FH[15]. However, *APOB* mutation was very rare in Japan. *APOB* c.10579 C>T had not been reported in Japan before[16, 17].

The *APOB* c.10580 G>A (p.R3527Q) mutation has not been reported in Taiwan before, but was a quite prevalent mutation in the Caucasian population[4, 18, 19]. By performing targeted NGS of 27 genes involved in lipid metabolism in 1,528 referral patients with LDL-C levels greater than 5 mmol/L, Reeskamp *et al.* reported heterozygous FH mutations in 227 participants, including *LDLR* mutations in 182 participants (80.2%), *APOB* in 33 (14.5%), and *PCSK9* in 12 (5.3%)[18]. In the Copenhagen General Population Study, the prevalence of the *APOB* c.10580 G>A variant was 0.11% (1:884) among nearly 100,000 Danish subjects[19].

In Hong Kong, both *APOB* c.10579 C>T and *APOB* c.10580G>A were identified in 6 (6.3%) index patients among 96 with clinical FH[15]. Recently, a Japanese study reported the first case of *APOB* c.10580G>A pathogenic variants as identified by whole-exome sequencing in patients with FH with no pathogenic variants in the *LDLR* and *PCSK9* genes[20]. *APOB* c.10580 G>A has not been reported in a Taiwan series[14] and it was, for the first time, detected in two probands (0.4%) by targeted NGS **(Table 2)**. Both probands were referred from the same lipid clinic in Tainan city; however, they did not have clear relationship with each other. The origin of this *APOB* mutation was unknown and might be imported from other countries, since southern Taiwan has been ruled by the Netherlands for 38 years from 1624 to 1662.

Mutations in *PCSK9* have been reported in around 1-5% of FH-mutation positive patients in Western countries, and this rate varies geographically[4, 18]. *PCSK9* mutations are common in Japan but are uncommon in China. In a study conducted in the Hokuriku district of Japan, 25 patients with clinical homozygous FH received genetic analysis. A *PCSK9* mutation was identified in five patients (*PCSK9* c.94G>A, p.E32K), including two true homozygotes and three compound heterozygotes (*LDLR* and *PCSK9*)[21]. In another recent study from Japan, 801 clinically diagnosed patients with heterozygous FH were analyzed for *LDLR* and *PCSK9* mutations. *PCSK9* pathogenic variants were identified in 51 patients (7.8%) out of the 650 unrelated patients with FH. The study found that *PCSK9* c.94G>A (p.E32K) was the most frequently detected pathogenic *PCSK9* variant in the Japanese FH population[5]. In a multi-center study in China, *PCSK9* gene variants were identified in 4 (2.70%) index cases among 148 cases with detected mutations[22]. Until now, there is still no *PCSK9* pathogenic mutation detected in Taiwan.

The *ABCG5* or *ABCG8* variation is associated with increased intestinal absorption of cholesterol, contributing to hypercholesterolemia[23]. Sitosterolemia is a rare autosomal recessive disorder caused by several functional mutations in *ABCG5/G8*, which are characterized by an increase of phytosterols in the blood, up to 30 times greater than normal[24]. In a Spanish FH cohort, *ABCG5/G8* gene mutations were detected in 8 patients (3.73%) out of 214 patients with mutation-negative FH[25]. In a large European cohort of 3,031 patients with clinical FH referred to the Amsterdam University Medical Center, about 2.44% carried putative pathogenic *ABCG5* (1.48%) and *ABCG8* (0.96%) variants. The study found that heterozygous carriers of *ABCG5* or *ABCG8* variants had lower LDL-C levels compared to heterozygous carriers of an *LDLR* variant. Furthermore, heterozygosity for mutations in *ABCG5* or *ABCG8* did not contribute to a significant effect on LDL-C levels on top of an *LDLR* mutation[26]. In a Japanese cohort of 487 subjects who meet the Japanese clinical diagnostic criteria of FH, 8% of the subjects had deleterious mutations in *ABCG5/ABCG8* but not in FH genes (*ABCG5/ABCG8* mutation carriers) and 4% of the subjects had deleterious mutations in an FH gene and *ABCG5/ABCG8* (*ABCG5/ABCG8*-oligogenic FH). The study found that subjects with oligogenic FH had significantly higher LDL-C levels than the monogenic FH subjects, suggesting that *ABCG5/ABCG8* contributes substantially to mimicking and exacerbation of the FH phenotype[27]. Recently, there were some case reports of sitosterolemia from China[28, 29]. In a recent study from China, 2 cases of sitosterolemia were identified from 208 unrelated Chinese with possible/probable or definite FH probands by a targeted genetic panel[30]. In Taiwan, five cases of sitosterolemia

had been reported, including four cases of compound heterozygous mutations and one homozygous mutation[31]. In the present study, we identified two additional cases of compound heterozygotes with three pathogenic *ABCG5* mutations detected by targeted NGS, including a novel *ABCG5* c.1337 G>A (p.R446Q) mutation. The incidence of sitosterolemia was 2/445 (0.45%) in all cases with genetic mutations and 2/62 (3.22%) in cases with negative mass spectrometry results and LDL-C levels >250 mg/dL. Our finding confirmed the presence of sitosterolemia in Taiwanese population and highlighted the clinical significance of genotyping for *ABCG5* in Taiwan. Further studies are necessary to investigate its impacts on the clinical outcome of patients with sitosterolemia.

There are some limitations in the present study. First, the FH mutation detection rate was 445/750 (59%), indicating that some other unknown loci or genes were undetected. Further genetic testing, such as whole exome or genome sequencing, may be necessary; however, they can only be applied to special cases due to its cost. Second, the mutation detection rate of 415/750 (55%) by the custom-made mass spectrometry indicates that it is a feasible approach for the initial genetic screening of patients with severe hypercholesterolemia. However, it is still necessary to update its panels to identify more novel mutations. Third, targeted NGS and MLPA were analyzed only in those with negative mass spectrometry results and LDL-C levels >250 mg/dL due to limited budget, which might result in underdiagnoses. It is necessary to complete surveys of genetic mutations by performing both targeted NGS and MLPA in all patients with negative mass spectrometry in the future. Fourth, measurement of plant sterols should be performed to confirm the diagnosis of sitosterolemia in the cases of *ABCG5* mutations.

Conclusion

LDLR and *APOB* mutations are the major causes of FH, but they have very high heterogeneity rates in Taiwan. To date, *PCSK9* mutations were not detected. Four novel mutations in either the *LDLR* or *ABCG5* genes were identified by targeted NGS in this series. The unique mass genetic screening using a custom-made mass spectrometry technique followed by targeted NGS and MLPA analysis provided an efficient algorithm in the genetic testing for FH in Taiwan.

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Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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