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=3), 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 Â² T>C (IVS 7 Â²), 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.

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 mortality1,2. 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 condition3. Mutations in a third gene, Proprotein Convertase Subtilisin Kexin type 9 (PCSK9), which degrades the LDLR, have
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\(^9\) 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 mass spectrometry. 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.

**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. 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 target DNA sequences of the candidate regions: coding exons of 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°C for 15 min, followed by 18 cycles of 94°C (30s), 60°C (3 min), and 72°C (1 min), and a final extension of 72°C 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 (ddH2O) and denatured at 98°C 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°C 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-600 size standards (Applied Biosystems). The procedure was performed according to the manufacturer’s instructions.

Data analysis of the MLPA was performed using
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

Table 1. Clinical characteristics of the probands

| Characteristics  | n = 750 |
|------------------|---------|
| Age at recruitment (years) | 52.4 ± 15.1 |
| Sex (% male) | 40.9 |
| BMI (kg/m²) | 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% |
| Ezetimibe | 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.
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).

**Homozogous 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
| Gene      | Mutation          | Designation | Region affected | Frequency (n, %) | Polyphen-2                  | SIFT                | ACMG   | dbSNP          | Methodology*          |
|-----------|-------------------|-------------|----------------|-----------------|---------------------------|---------------------|--------|----------------|-----------------------|
| APOB      | NM_000384.2: c.10579 T>C | R3527W      | Exon 26        | 56 (12.6%)      | Probably damaging (score: 1.000) | Damaging (score: 0.056) | Pathogenic | rs144467873 | 1                      |
| LDLR      | NM_000527: c.986 G>A | C329Y       | Exon 07        | 51 (11.5%)      | Probably damaging (score: 1.000) | Damaging (score: 0)   | Pathogenic | rs761954844 | 1                      |
| LDLR      | NM_000527: c.1747 C>T | H583Y       | Exon 12        | 48 (10.8%)      | Probably damaging (score: 1.000) | Damaging (score: 0)   | Pathogenic | rs730882109 | 1                      |
| LDLR      | NM_000527: c.268 G>A | D90N        | Exon 03        | 26 (5.8%)       | Probably damaging (score: 1.000) | Damaging (score: 0)   | Pathogenic | rs749038326 | 1                      |
| LDLR      | NM_000527: 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: c.769 C>T+ c.1765 G>A | R257W+ D589N | Exon 05+12     | 23 (5.2%)       | Possibly damaging (score: 0.993) | Possibly damaging (score: 0.998) | Pathogenic | rs200990725 | +                       |
| LDLR      | NM_000527: c.2054 C>T     | P685L       | Exon 14        | 15 (3.4%)       | Probably damaging (score: 1.000) | Damaging (score: 0)   | Pathogenic | rs28942084 | 1                      |
| LDLR      | NM_000527: c.1211 T>G     | L414R       | Exon 09        | 13 (2.9%)       | Probably damaging (score: 0.957) | Damaging (score: 0)   | Pathogenic | rs748554592 | 1                      |
| LDLR      | NM_000527: c.1246 C>T     | R416W       | Exon 09        | 11 (2.5%)       | Probably damaging (score: 0.998) | Damaging (score: 0)   | Pathogenic | rs570942190 | 1                      |
| LDLR      | NM_000527: c.1953,1954 del [TA] | M652Fs      | Exon 09        | 11 (2.5%)       | -                         | -                   | Pathogenic | rs875989935 | 1                      |
| LDLR      | NM_000527: 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: c.1016 T>G     | L393L       | Exon 09        | 7 (2.0%)        | Benign (score: 0)          | Tolerated (score: 0.902) | Likely Pathogenic | rs555292896 | 1                      |
| LDLR      | NM_000527: c.1867 A>G     | J623V       | Exon 10        | 6 (1.6%)        | Benign (score: 0.449)      | Tolerated (score: 0.056) | Pathogenic | rs872954813 | 1                      |
| LDLR      | NM_000527: c.1174 Ins[T]  | C392Fs      | Exon 08        | 6 (1.6%)        | -                         | -                   | Pathogenic | rs872954862 | 1                      |
| LDLR      | NM_000527: c.2389 G>A     | V797M       | Exon 10        | 6 (1.3%)        | Benign (score: 0.449)      | Tolerated (score: 0.056) | Pathogenic | rs879254862 | 1                      |
| LDLR      | NM_000527: c.510 del [C]  | P171Fs      | Exon 04        | 5 (1.1%)        | -                         | -                   | Pathogenic | rs875989921 | 1                      |
| LDLR      | NM_000527: c.1448 G>A     | W483X       | Exon 10        | 5 (1.1%)        | -                         | -                   | Pathogenic | rs879254862 | 1                      |
| LDLR      | NM_000527: 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: 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: c.599 T>G      | F200C       | Exon 04        | 4 (0.9%)        | Possibly damaging (score: 0.796) | Tolerated (score: 0.1) | Likely Pathogenic | rs779921498 | 2                      |
| LDLR      | NM_000527: c.1186 + 2 T>G | IV58 + 2 T>G | Intron 08      | 4 (0.9%)        | -                         | -                   | Pathogenic | rs879254849 | 1                      |
| LDLR      | NM_000527: c.1268 T>C     | I423T       | Exon 09        | 4 (0.9%)        | Possibly damaging (score: 0.845) | Damaging (score: 0.001) | Pathogenic | rs1205480064 | 1                      |
| LDLR      | NM_000527: c.1723 C>T     | L575F       | Exon 11        | 4 (0.9%)        | Probably damaging (score: 1.000) | Damaging (score: 0.001) | Pathogenic | rs879254557 | 1                      |
| LDLR      | NM_000527: c.516 D172E    |            | Exon 04        | 3 (0.7%)        | Probably damaging (score: 0.997) | Damaging (score: 0.045) | Pathogenic | rs200238879 | 2                      |
| LDLR      | NM_000527: c.694 C>T     |            | Exon 04        | 3 (0.7%)        | -                         | -                   | Pathogenic | rs200238879 | 2                      |
| LDLR      | NM_000527: c.1016 T>C     | L339P       | Exon 07        | 3 (0.7%)        | Probably damaging (score: 0.985) | Damaging (score: 0)   | Pathogenic | rs28942079 | 1                      |
| LDLR      | NM_000527: c.1291 G>A     | A431T       | Exon 09        | 3 (0.7%)        | Probably damaging (score: 1.000) | Damaging (score: 0.001) | Pathogenic | rs758194385 | 1                      |
| LDLR      | NM_000527: c.1691 A>G     | N564S       | Exon 11        | 3 (0.7%)        | Probably damaging (score: 1.000) | Damaging (score: 0.039) | Pathogenic | rs555292896 | 1                      |
### Table 2: Gene Mutation Designation, Region Affected, Frequency, and Analysis Results

| Gene   | Mutation Details | 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 [AGTITG] ins T | E113Fs | Exon 04 | 2 (0.4%) | - | - | Pathogenic | - | 1 |
| LDLR   | NM_000527.4: c.940 IVS6 + 1 G > A + 1 G > R | R406W | Exon 09 | 2 (0.4%) | Probably damaging (score: 1.000) | Tolerated (score = 1) | Pathogenic | rs879254729 | 1 |
| LDLR   | NM_000527.4: c.1216 C > T | A431T + I623V | Exon 09 + 13 | 2 (0.4%) | Probably damaging (score: 1.000) + Benign (score: 0.001) | Tolerated (score = 0.902) | Pathogenic + Likely Pathogenic | rs28942079 / rs555292896 | 1 |
| 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 > G | 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.1420 C > Y | Q474X | Exon 10 | 2 (0.4%) | - | - | Pathogenic | rs87954393 | 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 | rs879254046 | 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 > G | K603X | Exon 12 | 2 (0.4%) | - | - | Pathogenic | rs879255029 | 1 |
| LDLR   | NM_000527.4: c.1420 C > Y | Q474X | Exon 10 | 2 (0.4%) | - | - | Pathogenic | rs87954393 | 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 | rs879254046 | 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 > G | K603X | Exon 12 | 2 (0.4%) | - | - | Pathogenic | rs879255029 | 1 |
| LDLR   | NM_000527.4: c.1420 C > Y | Q474X | Exon 10 | 2 (0.4%) | - | - | Pathogenic | rs87954393 | 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 | rs879254046 | 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 > G | K603X | Exon 12 | 2 (0.4%) | - | - | Pathogenic | rs879255029 | 1 |
| LDLR   | NM_000527.4: c.1420 C > Y | Q474X | Exon 10 | 2 (0.4%) | - | - | Pathogenic | rs87954393 | 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 | rs879254046 | 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 > G | K603X | Exon 12 | 2 (0.4%) | - | - | Pathogenic | rs879255029 | 1 |

*Methodology: Novel, Likely Pathogenic, Pathogenic, Likely Benign, Benign, Tolerated*
(Cont. Table 2)

| Gene   | Mutation          | Designation | Region affected | Frequency (n, %) | Polyphen-2 | SIFT               | ACMG       | dbSNP          | Methodology* |
|--------|-------------------|-------------|----------------|-----------------|------------|--------------------|------------|----------------|--------------|
| LDLR   | NM_000527.4:c.817 | IVS5 + 1 G > A | Intron 05     | 1 (0.2%)        | -          | -                  | Pathogenic | rs879254685  | 1            |
| LDLR   | NM_000527.4:c.828 | C276X       | Exon 06       | 1 (0.2%)        | -          | -                  | Likely Pathogenic | rs146651743 | 1            |
| LDLR   | NM_000527.4:c.947 | N316S       | Exon 07       | 1 (0.2%)        | Probably damaging (score: 1.000) | Damaging (score: 0.002) | Pathogenic | rs730882094 | 1            |
| LDLR   | NM_000527.4:c.1048 | R350X       | Exon 07       | 1 (0.2%)        | -          | -                  | Pathogenic | -              | 1            |
| LDLR   | NM_000527.4:c.1054 | C352S       | Exon 07       | 1 (0.2%)        | Probably damaging (score: 1.000) | Damaging (score: 0) | Pathogenic | rs879254769 | 1            |
| LDLR   | NM_000527.4:c.1057 | E353K       | Exon 07       | 1 (0.2%)        | Benign (score: 0.437) | Tolerated (score=0.187) | Likely Pathogenic | rs370471092 | 1            |
| LDLR   | NM_000527.4:c.1060 | IVS7 + 2 T > C | Intron 07 | 1 (0.2%)        | -          | -                  | Pathogenic | rs774069731  | 2 Novel      |
| LDLR   | NM_000527.4:c.1139 | E380A       | Exon 08       | 1 (0.2%)        | Benign (score: 0.009) | Tolerated (score=0.411) | Likely Pathogenic | 2 Novel      | 2 Novel      |
| LDLR   | NM_000527.4:c.1187 | IVS8-10 G > A | Intron 08 | 1 (0.2%)        | -          | -                  | Likely Pathogenic | rs765690608 | 2            |
| LDLR   | NM_000527.4:c.1195 | A399T       | Exon 09       | 1 (0.2%)        | Probably damaging (score: 0.993) | Damaging (score: 0.006) | Pathogenic | rs730882099 | 1            |
| LDLR   | NM_000527.4:c.1222 | E408K       | Exon 09       | 1 (0.2%)        | Probably damaging (score: 0.995) | Damaging (score: 0.004) | Pathogenic | rs137943601 | 2            |
| LDLR   | NM_000527.4:c.1247 | R416L       | Exon 09       | 1 (0.2%)        | Benign (score: 0.144) | Damaging (score: 0) | Pathogenic | -              | 1            |
| LDLR   | NM_000527.4:c.1285 | V429L       | Exon 09       | 1 (0.2%)        | Benign (score: 0.307) | Damaging (score: 0.016) | Pathogenic | rs28942078  | 2            |
| LDLR   | NM_000527.4:c.1384 | V462I       | Exon 10       | 1 (0.2%)        | Benign (score: 0.005) | Tolerated (score=0.434) | Uncertain Significance | rs750363970 | 1            |
| LDLR   | NM_000527.4:c.1552 | K518E       | Exon 10       | 1 (0.2%)        | Possibly damaging (score: 0.5166) | Tolerated (score=1) | Likely Pathogenic | rs879254937 | 1            |
| LDLR   | NM_000527.4:c.1597 | W533R       | Exon 11       | 1 (0.2%)        | Probably damaging (score: 1.000) | Damaging (score: 0) | Pathogenic | rs879254951 | 1            |
| LDLR   | NM_000527.4:c.1661 | 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 | G565R       | Exon 11       | 1 (0.2%)        | Probably damaging (score: 1.000) | Damaging (score: 0) | Pathogenic | -              | 1            |
| LDLR   | NM_000527.4:c.1706 | IVS12-1 G > A | Intron 11     | 1 (0.2%)        |  | -                  | Pathogenic | rs879254996 | 2            |
| LDLR   | NM_000527.4:c.1721 | R574H       | Exon 12       | 1 (0.2%)        | Probably damaging (score: 1.000) | Damaging (score: 0) | Pathogenic | rs777188764 | 2            |
| LDLR   | NM_000527.4:c.1726 | Y576Fs      | Exon 12       | 1 (0.2%)        | -          | -                  | Pathogenic | -              | 1            |
| LDLR   | NM_000527.4:c.1783 | R595W       | Exon 12       | 1 (0.2%)        | Probably damaging (score: 1.000) | Damaging (score: 0) | Likely Pathogenic | rs373371572 | 1            |
| LDLR   | NM_000527.4:c.1851 | 597-600delVF WT | Exon 13     | 1 (0.2%)        | -          | -                  | Likely Pathogenic | -              | 1            |
| LDLR   | NM_000527.4:c.1988 | IVS14-1 G > C | Intron 13     | 1 (0.2%)        | -          | -                  | Pathogenic | rs1555807335 | 1            |
| LDLR   | NM_000527.4:c.2096 | 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 | 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 | E714X       | Exon 14       | 1 (0.2%)        | -          | -                  | Pathogenic | rs869320652 | 2            |
| LDLR   | NM_000527.4:c.2215 | 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.817-2_3del | Exon 6-18 del | - | 1 (0.2%) | - | - | Pathogenic | - | 3 |
| LDLR      | NM_000527.4: c.941-1_1186+3del | Exon 7-8 del | - | 1 (0.2%) | - | - | Pathogenic | - | 3 |
| LDLR      | NM_000527.4: c.1187-2_2140+3del | Exon 9-14 del | - | 1 (0.2%) | - | - | Pathogenic | - | 3 |
| LDLR      | NM_000527.4: c.68-3_940+3dup | Exon 1-6 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: 0.001) | Uncertain Significance | - | 2 Novel |

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

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.1623V) 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.
**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.

**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.
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 \textit{ABCG5} c.1166 G \textgreater A (p.R389H) and \textit{ABCG5} c.1337 G \textgreater 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 \textit{LDLR} (395 cases, 86%), \textit{APOB} (58 cases, 13%), but not in \textit{PCSK9}. The three most common genetic mutations were \textit{APOB} c.10579 C \textgreater T (p.R3527W) (12.6%),
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, however it was first identified in our series by targeted NGS.

The linked **LDLR** c.1181 G>A (p.A431T) 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. 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. However, the mutation profiles are different from those in Japanese patients with heterozygous FH.

**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 | H583Y | R257W+D589N | D90N APOB-R3527W | G478R | D90N |
| FH classification | Simple | Compound | Simple | Compound | Double | Compound | Double |
| Genetic tests | MS | MS | MS+NGS | MS | 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 | R257W+D589N | APOB-R3527W | IVS2+4 | L393P | R1416W | 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.
Two novel LDLR mutations were identified by targeted NGS in this series. The LDLR c.1060+2T>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.1139A>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 mutation was very rare in Japan. APOB c.10580G>A (p.E32K) was identified in 6 (6.3%) probands among 96 Chinese participants with clinical FH. However, APOB mutation was very rare in Japan. APOB c.10579 C>T had not been reported in Japan before.

The APOB c.10580G>A (p.R3527Q) mutation has not been reported in Taiwan before, but was a quite prevalent mutation in the Caucasian population. 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%). In the Copenhagen General Population Study, the prevalence of the APOB c.10580G>A variant was 0.11% (1:884) among nearly 100,000 Danish subjects.

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. 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. APOB c.10580 G>A has not been reported in a Taiwan series and it was, for the first time, detected in two probands (0.4%) by targeted NGS. 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. 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). 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.

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. In Taiwan, five cases of sitosterolemia were identified from 208 unrelated Chinese with possible/probable or definite FH probands by a targeted genetic panel.
had been reported, including four cases of compound heterozygous mutations and one homozygous mutation. 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 generic 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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