Rare and common variants of APOB and PCSK9 in Korean patients with extremely low low-density lipoprotein-cholesterol levels

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Abstract

Background
Screening of variants, related to lipid metabolism in patients with extreme cholesterol levels, is a tool used to identify targets affecting cardiovascular outcomes. The aim of this study was to examine the prevalence and characteristics of rare and common variants of APOB and PCSK9 in Korean patients with extremely low low-density lipoprotein-cholesterol (LDL-C) levels.

Methods
Among 13,545 participants enrolled in a cardiovascular genome cohort, 22 subjects, whose LDL-C levels without lipid-lowering agents were ≤ 1 percentile (48 mg/dL) of Korean population, were analyzed. Two target genes, APOB and PCSK9, were sequenced by targeted next-generation sequencing. Prediction of functional effects was conducted using SIFT, PolyPhen-2, and Mutation Taster, and matched against a public database of variants.

Results
Eight rare variants of the two candidate genes (five in APOB and three in PCSK9) were found in nine subjects. Two subjects had more than two different rare variants of either gene (one subject in APOB and another subject in APOB/PCSK9). Conversely, 12 common variants (nine in APOB and three in PCSK9) were discovered in 21 subjects. Among all variants, six in APOB and three in PCSK9 were novel. Several variants previously reported functional, including c.C277T (p.R93C) and c.G2009A (p.G670E) of PCSK9, were found in our population.

Conclusions
Rare variants of APOB or PCSK9 were identified in nine of the 22 study patients with extremely low LDL-C levels, whereas most of them had common variants of the two genes.
The common novelty of variants suggested polymorphism of the two genes among them. Our results provide rare genetic information associated with this lipid phenotype in East Asian people.

Introduction

Individuals with hypobetalipoproteinemia have 30–40% reduction in low-density lipoprotein-cholesterol (LDL-C) levels, when they are heterozygous. Additionally, they can show LDL-C levels <5 percentile or <50 mg/dL without lipid-lowering therapy. In patients with homozygous variants, LDL-C levels are known to be much lower. Although the patients are often asymptomatic, some have increased risk for steatohepatitis. This phenotype is associated with variants of APOB or PCSK9. Even common variants of the two genes may affect the levels of LDL-C, although this effect can be modest.

In a Canadian database, 120 variants in APOB and 29 variants in PCSK9, with disease-causing potential, have been reported. In affected patients, mutated APOB can induce truncated forms of apolipoprotein B (apoB), which are short enough to be degraded. Mutated PCSK9 causes less degradation of LDL receptors and increases the number of these receptors on the cell, thereby reducing blood levels of LDL-C. Although the reduction in LDL-C by a variant of a specific gene may not be large, its impact on the cardiovascular outcome can be greater than that of the LDL-C level assessed in adulthood. Therefore, screening of variants related to lipid metabolism in a population with extreme LDL-C levels can be a tool to identify an important target affecting clinical outcomes.

Here, the aim of our study was to examine the prevalence and characteristics of rare and common variants of APOB and PCSK9 underlying the phenotype of hypobetalipoproteinemia in Korean subjects. We used targeted next-generation sequencing, which is becoming widespread in genetic studies.

Materials and methods

Study population

The Institutional Review Board of Severance Hospital approved the study protocols, and all subjects provided written informed consent. Subjects with extremely low levels of LDL-C were included in this study. Between November 2000 and March 2011, 13,545 subjects were enrolled in the Cardiovascular Genome Center Cohort, Yonsei University College of Medicine, Seoul, Korea. Men and women ≥18 years were recruited in this cohort when they visited Severance Hospital for cardiovascular diseases, control of risk factors, or health check-up. Participants were interviewed about their medical histories, and then underwent physical examinations. Among the total number of subjects, 22 subjects, whose LDL-C levels were ≤1 percentile (48 mg/dL) of the general Korean population, were finally analyzed. These 22 subjects were free from hypolipidemic treatment before or after enrollment to our study. This cut-off value is based on the data from 2011 Korea National Health and Nutrition Examination Survey. The level of LDL-C was assessed by direct measurement. Individuals with diagnosis of thyroid-, liver-, or kidney disease, pregnancy, cancer, or prescribed regimens that could affect lipid profiles (such as lipid-modifying agents, corticosteroids, or oral estrogen) at the time of blood sampling were excluded.
Laboratory assessment

The levels of total cholesterol, triglyceride, high-density lipoprotein-cholesterol (HDL-C), and LDL-C were measured in all the subjects. The subjects fasted and avoided alcohol for at least 12 hours before blood sampling. Samples were analyzed within 4 hours by a laboratory that was certified by the Korean Society of Laboratory Medicine. Circulating apoB (Roche, Basel, Switzerland) and proprotein convertase subtilisin/kexin type 9 (PCSK9) levels (R&D Systems, Minneapolis, MN, USA) were measured using ELISA assays.

Targeted sequencing and variant analysis

Two target genes were sequenced: APOB (MIM 107730) and PCSK9 (MIM 607786). Genomic DNA was extracted from blood using the QiagenDNeasy kit (Qiagen, Valencia, CA, USA). For mutation analysis, a panel for targeted DNA capture and sequencing was developed by Celemics, Inc. (Seoul, Korea). Targeted sequencing and variant analysis were conducted as described. Briefly, DNA fragments, containing all coding exons and exon-intron junctions, were enriched by solution-based hybridization capture, followed by sequencing using the Illumina HiSeq 2000 platform (Illumina, Inc., San Diego, CA, USA). The quality of next-generation sequencing data including coverage information is presented in S1 Fig. Analysis of sequencing data was performed using an in-house analysis pipeline. Briefly, sequencing reads from the HiSeq 2000 raw data were sorted by index and barcode sequences. Sorted fastq files were aligned to the hg19 reference genome using the Burrows-Wheeler Aligner (BWA; ver. 0.7.12) BWA-MEM algorithm. Output SAM files were converted into BAM files and sorted using SAMtools (ver. 1.1). Duplicate removal was performed with Picard tools (ver. 1.128) MarkDuplicates. Realignment around known indel sites and Base Quality Score Recalibration (BQSR) were performed using GATK (v3.3.0) to create the final BAM files. Variants were called using the GATK v3.3.0 Unified Genotyper algorithm for loci with sequencing depth greater than or equal to 50X. Analysis of the splice regions, including sufficient intronic bases, was performed using Human Splicing Finder. Functional annotation of genetic variants was performed by ANNOVAR (ver. 2014-11-12). Functional effect predictions for single nucleotide variants were performed using SIFT, PolyPhen-2 and MutationTaster, and matched against the Korean population exome data (n = 476) and public databases of variants (dbSNP 138, Exome Variant Server and 1000 Genome project SNP [April 2012 release] from both Asian and all-population databases). We then prioritized variants according to the following criteria: 1) variants that were reported to be disease-causing in the Human Gene Mutation Database; 2) disruptive variants (nonsense, splice-site [two nucleotides on either side of the intron/exon boundary] and frameshift) that were novel or rare; and 3) novel or rare missense variants that were predicted to be deleterious by SIFT, Polyphen-2 (HumVar), or MutationTaster. Variants that met these criteria were validated by bidirectional Sanger sequencing of PCR amplicons. Databases used for identity and frequency of the variants included 1000 Genomes Project, Exome Sequencing Project 6500, and gnomAD browser (http://gnomad.broadinstitute.org/). Variants were classified as rare when minor allele frequency (MAF) <1%, whereas classified as common when MAF ≥5% in public databases.

Results

Clinical characteristics of study subjects

Clinical characteristics of the study subjects are described in Table 1. Mean patient age was 52 years and 64% of the patients were males; 14% of the subjects had type 2 diabetes for 0 to 27 years; the mean level of LDL-C was 39.2 mg/dL. The patients’ median apoB level was 53 mg/
This median value was much lower than 90–97 mg/dL, which has been reported in prior studies in healthy Koreans.[9,10] The median PCSK9 level was 251 ng/mL (interquartile range: 190–342 ng/mL) (Table 1). The characteristics of the total cohort are shown in Table A in S1 File.

Analysis of candidate genes

Eight rare variants (five in APOB and three in PCSK9) of the two candidate genes were identified in nine subjects. Among all the rare variants, five were novel and five were suspected of being disease-causing (Table 2). Two subjects had more than two different rare variants in either gene (one subject in APOB and another subject in APOB/PCSK9). Conversely, 12 common variants (nine in APOB and three in PCSK9) of the two genes were discovered in 21 subjects. Among all the common variants, one in APOB was novel. Five common variants in APOB (e.g., c.C8216T [p.P2739L]) and two in PCSK9 (e.g. c.G2009A [p.G670E]) were frequent and found in more than 10 individuals (Fig 1 and Tables 2 and 3). Three variants of unknown frequency (one in APOB and two in PCSK9) were identified in 10 subjects. One subject did not possess any variants of the two genes. Analysis of the splice regions revealed no variants. The type of variants in non-diabetic and diabetic subjects was analyzed and there was no significant difference therein between the two groups (Table B in S1 File).

APOB

Five rare variants of APOB were discovered in seven subjects: c.G12016A (p.V4006I), c.C11120T (p.A370V), c.C2398A (p.L800M), c.G1342A (p.A448T), and c.T35C (p.L12P). All the rare variants were present in heterozygous form. One subject showed three different rare heterozygous variants of APOB, while one subject showed two rare heterozygous variants of APOB and PCSK9. Four of five rare variants were novel, and c.G10216A, c.G2398A, and c.T35C variants were predicted to be damaging. Meanwhile, seven common variants of this
| Gene | Genomic coordinate | Nucleotide change | Mutation type | Amino acid change (rs number in dbSNP) | Allele frequency | Frequency in gnomAD Database (East Asian) | Affected patients (homo/hetero) | Report | Effect | SIFT/Polyphen/ Mutation taster prediction (Clinical significance based on clinVAR) |
|------|-------------------|------------------|---------------|-------------------------------------|-----------------|------------------------------------------|-----------------------------|--------|-------|-----------------------------------------------------------------------------|
| APOB | chr2: 21,227,212  | c.G12016A        | nonsynonymous SNV | p.V4006I (rs183117027) | 0.045 | 0.006 | 0/2 | Yes* | Unknown | Tolerated/ benign/ disease_causing (Likely benign) |
|      | chr2: 21,228,620  | c.C11120T        | nonsynonymous SNV | p.A3707V (rs756381590) | 0.023 | <0.001 | 0/1 | No | Unknown | Tolerated/ Benign/ Polymorphism (NA) |
|      | chr2: 21,247,843  | c.C2398A         | nonsynonymous SNV | p.L800M (rs183950016) | 0.045 | <0.001 | 0/2 | No | Unknown | Tolerated/ possibly damaging/ disease_causing (NA) |
|      | chr2: 21,255,236  | c.G1342A         | nonsynonymous SNV | p.A448T (rs752032737) | 0.023 | <0.001 | 0/1 | No | Unknown | Tolerated/ Benign/ polymorphism (NA) |
|      | chr2: 21,266,783  | c.T35C           | nonsynonymous SNV | p.L12P (rs758450840) | 0.068 | <0.001 | 0/3 | No | Unknown | Deleterious/ benign/ polymorphism (NA) |
|      | chr2: 21,225,281  | c.G13013A        | nonsynonymous SNV | p.S4338N (rs1042034) | 0.273 | 0.273 | 1/10 | Yes[1] | Unknown | Tolerated/ benign/ polymorphism_ automatic (Benign/ Likely benign) |
|      | chr2: 21,231,387  | c.A8353C         | nonsynonymous SNV | p.N2175H (rs2136204) | 0.045 | 0.059 | 0/2 | Yes* | Unknown | Tolerated/ Benign/ polymorphism (Conflicting interpretations of pathogenicity) |
|      | chr2: 21,231,524  | c.G8216T         | nonsynonymous SNV | p.P2739L (rs676210) | 0.364 | 0.725 | 3/10 | Yes[1] | Familial hypercholesterolemia; hypocholesterolemia | Deleterious/probably damaging/ Possibly damaging (Benign/ Likely benign) |
|      | chr2: 21,232,803  | c.A6937G         | nonsynonymous SNV | p.I2313V (rs584542) | 0.455 | 0.999 | 10/0 | Yes* | Unknown | Tolerated/ Benign/ Possibly damaging (Benign) |
|      | chr2: 21,235,475  | c.A4265G         | nonsynonymous SNV | p.Y1422C (rs568413) | 0.545 | 1.000 | 12/0 | No | Unknown | Tolerated/ Benign/ Possibly damaging (NA) |
|      | chr2: 21,250,914  | c.C1853T         | nonsynonymous SNV | p.A6118V (rs679899) | 0.545 | 0.851 | 11/2 | Yes[1] | Hypocholesterolemia | Tolerated/probably damaging/ Polymorphism_ automatic (Benign/ Likely benign) |
|      | chr2: 21,252,534  | c.C1594T         | nonsynonymous SNV | p.R532W or R505W (rs13306194) | 0.159 | 0.135 | 0/6 | Yes(Yilmaz) | Familial hypobetalipoproteinemia | Deleterious/probably damaging/ Disease_causing (Likely benign) |
|      | chr2: 21,260,084  | c.C581T          | nonsynonymous SNV | p.T194M (rs13306198) | 0.045 | 0.055 | 0/2 | Yes* | Unknown | Deleterious/probably damaging/ polymorphism (Likely benign) |

(Continued)
### Table 2. (Continued)

| Gene | Genomic coordinate | Nucleotide change | Mutation type | Amino acid change (rs number in dbSNP) | Allele frequency | Frequency in gnomAD Database (East Asian) | Affected patients (homo/hetero) | Report | Effect | SIFT/Polyphen/Mutation taster prediction (Clinical significance based on clinVar) |
|------|--------------------|------------------|---------------|------------------------------------------|------------------|------------------------------------------|-----------------------------|--------|-------|----------------------------------------------------------------------------------|
| chr2: 21,283,900 | c.C293T | nonsynonymous SNV | p.T98I (rs1367117) | 0.068 | 0.127 | 0/3 | Yes [11] | Hypocholesterolemia | Tolerated/Benign/Polymorphism automatic (Benign/Likely benign) |
| chr2: 21,266,774 | c.35_44TGGCGCTGC | frameshift substitution | NA | 0.136 | NA | 0/6 | No | Unknown |

**PCSK9**

| Gene | Genomic coordinate | Nucleotide change | Mutation type | Amino acid change (rs number in dbSNP) | Allele frequency | Frequency in gnomAD Database (East Asian) | Affected patients (homo/hetero) | Report | Effect | SIFT/Polyphen/Mutation taster prediction (Clinical significance based on clinVar) |
|------|--------------------|------------------|---------------|------------------------------------------|------------------|------------------------------------------|-----------------------------|--------|-------|----------------------------------------------------------------------------------|
| chr1: 55,505,520 | c.G10A | nonsynonymous SNV | p.V4I (rs186669805) | 0.023 | 0.002 | 0/1 | Yes [6,12] | Hypercholesterolemia | Tolerated/Benign/B/polymorphism (NA) |
| chr1: 55,509,585 | c.C277T | nonsynonymous SNV | p.R93C (rs151193009) | 0.023 | 0.009 | 0/1 | Yes [12,13] | Hypocholesterolemia | Tolerated/probably damaging/D/polymorphism (Benign) |
| chr1: 55,524,312 | c.C1495T | nonsynonymous SNV | p.R499C (rs201395805) | 0.023 | <0.001 | 0/1 | No | Unknown | Tolerated/probably damaging/polymorphism (NA) |

**Common variants**

| Gene | Genomic coordinate | Nucleotide change | Mutation type | Amino acid change (rs number in dbSNP) | Allele frequency | Frequency in gnomAD Database (East Asian) | Affected patients (homo/hetero) | Report | Effect | SIFT/Polyphen/Mutation taster prediction (Clinical significance based on clinVar) |
|------|--------------------|------------------|---------------|------------------------------------------|------------------|------------------------------------------|-----------------------------|--------|-------|----------------------------------------------------------------------------------|
| chr1: 55,505,668 | c.C158T | nonsynonymous SNV | p.A53V (rs11583680) | 0.091 | 0.122 | 0/4 | Yes [13] | Unknown | Tolerated/Benign/Polymorphism automatic (Benign/Likely benign) |
| chr1: 55,524,237 | c.G1420A | nonsynonymous SNV | p.V474I (rs562556) | 0.409 | 0.993 | 8/2 | Yes [6,14] | Hypocholesterolemia; familial hypercholesterolemia | Tolerated/Benign/Polymorphism automatic (Benign/Likely benign) |
| chr1: 55,529,187 | c.G2009A | nonsynonymous SNV | p.G670E (rs505151) | 0.500 | 0.948 | 10/2 | Yes [6,12,13,15] | Hypocholesterolemia | Tolerated/Benign/Polymorphism automatic (Benign/Likely benign) |

**Variants of unknown frequency**

| Gene | Genomic coordinate | Nucleotide change | Mutation type | Amino acid change (rs number in dbSNP) | Allele frequency | Frequency in gnomAD Database (East Asian) | Affected patients (homo/hetero) | Report | Effect | SIFT/Polyphen/Mutation taster prediction (Clinical significance based on clinVar) |
|------|--------------------|------------------|---------------|------------------------------------------|------------------|------------------------------------------|-----------------------------|--------|-------|----------------------------------------------------------------------------------|
| chr1: 55,505,552 | c.42_43insCTGCTGCTG | nonframeshift substitution | p.P14delinsPLL | 0.091 | NA | 0/4 | No | Unknown |
| chr1: 55,529,225 | c.2048dupA | frameshift substitution | p.H838fs | 0.023 | NA | 0/1 | No | Unknown |

SNV: single nucleotide variant, NA: not available
*: reported in gnomAD browser

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gene were found in 19 subjects. Common homozygous variants were identified in 17 subjects, whereas common heterozygous variants were shown in 14 individuals. The C8216T (p.P2739L), c.C1853T (p.A618V), c.A4265G (p.Y1422C), c.G13013A (p.S4338N), and c.A6937G (p.I2313V) variants were relatively frequent and identified in 13, 13, 12, 11, and 10 individuals, respectively. Among the common variants, c.C8216T, c.A6937G, c.A4265G, c.C1853T, c.C1594T (p.R532W), and c.C581T (p.T194M) were suspected to be disease-causing as assessed using in silico analysis.

Fig 1. Proportion of carriers who had variants of each gene identified in 22 study subjects. With regard to APOB, seven subjects carried rare heterozygous variants, 17 common homozygous variants, and 14 had common heterozygous variants. Six subjects carried an APOB variant of unknown frequency. Conversely, with regard to PCSK9, three subjects carried rare heterozygous variants, 12 had common homozygous variants, and seven had common heterozygous variants. Five subjects carried PCSK9 variants of unknown frequency.

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Table 3. Summary of genetic variants of target genes identified in each individual.

| Patients | Sex | Age | TC (mg/dL) | TG (mg/dL) | HDL-C (mg/dL) | LDL-C (mg/dL) | Non-HDL-C (mg/dL) | Remnant-C (mg/dL) | apoB (mg/dL) | PCSK9 (ng/mL) | Numbers of variants |
|----------|-----|-----|------------|------------|---------------|---------------|------------------|-----------------|-------------|-------------|-------------------|
|          |     |     |            |            |               |               |                  |                 |             |             | APOB              |
|          |     |     |             |            |               |               |                  |                 |             |             | Rare | Common | Unknown |
|          |     |     |             |            |               |               |                  |                 |             |             |          |          |          |
|          |     |     |             |            |               |               |                  |                 |             |             |          |          |          |
|          |     |     |             |            |               |               |                  |                 |             |             |          |          |          |
|          |     |     |             |            |               |               |                  |                 |             |             |          |          |          |
|          |     |     |             |            |               |               |                  |                 |             |             |          |          |          |
|          |     |     |             |            |               |               |                  |                 |             |             |          |          |          |
|          |     |     |             |            |               |               |                  |                 |             |             |          |          |          |
|          |     |     |             |            |               |               |                  |                 |             |             |          |          |          |
|          |     |     |             |            |               |               |                  |                 |             |             |          |          |          |
|          |     |     |             |            |               |               |                  |                 |             |             |          |          |          |
|          |     |     |             |            |               |               |                  |                 |             |             |          |          |          |
|          |     |     |             |            |               |               |                  |                 |             |             |          |          |          |
| TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein-cholesterol; LDL-C: low-density lipoprotein-cholesterol

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One variant of unknown frequency, c.35_44TGGCGCTGC was identified in six subjects (Fig 1 and Tables 2 and 3; Table C in S1 File). Circulating apoB levels did not show correlations with any specific variants in an individual.

**PCSK9**

Three rare variants of PCSK9 were found in three subjects: c.G10A (p.V4I), c.C277T (p.R93C), and c.C1495T (p.R499C). All the rare variants were heterozygous. The c.C1495T variant was novel, whereas c.C277T and c.C1495T were predicted to be damaging. Conversely, three common variants were discovered in 17 participants. Common homozygous variants were discovered in 12 subjects, while common heterozygous variants were shown in seven subjects. Among them, c.G2009A (p.G670E) and c.G1420A (p.V474I) were frequent and found in 12 and 10 individuals, respectively. The disease causality of the three common variants of PCSK9 was not certain as assessed by *in silico* analysis. Two variants of unknown frequency were discovered in 5 individuals: c.42_43insCTGCTGCTG and c.2048dupA (p.H683fs) (Fig 1 and Tables 2 and 3; Table C in S1 File). Circulating PCSK9 levels were not associated with any specific variants in study subjects.

**Discussion**

In our study population with extremely low LDL-C levels, rare variants of either APOB or PCSK9 were found in nine of all subjects: seven had rare variants in APOB, whereas three showed rare variants in PCSK9. Two subjects had more than two different rare variants of either gene: one in APOB and one in APOB/PCSK9. Most of the study subjects had more than one common variant of the two genes: 19 had variants in APOB and 17 had variants in PCSK9. Eleven of 15 rare or common variants of APOB were novel, while five of six variants of PCSK9 were known. These results provide rare and informative data about variants associated with extremely low levels of LDL-C in East Asian population.

In previous studies, the prevalence of APOB mutations in hypobetalipoproteinaemia ranged from 44% to 64%. However, genetic data for this disease in Asian patients has been scarce. The prevalence of rare variants in APOB, detected in our study, was 41%, which indicates a lower tendency than that in Western studies. This rate was higher than the 14% demonstrated in a Japanese study, although it is difficult to compare those results with ours because of the levels of different LDL-C at enrollment. On the other hand, a considerable proportion of subjects with the phenotype did not have rare variants of the two genes. These individuals are probably influenced by the polygenic effects of lipid-related genes.

More than 60 rare variants of APOB have been reported in prior studies. In our study, four of five rare APOB variants identified in the study subjects did not overlap with any of the variants reported previously. The rate of novel rare variants in APOB was greater than that in PCSK9 (80% and 33%, respectively) in our results. Meanwhile, eight of nine common APOB variants were previously identified. Among them, the c.C1594T variant, which was recently reported in a Turkish case, is known to be of much higher minor allele frequency in East Asian population than those of other ethnicities. Four other common APOB variants, c.C293T, c.C1853T, c.C8216T, and c.G13013A, were found in a Dutch study.

The LDL-C reducing effect of c.C277T (R93C), a rare variant of PCSK9, has been shown in studies conducted in Japan and Canada. We also discovered this variant in one Korean individual with this phenotype. Accordingly, the c.C277T variant may be one of the influential variants in East Asians with very low levels of LDL-C. The c.G10A (p.V4I) variant, another rare variant of PCSK9 found in our study, was also reported in Japan and Canada. In the study by Miyake et al, this variant was shown only in subjects with high levels of LDL-C.
Conversely, it did not impact the lipid profile in individuals without LDLR mutation,[21] and the function of this variant is not clear to date. Similar to APOB, diverse variants of PCSK9 have been reported, and this gene is also considered highly polymorphic.[13,22] The c. G2009A (p.G670E) variant previously demonstrated in the United States,[15] Canada,[7,13] and Japan,[12] has shown a phenotype similar to that observed in our study. Because this variant was the most frequent among the common variants of PCSK9 in this study, it may have considerable effect in Koreans with extremely low levels of LDL-C. Additionally, the c.C158T (p.A53V) variant, found in our study, has also been reported in a Canadian study.[13] The c. G1420A (p.V474I) variant is the second most frequent among the common variants of PCSK9, as observed in our study. However, it was associated with high levels of LDL-C in a Japanese study,[14] and its biological effect is incompletely proven.

Interestingly, in our analysis of the effect of allele number on body mass index, we found a positive association between the number of variant alleles in PCSK9 and the index (r = 0.47, p = 0.03). However, there is controversy on the relationship between PCSK9 and fat accumulation.[23–25] In addition, we compared the triglyceride levels in carriers versus non-carriers of APOB or PCSK9 variants and found that the levels were different with the presence of a few variants. With regard to APOB, the median triglyceride levels were lower in the carriers of c. C1853T (p.A618V) than in the non-carriers (174 mg/dL vs. 264 mg/dL, p = 0.04). Likewise, the levels were lower, but not significantly, in the carriers of c.T35C (p.L12P) (76 mg/dL vs. 206 mg/dL, p = 0.052) or c.C581T (p.T194M) (79 mg/dL vs. 206 mg/dL, p = 0.09) than in the non-carriers. On the contrary, the median triglyceride levels in the carriers of c.G2009A (p. G670E) in PCSK9 tended to be higher than those in the non-carriers (227 mg/dL vs. 116 mg/dL, p = 0.06).

Our study has potential limitations. Information on the family history of the study subjects was not sufficiently available. If we could have analyzed the variants by co-segregation or functional tests, it may have provided further insight into their biological effects. In addition, many individuals showed multiple common variants in both genes, and this may cause confusion about their functionality. Although we tried to predict disease-causality of these variants by public analysis tools, we recognized that it was not perfect and was a limitation of our study. As mentioned above, variants such as c.C277T (R93C) found in our subjects and other studies are assumed to have a damaging effect on protein function. However, because the effect of most PCSK9 variants in our study was only predicted by in silico analysis (Table 2), their influence on protein functionality might not be sufficiently understood in our study. We did not compare the prevalence of variants in the total cohort population and that of the study subjects. Such a comparison may have suggested an additional clinical relevance of the variants. At the same time, it was difficult to estimate per-allele LDL-C reduction effects using our data. However, we completed the main purpose of our study, characterizing the variants of the two genes in our population. Conversely, we investigated the genetic background of individuals with extremely low levels of LDL-C, and the subjects with that extreme lipid phenotype were appropriate for the aim of our study. Therefore, the number of people, who met the phenotypic criteria, could not be very large. However, the number of our study subjects was relatively large, compared with those in other studies,[4] particularly studies conducted with respect to Asian ethnicities. Finally, because of the study design and inclusion criteria, the range of LDL-C levels was quite narrow in our study. Thus, it was difficult to obtain statistical significance when we examined the association between a specific variant with the levels of LDL-C within our population. Likewise, it might be hard to find associations between circulating apoB or PCSK9 levels and specific variants in our homogenous subjects that do not have sufficient controls for comparison. Analyses, using co-segregation or comparison with total cohort population mentioned above, would be helpful for such an examination in future studies.
Conclusion

Taken together, rare variants of either APOB or PCSK9 were identified in nine of the 22 study subjects with extremely low LDL-C levels: carriers of rare variants were more frequent for APOB than PCSK9. Most of the study population had common variants in at least one of the two genes. The common novelty of variants suggested polymorphism of the two genes in this phenotype. Our results provide rare genetic information associated with extremely low levels of LDL-C in East Asian people.

Supporting information

S1 Fig. NGS data statistics of targeted sequencing. (A) Blue diamonds indicate the average sequencing depth of the target region. (B) The blue histogram represents the coverage of the target region in each sample.

S1 File. Clinical characteristics of the total cohort and study subjects (Table A). Variants of APOB and PCSK9 in non-diabetic and diabetic subjects (Table B). Genetic variants of target genes identified in each individual (Table C).

Author Contributions

Conceptualization: Ji Hyun Lee, Sang-Hak Lee.

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References

1. Welty FK. Hypobetalipoproteinemia and abetalipoproteinemia. Curr Opin Lipidol. 2014; 25: 161–168. https://doi.org/10.1097/MOL.0000000000000072 PMID: 24751931

2. Hooper AJ, Burnett JR. Update on primary hypobetalipoproteinemia. Curr Atheroscler Rep. 2014; 16: 423. https://doi.org/10.1007/s11883-014-0423-3 PMID: 24781598

3. Ramasamy I. Update on the molecular biology of dyslipidemias. Clin Chim Acta. 2016; 454: 143–185. https://doi.org/10.1016/j.cca.2015.10.033 PMID: 26546829

4. Rimbert A, Pichelin M, Lecointe S, Marrec M, Le Scouarnec S, Barrak E, et al. Identification of novel APOB mutations by targeted next-generation sequencing for the molecular diagnosis of familial hypobetalipoproteinemia. Atherosclerosis. 2016; 250: 52–56. https://doi.org/10.1016/j.atherosclerosis.2016.04.010 PMID: 27179706

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5. Kotowski IK, Pertsemidis A, Luke A, Cooper RS, Vega GL, Cohen JC, et al. A spectrum of PCSK9 alleles contributes to plasma levels of low-density lipoprotein cholesterol. Am J Hum Genet. 2006; 78: 410–422. https://doi.org/10.1086/500615 PMID: 16465619

6. Fu J, Kwok S, Sinai L, Abdel-Razek O, Babula J, Chen D, et al. Western Database of Lipid Variants (WDLV): a catalogue of genetic variants in monogenic dyslipidemias. Can J Cardiol. 2013; 29: 934–939. https://doi.org/10.1016/j.cjca.2013.01.008 PMID: 23623477

7. Cohen JC, Boerwinkle E, Mosley TH Jr., Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med. 2006; 354: 1264–1272. https://doi.org/10.1056/NEJMoa054013 PMID: 16554528

8. Benn M, Nordestgaard BG, Grande P, Schnohr P, Tybjaerg-Hansen A. PCSK9 R46L, low-density lipoprotein cholesterol levels, and risk of ischemic heart disease: 3 independent studies and meta-analyses. J Am Coll Cardiol. 2010; 55: 2833–2842. https://doi.org/10.1016/j.jacc.2010.02.044 PMID: 20579540

9. Seo MH, Bae JC, Park SE, Rhee EJ, Park CY, Oh KW, et al. Association of lipid and lipoprotein profiles with future development of type 2 diabetes in nondiabetic Korean subjects: a 4-year retrospective, longitudinal study. J Clin Endocrinol Metab. 2011; 96: E2050–2054. https://doi.org/10.1210/jc.2011-1857 PMID: 21994961

10. Yang MH, Sung J, Gwak GY. The associations between apolipoprotein B, A1, and the B/A1 ratio and nonalcoholic fatty liver disease in both normal-weight and overweight Korean population. J Clin Lipidol. 2016; 10: 289–298. https://doi.org/10.1016/j.jcl.2015.11.017 PMID: 27059595

11. Huijgen R, Sjouke B, Vis K, de Randamie JS, Defesche JC, Kastelein JJ, et al. Genetic variation in the proprotein convertase subtilisin/kexin 9 (PCSK9) gene in cholesterol metabolism and disease. Hum Mutat. 2009; 30: 520–529. https://doi.org/10.1002/humu.21660 PMID: 22095935

12. Miyake Y, Kimura R, Kubo Y, Okayama Y, Tomaiko H, Yamamura T, et al. Genetic variants in PCSK9 in the Japanese population: rare genetic variants in PCSK9 might collectively contribute to plasma LDL cholesterol levels in the general population. Atherosclerosis. 2008; 196: 29–36. https://doi.org/10.1016/j.atherosclerosis.2008.12.039 PMID: 17316651

13. Mayne J, Ooi TC, Raymond A, Cousins M, Bernier L, Dewpura T, et al. Differential effects of PCSK9 loss of function variants on serum lipid and PCSK9 levels in Caucasian and African Canadian populations. Lipids Health Dis. 2013; 12: 70. https://doi.org/10.1186/1476-511X-12-70 PMID: 23663680

14. Shioji K, Mannami T, Takami K, Takagi S, Goto Y, et al. Genetic variants in PCSK9 affect the cholesterol level in Japanese. J Hum Genet. 2004; 49: 109–114. https://doi.org/10.1007/s10038-003-0114-3 PMID: 14727186

15. Chen SN, Ballantyne CM, Gotto AM Jr., Tan Y, Willerson JT, Marian AJ. A common PCSK9 haplotype, encompassing the E670G coding single nucleotide polymorphism, is a novel genetic marker for plasma low-density lipoprotein cholesterol levels and severity of coronary atherosclerosis. J Am Coll Cardiol. 2005; 45: 1611–1619. https://doi.org/10.1016/j.jacc.2005.01.051 PMID: 15893176

16. Fouchier SW, Sankatsing RR, Peter J, Castillo S, Pocovi M, Alonso R, et al. High frequency of APOB, PCSK9, and ANGPTL3 in carriers of pathogenic autosomal dominant hypercholesterolemics with unexpected low LDL-C Levels. Hum Mutat. 2012; 33: 448–455. https://doi.org/10.1002/humu.21660 PMID: 22095935

17. Taniguchi T, Abe S, Hori M, Takahashi A, Ogura M, Makino H, Tamanaha T, et al. Proprotein convertase subtilisin/kexin 9 variant with LDLR mutations modifies the phenotype of familial hypercholesterolemia. J Clin Lipidol. 2016; 10: 289–298. https://doi.org/10.1016/j.jcl.2015.11.017 PMID: 27059540

18. Huijgen R, Sjouke B, Vis K, de Randamie JS, Defesche JC, Kastelein JJ, et al. Genetic variation in the proprotein convertase subtilisin/kexin 9 V4I variant with LDLR mutations modifies the phenotype of familial hypercholesterolemia. J Clin Lipidol. 2016; 10: 289–298. https://doi.org/10.1016/j.jcl.2015.11.017 PMID: 27059540

19. Kotowski IK, Pertsemidis A, Luke A, Cooper RS, Vega GL, Cohen JC, et al. A spectrum of PCSK9 alleles contributes to plasma levels of low-density lipoprotein cholesterol. Am J Hum Genet. 2006; 78: 410–422. https://doi.org/10.1086/500615 PMID: 16465619

20. Fu J, Kwok S, Sinai L, Abdel-Razek O, Babula J, Chen D, et al. Western Database of Lipid Variants (WDLV): a catalogue of genetic variants in monogenic dyslipidemias. Can J Cardiol. 2013; 29: 934–939. https://doi.org/10.1016/j.cjca.2013.01.008 PMID: 23623477

21. Cohen JC, Boerwinkle E, Mosley TH Jr., Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med. 2006; 354: 1264–1272. https://doi.org/10.1056/NEJMoa054013 PMID: 16554528

22. Arfifadel M, Rabes JP, Devillers M, Munirich A, Erlich D, Junien C, et al. Mutations and polymorphisms in the proprotein convertase subtilisin kexin 9 (PCSK9) gene in cholesterol metabolism and disease. Hum Mutat. 2009; 30: 520–529. https://doi.org/10.1002/humu.20882 PMID: 19191301

23. Arsenault BJ, Pelletier-Beaumont E, Almeras N, Tremblay A, Poirier P, Bergeron J, et al. PCSK9 levels in abdominally obese men: association with cardiometabolic risk profile and effects of a one-year
lifestyle modification program. Atherosclerosis. 2014; 236: 321–326. https://doi.org/10.1016/j.atherosclerosis.2014.07.010 PMID: 25128757

24. Levenson AE, Shah AS, Khoury PR, Kimball TR, Urbina EM, de Ferranti SD, et al. Obesity and type 2 diabetes are associated with elevated PCSK9 levels in young women. Pediatr Diabetes. 2017. 2017/01/18. https://doi.org/10.1111/pedi.12490 PMID: 28093849

25. Baragetti A, Balzarotti G, Grigore L, Pellegatta F, Guerrini U, Pisano G, et al. PCSK9 deficiency results in increased ectopic fat accumulation in experimental models and in humans. Eur J Prev Cardiol. 2017. 2017/08/02. https://doi.org/10.1177/2047487317724342 PMID: 28758421