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Resequencing of the *CETP* gene in American whites and African blacks: Association of rare and common variants with HDL-cholesterol levels

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Abstract

Background—Cholesteryl ester transfer protein (CETP) plays a crucial role in lipid metabolism. Associations of common *CETP* variants with variation in plasma lipid levels, and/or CETP mass/activity have been extensively studied and well-documented; however, the effects of uncommon/rare *CETP* variants on plasma lipid profile remain undefined. Hence, resequencing of the gene in extreme phenotypes and follow-up rare-variant association analyses are essential to fill this gap.

Objective—To identify common and uncommon/rare variants in the *CETP* gene by resequencing the entire gene and test the effects of both common and uncommon/rare *CETP* variants on plasma lipid traits in two genetically distinct populations.

Methods and Results—The entire *CETP* gene plus flanking regions were resequenced in 190 individuals comprising 95 non-Hispanic Whites (NHWs) and 95 African blacks with extreme HDL-C levels. A total of 279 sequence variants were identified, of which 25 were novel. Selected variants were genotyped in the entire samples of 623 NHWs and 788 African blacks and 184 QC-passed variants were tested in relation to plasma lipid traits by using gene-based, single-site,

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DISCLOSURES

None.

haplotype and rare variant association analyses (SKAT-O). Two novel and independent associations of rs1968905 and rs289740 with HDL-C were identified in African blacks. Using SKAT-O analysis, we also identified rare variants with minor allele frequency <0.01 to be associated with HDL-C in both NHWs (P=0.024) and African blacks (P=0.009).

Conclusions—Our results point out that in addition to the common *CETP* variants, rare genetic variants in the *CETP* gene also contribute to the phenotypic variation of HDL-C in the general population.

Keywords

CETP; lipid metabolism; HDL-C; genetic association; rare variants; sequencing

1. INTRODUCTION

Human cholesteryl ester transfer protein (CETP protein; *CETP* gene) plays a crucial role in lipid metabolism by mediating the transfer of cholesteryl esters from high density lipoprotein (HDL) to apolipoprotein (apo) B rich lipoproteins in exchange for triglycerides (TG) [1]. High CETP activity contributes to unfavorable plasma lipoprotein profile by lowering HDL-cholesterol (HDL-C) and increasing low-density lipoprotein cholesterol (LDL-C) [1–3]. However, it has been shown that reduced CETP activity is associated with increased CAD risk despite resulting high levels of HDL-C (4–6). Even though the effects of CETP inhibition in the therapy of CAD are conflicting, inhibition of CETP activity has been used as a new approach to raise HDL-C levels (7–8). In order to understand the role of CETP in CAD and to develop therapeutic approaches, the elucidation of genetic variation in the *CETP* gene is important.

Several common variants in *CETP* have been reported to be associated with variation in plasma lipid levels, CAD risk and/or CETP mass/activity [9]. However, reported common *CETP* variants explain only 5–8% of the overall genetic contribution to lipid levels [6]. The so called 'missing heritability' likely lies in low-frequency and rare variants and they can only be uncovered by resequencing individuals with extreme lipid phenotypes [10–11]. This strategy has already been proven to be useful in identifying multiple rare variants in lipid genes affecting variation in plasma lipid levels (12–16). Toward this effort, Khovidhunkit et al. [16] sequenced the exons and exon-intron junctions of the *CETP* gene in 64 Thai individuals with hyperalphalipoproteinemia and identified rare *CETP* variants contributing to HDL-C. However, the contribution of rare *CETP* variants in regulating lipid levels in the general population requires further investigation.

The objective of this study was to resequence the entire *CETP* gene plus flanking regions in selected individuals having extreme HDL-C levels derived from two well-characterized population-based samples of U.S. non-Hispanic whites (NHWs) and African blacks in order to identify both common (minor allele frequency (MAF)≥5%), and rare and uncommon *CETP* variants (MAF <5%) followed by genotype-phenotype association analyses with lipid levels in the entire samples of 788 African blacks and 623 NHWs.

2. METHODS

2.1. Subjects

The study was carried out on two well-characterized and population-based epidemiological samples comprising unrelated 623 non-Hispanic whites (NHWs) from the US and 788 African blacks from Nigeria. NHWs were collected as a part of the San Luis Valley Diabetes Study, a population-based case-control study of type 2 diabetes in the San Luis Valley, Southern Colorado [17,18]. The 623 NHWs subjects used in the current study did not have diabetes and a detailed description of this sample set can be found elsewhere [19]. African black samples were drawn from a study on coronary heart disease (CHD)-related risk factors in Benin City, Nigeria [20,21]. The characteristics of African black samples have been described in detail in Harris et al. (1998). Biometric and quantitative data of 623 NHWs and 788 African Blacks are summarized in Table 1. For the resequencing discovery stage, we selected 95 NHWs and 95 African blacks falling in the upper (47 NHWs, 48 African blacks) and lower (48 NHWs, 47 African blacks) 10^{th} percentile distribution of plasma HDL-C levels (Table 2). This study was approved by the University of Pittsburgh and University of Colorado Denver Institutional Review Boards. All study participants provided written informed consent.

2.2. Lipid measurements

Fasting total cholesterol (TC) was determined by esterase-oxidase method [22]. Serum HDL-C and TG concentrations were measured by enzymatic procedures [23]. LDL-C was calculated by using the Friedewald equation if TG levels were less than 400 mg/dl (4.5 mmol/l) [24].

2.3. DNA sequencing

DNA samples were extracted from blood clots and buffy coats from African blacks and NHWs, respectively, by using standard DNA extraction procedures. Polymerase chain reaction (PCR) was performed to amplify the entire *CETP* gene, including 16 exons and adjacent introns plus 1 kb from both 3' and 5' flanking regions of the gene (total ~26 kb genomic fragment). The Primer 3 software (http://frodo.wi.mit.edu/primer3/) was used for designing primers. The accession number of *CETP* reference sequence used in this study is: NC_000016 derived from Genbank in NCBI site (http://www.ncbi.nlm.nih.gov). A total of 47 overlapping resequencing amplicons were sequenced in both directions in selected 95 NHW and 95 African black individuals with extreme HDL-C levels. PCR conditions and primer sequences are available upon request. After PCR amplification, plates were sent to a commercial lab (Beckman Coulter Genomics, Danvers, MA) where automated Sanger sequencing was performed on ABI 3730x1DNA Analyzers. Sequencing data analysis was performed in our lab by using Variant Reporter (Applied Biosystems, Foster City, CA) and Sequencher (Gene Codes Corporation, Ann Arbor, MI) softwares. All amplicons were successfully analyzed except a repetitive region in intron 2 which did not pass the quality control (QC) and was not analyzed (positions between 6938 bp and 8664 bp).

2.4. Variant selection and Genotyping

Single-nucleotide variant (SNV) selection for genotyping in the total samples of 623 NHWs and 788 African blacks was based on our sequencing data plus information obtained from public databases, and literature including candidate and genome-wide association studies (GWAS) [25–31]. Sequencing-derived SNVs included tagSNPs and uncommon/rare variants that were selected based on their locations in the gene and their frequency distribution among individuals with extreme HDL-C included in resequencing. TagSNP selection was performed by tagger and LD analyses in Haploview by using the following parameters: $r^2 \geq 0.9$, MAF≥0.05. Forty-one common tagSNPs in NHWs and 83 tagSNPs in African blacks were identified which capture 81 and 126 common variants (MAF≥0.05) in NHWs and African blacks, respectively (see Supplementary Tables 1–2**).** We compared our sequencing-derived tagSNPs with the tagSNPs of HapMap data for CEU (Utah residents with ancestry from northern and western Europe) and YRI (Yoruba in Ibadan, Nigeria) populations and determined that all of the HapMap SNVs (MAF≥0.05) in the same region were captured by our tagSNPs. In addition to tagSNPs, 40 uncommon and low frequency (MAF< 0.05) variants in NHWs and 77 in African blacks were selected from our sequencing data. Detailed information for selection criteria of SNVs included in association analyses can be found in Supplementary Table 3 for NHWs and Supplementary Table 4 for African blacks. In addition, we selected 16 SNVs for genotyping (8 in NHWs and 8 in African blacks) that were not detected in our sequencing samples but they have been previously reported in dbSNP build 137 in European and African descent populations. We also genotyped 26 additional SNVs in NHWs and 7 in African blacks that were not identified tagSNPs in our sequencing cohort but previously they have been shown to be associated with lipid levels in candidate or GWAS. Altogether, 115 variants in NHWs and 175 variants in African blacks were selected for genotyping and 251 of them were successfully genotyped in at least one population (111 SNVs in NHWs and 140 SNVs in African blacks).

2.5. Genotyping

Selected variants were genotyped in the total sample of 623 NHWs and 788 African blacks by using either TaqMan (Applied Biosystems) or iPLEX Gold (Sequenom, San Diego, CA) genotyping methods following manufacturer's protocols. The 384-well plates containing dried whole genome amplified DNAs were used in TaqMan and iPLEX Gold genotyping methods. The ABI Prism 7900HT Sequence Detection Systems was used for endpoint fluorescence reading of the 384-well plates after TaqMan protocol. The iPLEX Gold genotyping was performed in the Genomics and Proteomics Core laboratories of the University of Pittsburgh. Sequences of primers and probes used for custom TaqMan and iPLEX Gold genotyping are available upon request.

2.6. Statistical Analysis

Variants identified by sequencing were analyzed by using Haploview [32] (www.broadinstitute.org/haploview) to test the concordance of the genotype distribution with Hardy-Weinberg equilibrium, to determine allele frequencies and their distributions among high and low HDL groups and their linkage disequilibrium (LD) patterns. For those SNVs that were genotyped in the entire sample, the additive linear regression model was

used to test for the effects of genotypes on the means of plasma HDL-C, TG, LDL-C, and TC levels. While all plasma lipid levels were transformed to natural logarithms using Box-Cox transformation to improve normality in African blacks, only HDL-C and TG were transformed in NHWS. The significant covariates were identified using stepwise regression in both directions. In NHWs, the covariates included in the analyses were gender, age, BMI, smoking and, in African blacks, they were gender, age, BMI, smoking, waist (waist measurement (cm) at the narrowest point), staff level (junior/senior) and exercise (minutes walking or bicycling to work each day (min)). The R statistical software package (http://www.r-project.org) was used to perform all computations. False discovery rate (FDR) method [33] was used to control for multiple testing. A P <0.05 was considered as suggestive evidence of association and FDR value (q-value) of <0.05 as statistically significant. The versatile gene-based associations (VEGAS) were performed to assess the relationship between traits and *CETP* [34]. For haplotype association analysis, the generalized linear model (GLM) was used [35]. Since including too many haplotypes can make GLM inefficient and impractical, we used sliding windows consisting of 4 SNVs per window in order to reduce the number of haplotypes considered in association analysis. A global P-value for testing overall effect of haplotypes with frequency >0.01 was used to assess the association between the trait and haplotypes in each window. Sliding-window haplotype analysis was performed using the haplo.glm function in the Haplo.Stats R package (version 1.5.0). We analyzed the cumulative effects of uncommon/rare variants by using burden tests and the SKAT-O method [36]. SKAT-O method has been proposed to be the optimal test for rare variant analysis over the SKAT and burden tests. Three different minor allele frequency bin thresholds (<1%, <2% and <5%) were used to perform the analyses. The SKAT-O method was implemented using the “SKAT” R package.

2.7. Functional annotation of significant SNVs

We used information from RegulomeDB [37] online database (http://regulome.stanford.edu/) version 1.0 to retrieve the regulatory annotations for the identified significant SNVs.

3. RESULTS

3.1. DNA sequencing

We identified a total of 279 variants by resequencing the *CETP* gene in 190 NHWs and African blacks, including 263 diallelic (181 transitions vs. 82 transversions), 2 triallelic and 14 indels. Of 279 variants, 254 are known and 25 are novel (submitted to dbSNP database: http://www.ncbi.nlm.nih.gov/SNP/snp_viewTable.cgi?handle=KAMBOH) (Supplementary Tables 5 and 6). Eighty of these variants were present in both population groups, 45 were found only in NHWs and 154 variants were found only in African blacks. The locations of the identified 279 variants were as follows: 224 in introns, 39 in the flanking regions, 1 in the 5'UTR of exon 1, 1 in the 3'UTR of exon 16, and 14 in coding exons. Of the identified 14 coding variants, 7 were non-synonymous and 7 were synonymous. The seven non-synonymous coding variants were: aspartic acid to valine (D6V) and alanine to glycine (A15G) in exon 1; arginine to tryptophan (R154W) in exon 5; alanine to proline (A390P), and valine to methionine (V385M) in exon 12; isoleucine to valine (I405V) in exon 14; and

arginine to glutamine (R451Q) in exon 15. All observed 14 indels were located in introns, including two insertions and 12 deletions, ranging in size from one to twelve bases. The characteristics of the identified variants are given in Fig. 1.

Of the 125 variants found in NHWs, 82 were common (MAF≥0.05), 20 were uncommon (0.01≤MAF<0.05) and 23 were rare (MAF<0.01) (see Supplementary Table 5). Of the 234 variants identified in African blacks, two were triallelic [rs289712 (intron 8) and rs7192120 (exon 12; synonymous)]. Among the 234 bi-allelic variants, 126 were common (MAF≥0.05), 67 were uncommon (0.01≤MAF<0.05) and 41 were rare (MAF<0.01) (see Supplementary Table 6).

3.2. Distribution of identified *CETP* variants in extreme HDL-C groups

Minor allele frequency distributions of the 125 variants in NHWs and 234 variants in African blacks between the two extreme HDL-C groups are shown in Supplementary Table 5 and Supplementary Table 6, respectively. In NHWs, 14 variants were present only in the low HDL-C group vs 11 only in the high HDL-C group; the remaining were present in both groups. In African blacks, 27 variants were present only in the low HDL-C group vs 29 only in the high HDL-C group; the remaining were present in both groups.

3.3. Genotyping of *CETP* variants

Two hundred and fifty one *CETP* variants were successfully genotyped in at least one population group (111 in NHWs and 140 in African blacks) and 51 of them were present in both groups. The genotype call rates, selection criteria and other features of the genotyped SNVs in NHWs (39 tagSNPs; 72 others) and African blacks (73 tagSNPs; 67 others) are shown in Supplementary Tables 3–4**.** The LD bins (r^2≥0.80) of the final QC passed SNVs are listed in Supplementary Tables 7–8. Of the 140 SNVs genotyped in African blacks, two triallelic SNVs (rs289712C/T/A and rs7192120C/G/T) were excluded from analysis. In NHWs, two SNVs (rs247615, rs12720918), which did not meet Hardy-Weinberg equilibrium after Bonferroni correction (*P*<10E-04), were excluded along with five monomorphic SNVs [rs4784741, rs9924087, rs1968905, rs189866004, rs5887]. In African blacks 7 SNVs were excluded from the analyses; rs158477 had low call rate (<85 %), and five SNVs (rs71383212, rs11860407, rs12708985, rs12720939, rs17231611) deviated from Hardy-Weinberg equilibrium (*P*<10E-04) and rs9936680 was non-polymorphic. Thus, a total of 104 SNVs (69 common and 35 uncommon/rare based on their MAF in the entire sample) in NHWs and 131 variants (86 common and 45 uncommon/rare) in African blacks were included in subsequent association analyses. The discrepancy rate was determined to be 0–0.5% for the genotyped variants based on the random repeats of ~10% of the samples.

3.4. Gene-based association analysis

Gene-based association test was performed to assess the joint effects of all successfully genotyped *CETP* variants on plasma lipid traits (HDL-C, TG, LDL-C, TG). Significant association was observed only with HDL-C in both NHWs (*P*=8.87E-04) and African blacks (*P*=9.00E-06) (Table 3). In view of the strong association of *CETP* with HDL-C, subsequent single-site, haplotype, and rare variant analyses were conducted with HDL-C only.

3.5. Association of common variants (MAF≥0.05) with HDL-C levels

In NHWs, we identified 26 common SNVs exhibiting associations with HDL-C levels (see Table 4). The most significant variant was rs247617 (β=0.043; *P*=7.41E-05; located ~3kb upstream of the *CETP* gene) and this was correlated (r^2>0.46) with twelve other HDL-C-associated SNVs (see Fig. 2) in NHWs. The remaining thirteen SNVs showed independent associations with HDL-C of the top SNV, rs247617. Among African blacks, 31 SNVs were associated with HDL-C; the most significant SNV was non-synonymous, rs34065661 (β=2.07, *P*=7.88E-10) and this was strongly correlated (r^2=0.99) with a promoter SNV rs17231520 (β=1.99, *P*=1.86E-09) (see Table 4 and Fig. 3). We also observed two novel independent associations of rs1968905 (β=0.63, *P*=0.002) and rs289740 (β=0.87, *P*=0.003) with HDL-C. Detailed results of the association of the *CETP* variants with HDL-C in NHWs and African blacks can be found in Supplementary Table 9 and Supplementary Table 10, respectively.

We identified twelve HDL-associated SNVs, which were significant in both populations, including rs183130, rs3764261 (GWAS significant), rs708272 (*Taq1*B), rs1800775 (-629C>A), rs3816117, rs711752, rs1864163, rs289718, rs289719, rs7205804, rs153624 and rs11076175 (see Table 4). The best SNV among the above-listed 12 HDL-associated SNVs was rs183130 (β=1.03; *P*=2.25E-06 in African blacks and β=0.040; *P*=1.91E-04 in NHWs) and this was in LD (r^2=0.49–0.94) with 7 significant SNVs in NHWs (rs3764261, rs1800775, rs3816117, rs711752, rs708272, rs7205804, rs1532624) and in LD (r^2=0.67–0.74) with 3 significant SNVs in African blacks (rs3764261, rs711752, rs708272) (see Fig. 4). The association of four remaining SNVs in NHWs (rs289719, rs289718, rs1864163 and rs11076175) and 8 remaining SNVs in African blacks (rs1800775, rs3816117, rs1864163, rs7205804, rs1532624, rs11076175, rs289718, rs289719) with HDL-C was independent of rs183130.

3.6. Association of uncommon/rare variants (MAF < 0.05) with HDL-C

Three MAF thresholds, (MAF<0.05; MAF<0.02; and MAF<0.01) were used to separate the uncommon/rare variants (35 variants in NHWs, 45 variants in African blacks) in the rare variant association analyses (Table 5). Rare variants with MAF <0.01 were associated with HDL-C in both NHWs (*P*=0.024) and African blacks (*P*=0.009). We also checked the LD of significant rare variants (MAF <0.01) with common variants (MAF≥0.05) and found them not to be in LD with common variants (r^2<0.20) that were associated with lipid traits in our NHW and African black samples.

3.7. Haplotype analyses

Sliding window approach was used to construct overlapping haplotypes of the genotyped SNVs and a global *P*-value was calculated for each 4-SNV window based on the comparison to the most common haplotype as a reference (R package-Haplostat). In both population groups, we observed multiple significant haplotype windows. Window 8 (rs17231506, rs12708968, rs4783961 and rs17245715) revealed the most significant signal for HDL-C (global *P*=3.60E-04) in NHWs, rs17231506 was the only significant SNV in single-site analysis in this haplotype window with a *P*-value of 1.25E-05. In African blacks,

window 14 (rs180075, rs17231520, rs34065661 and rs5884) had the most significant association with HDL-C (global *P*=1.70E-08).

In both population groups, the haplotypes in the 5' of the *CETP* gene showed highly significant association with HDL-C. Fig. 5 shows the plots of the haplotype-based association test results for HDL-C. Details for the haplotype association analyses with HDL-C are shown in Supplementary Tables 11–12.

3.8. Functional annotation of significant SNVs

The RegulomeDB [37] scores for all SNVs identified in NHWs and African blacks are listed in Supplementary Tables 5 and 6, respectively. We identified 34 HDL-C-associated SNVs with a RegulomeDB score of less than 6, and among those, 3 (rs9989419, rs1800775 and rs289743) had a RegulomeDB score of less than or equal to 3 (Table 4). One of our independent signals with HDL-C in NHWs, rs9989419 (located ~8kb upstream of the *CETP* gene) had a RegulomeDB score of 1f (known cis-eQTL which lies within a TF binding site or a DNase peak based on the experimental evidence). This SNV has shown genome-wide significant association with HDL-C (18). Two other SNVs with a RegulomeDB score ≤3, [rs1800775 (Regulome DB score=3a); rs289743 (RegulomeDB score=2b)] were located near the *CETP* gene and they were highly correlated with other HDL-C significant SNVs (Fig. 2 and Fig. 3).

4. DISCUSSION

Previous studies have examined the role of common variants and haplotypes in the *CETP* gene in relation to dysplidemia, atherosclerosis and coronary heart disease with inconsistent outcomes. To our knowledge, this is first study that has resequenced the *CETP* gene and its flanking regions in selected individuals with extreme HDL-C levels from two racial groups and then examined the association of identified common and rare variants with variation in HDL-C levels in the total samples of NHWs and African blacks.

The complete resequencing of the *CETP* gene in 95 NHWs and 95 African blacks revealed a total of 279 variants, of which 80 were found in both populations. We identified all but 16 common variants (8 in NHWs and 8 in African blacks) present in dbSNP (build 137) in populations of European and African descent and also in 1000 genome project. The 16 known variants were missed probably due to technical issues in resequencing and so these SNVs were genotyped in the entire sample of both populations in order to cover the full range of variation in this gene. In addition, we identified 25 low-frequency variants that were not reported in any public databases. In tandem with SNV discovery effort, 200 SNVs including tagSNPs, several low frequency variants (MAF <0.005) and significantly reported variants in and around the *CETP* were successfully genotyped in 623 NHWs and 788 African blacks. A total of 184 QC-passed variants (104 in NHWs and 131 in African blacks) were included in the subsequent association analyses.

In single-site association analysis, we identified 11 and 16 FDR-significant HDL-associated SNVs (FDR<0.05) in NHWs and African blacks, respectively (1.03E-07≤FDR≤0.041). This is consistent with published studies where *CETP* polymorphisms have been reported to

influence mostly HDL-C levels (6, 39–40). Notably, 12 SNVs (rs183130, rs3764261, rs1800775, rs3816117, rs711752, rs708272, rs1864163, rs7205804, rs1532624, rs11076175, rs289718, rs289719) were associated with HDL-C levels in both populations ($P<0.05$; 7.37E- 05≤FDR≤0.133). In NHWs, majority of these 12 SNVs were in strong LD with each other and with the GWAS significant variant, rs3764261 as well as with -629C>A (rs1800775) and *Taq1B* (rs70272), whereas little LD were observed among these SNVs among African blacks (Fig. 4A–4B).

Previously, three SNVs, *TaqIB* (rs708272; located in intron 1), -629C>A (rs1800775; located in promoter) and GWAS significant rs3764261 (5' flanking region), have been shown to be associated with lipid profile and/or CETP mass/activity in several studies [9,42]. It has been reported that up to 10% of the variation in the HDL-C can be explained by the *Taq*IB polymorphisim [9] and that *Taq1*B is a marker of promoter variant -629C>A (rs1800775) which is located in Sp2/SP2 binding sites of the proximal promoter [6, 43–46]. Although there is a strong LD between these two SNVs, their association with HDL-C has not been consistent in all studies [2,9]. In our study, -629C>A was strongly correlated with *Taq1B* in NHWs (r^2=0.75) but not in African blacks (r^2=0.19). Despite this, both *Taq1B* (β=0.033; P=2.42E-03 for NHWs; β=0.88; P=6.33E-05 for African blacks) and -629C>A (β=−0.027, P=1.25E-02 for NHWs; β=−0.53; P=0.006 for African blacks) were associated with plasma HDL-C levels in both populations. Recently, Lu et al. (2013) investigated the association of *Taq1*B and -629C>A polymorphisms with coronary heart disease and lipid levels in a multiethnic population of Singapore [47]. They found a weak correlation between the two polymorphisms and *Taq1*B showed stronger association with HDL-C and ApoA1 levels than -629C>A. Hence, they concluded that there could be additional functional sites, other than -629C>A, within or around the *CETP* gene that is in LD with *Taq1B* (47). In our study, rs183130 (located in ~3kb upstream) was the most significant HDL-associated SNV in both populations and that was also in LD with *Taq1*B and -629C>A. This has also shown consistent association with HDL-C across multiple ethnic groups including Europeans, Asians, and African Americans and it appears to be functional since it alters the consensus transcription binding site [42, 48].

Furthermore, an uncommon non-synonymous SNV, rs1800777 (R451Q), located in exon 15, that was previously reported to be associated with HDL-C levels [39] was found in only NHWs with a MAF of 0.035 (MAF= 0.00 % for both HapMAP YRI and our African blacks) and was associated with HDL-C (P=0.023) levels (see Supplementary Table 9).

Most of the significant SNVs identified in this study were located in the upstream and 5' half of the gene that were strongly correlated with each other and majority of these associations have been reported previously [9,38,41,48–49, 50–57]. However, we also observed two novel independent associations (rs1968905 and rs289740) with HDL-C in African blacks.

We also performed haplotype-based association analysis to evaluate whether combinations of C*ETP* SNVs are more strongly associated with HDL-C than individual SNVs. We found that HDL-C associated significant haplotypes were mainly located in the 5' region, including the promoter and first ~12 kb of the *CETP* gene, and all these significant haplotypes included the HDL-C associated SNVs in single-site analysis. Similar results were obtained

by a recent study in Latvians where a haploblock including SNVs located in ~0.6kb upstream of the *CETP* gene, promoter and first ~10kb of the *CETP* was associated with extreme HDL-C levels [41].

In addition to the single-site and haplotype association analyses, rare variant analyses (MAF<0.01) also revealed significant association with HDL-C levels. Although previous studies have shown the contribution of rare variants in other lipid genes in relation to lipid levels [12–15,38] to our knowledge, this is first study indicating that rare *CETP* variants make a substantial contribution to the variation of HDL-C levels in the general white and black populations. Our results are in concordance with a recent study published by ENGAGE consortium that highlights the aggregate effects of low-frequency/rare variants on inter-individual variation in lipid traits [59].

There are some limitations in our study. First, our sequencing sample in extreme HDL-C groups was relatively small in both ethnic groups and thus we may have missed some functional rare variants. Second, resequencing of the *CETP* failed to capture ~2kb in intron 2. Although we genotyped all reported significant and common SNVs in this un-sequenced region, some uncommon SNVs may have been missed and thus could not be analyzed.

In conclusion, this study provides credence to the "common disease-common variant" and "common disease-rare variant" hypotheses as both common and rare *CETP* variants were associated with HDL-C levels in American white and African black populations, and it further establishes the pivotal role of *CETP* in HDL metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CETP	cholesteryl ester transfer protein
SNP	single-nucleotide polymorphism
QC	quality control
TG	triglyceride
MAF	minor allele frequency
HDL-C	HDL cholesterol
LD	linkage disequilibrium
FDR	false discovery rate

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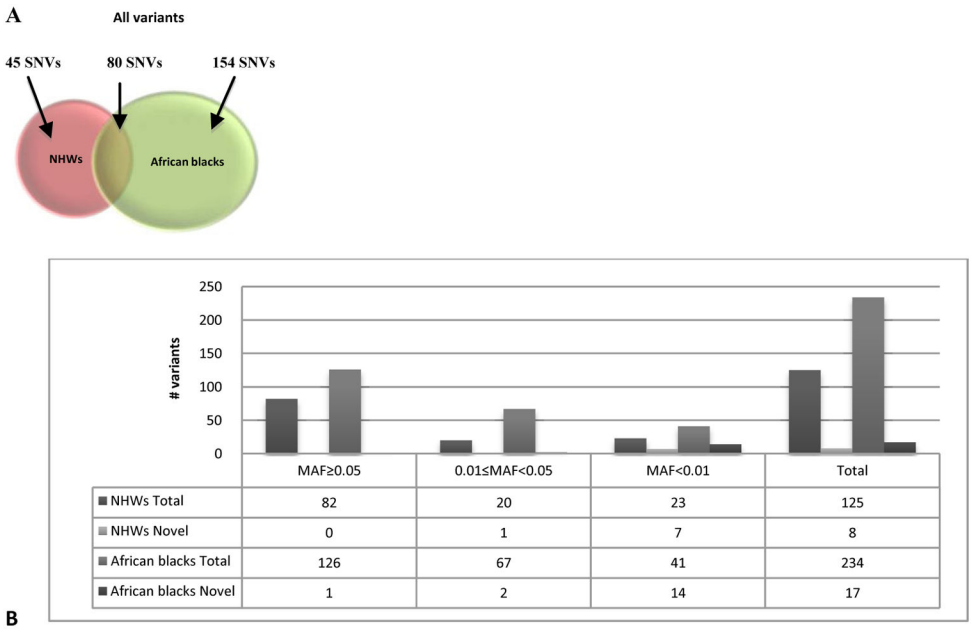

	MAF≥0.05	0.01≤MAF<0.05	MAF<0.01	Total
NHWs Total	82	20	23	125
NHWs Novel	0	1	7	8
African blacks Total	126	67	41	234
African blacks Novel	1	2	14	17

Fig. 1. Properties of the *CETP* variants identified in American whites (NHWs) and African blacks

a. Number of all SNVs identified in each population are shown by venn diagrams**. b.** Chart showing the distribution of the minor allele frequencies (MAF) of all variants

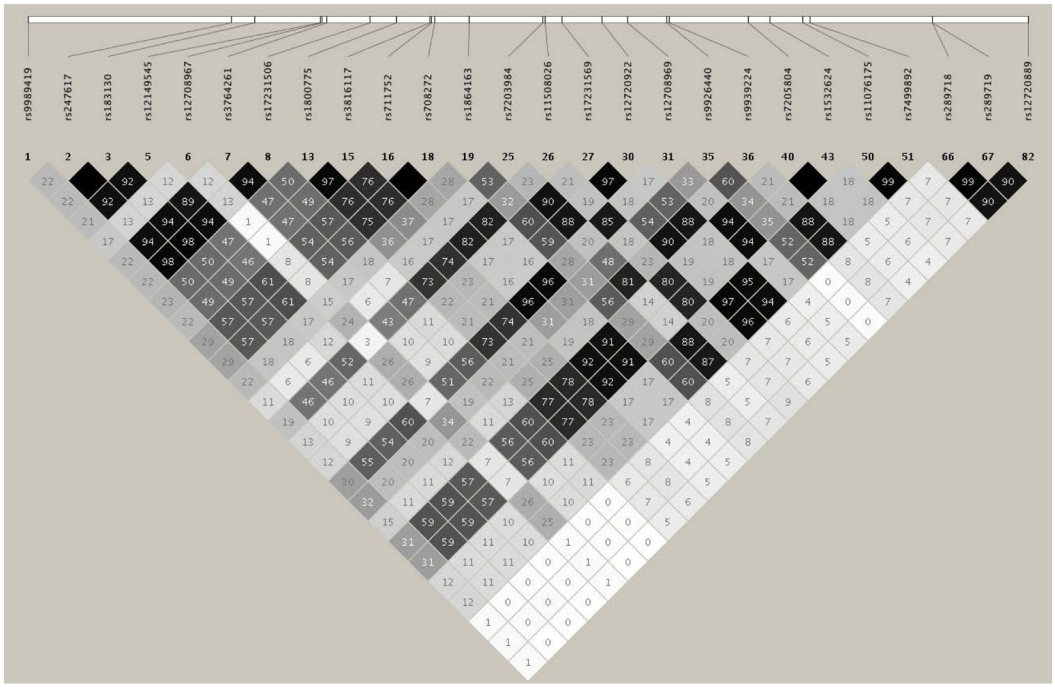

Fig. 2. LD structure of 26 common *CETP* SNVs associated with HDL-C in 623 NHWs

The degree of shades and values (r^2 x 100) in each square represent the pairwise LD between 26 genotyped SNPs: black indicating complete LD ($r^2 = 1$), white indicating no LD ($r^2 = 0$), and shade intensity indicating the degree of LD (r^2 between 0–1).

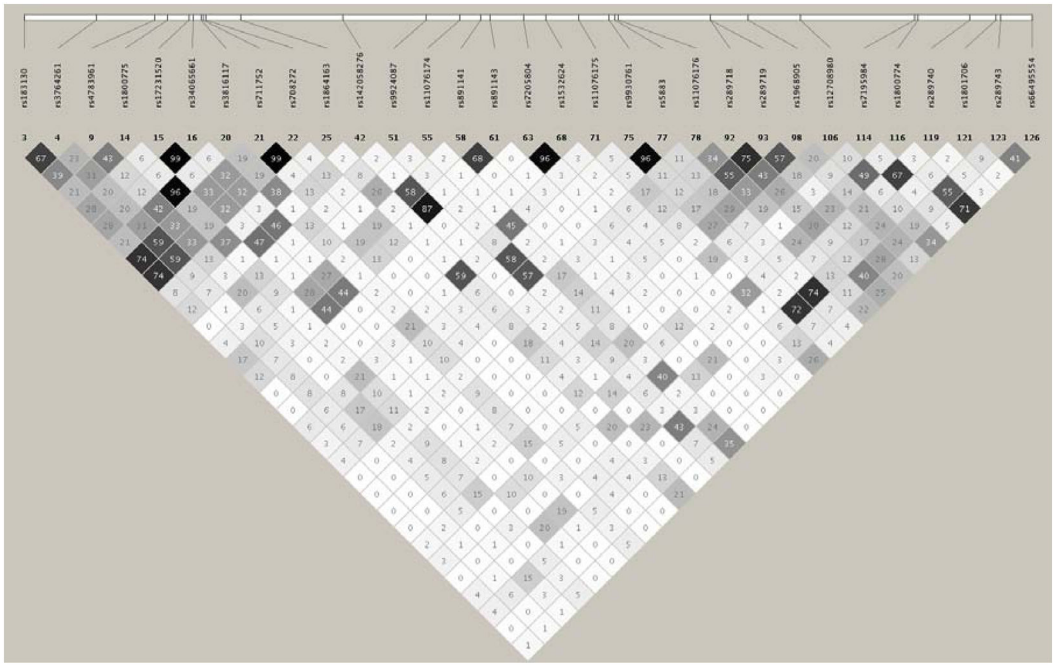

Fig. 3. LD structure of 31 common *CETP* SNVs associated with HDL-C in 788 African blacks

The degree of shades and values (r^2 x 100) in each square represent the pairwise LD between 31 genotyped SNPs: black indicating complete LD (r^2 = 1), white indicating no LD (r^2 = 0), and shade intensity indicating the degree of LD (r^2 between 0–1).

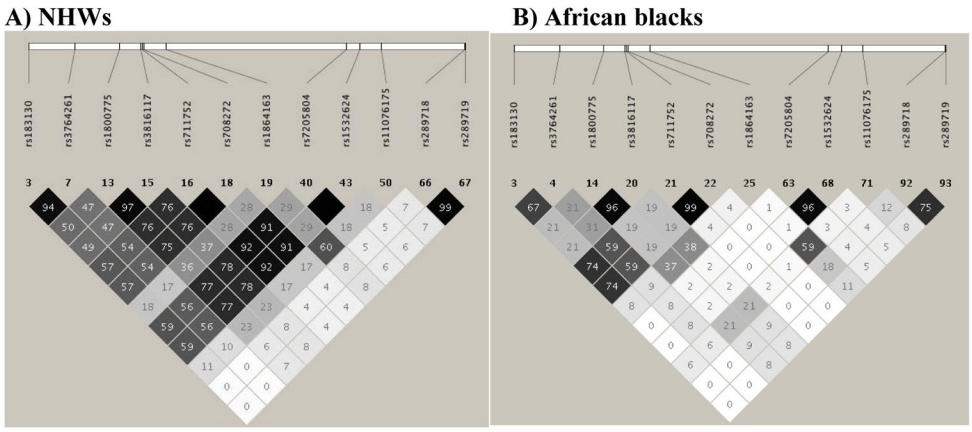

Fig. 4. LD structure of 12 common *CETP* SNVs associated with HDL-C in both populations. A) NHWs, B) African blacks

The degree of shades and values (r^2 x 100) in each square represent the pairwise LD between 12 genotyped SNPs: black indicating complete LD (r^2 = 1), white indicating no LD (r^2 = 0), and shade intensity indicating the degree of LD (r^2 between 0–1).

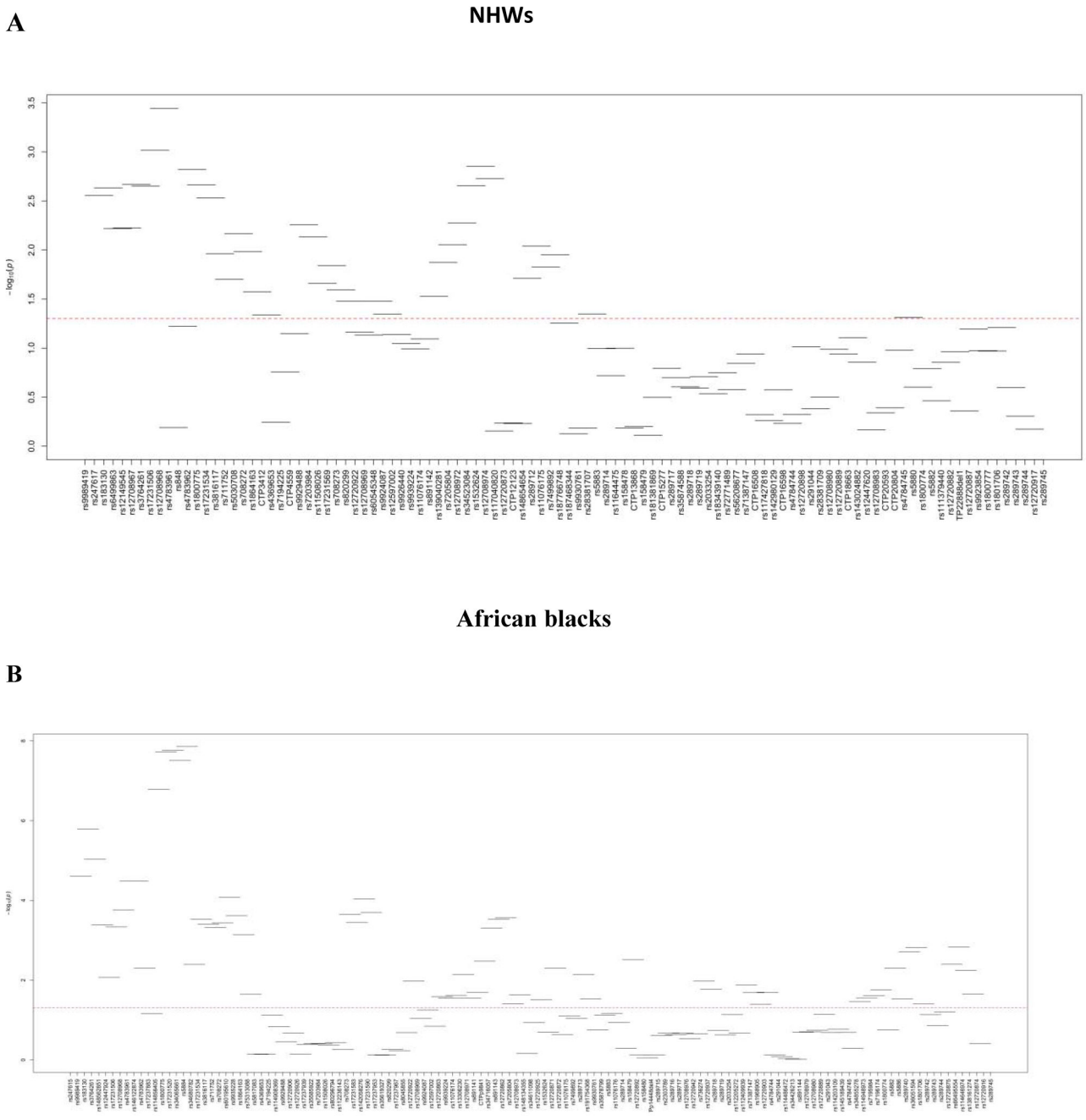

Fig. 5. *CETP* haplotype analysis with HDL-C levels in NHWs and African blacks

Haplotype windows for NHWs (A) and for African blacks (B). The global (-log)10 P values are presented in the y-axis. Horizontal lines represent the window tested (101 haplotype windows in NHWs and 128 haplotype windows in African blacks). Variants are shown on the x-axis. Red line shows the threshold for statistical significance (*P*=0.05).

Table 1

Biometric and quantitative data (mean±SD) of the entire American white (NHWs) and African black samples

Variable Mean±sd or percentage	NHWs (n=623)	African Blacks (n=788)
Males/Females (%)	47.35/52.64	62.81/37.18
Age (Yrs)	52.83 ± 11.41	40.95±8.39
BMI (kg/m^2)	25.51 ± 4.06	22.87±4.04
LDL-C (mg/dl)	136.9 ± 40.8	109.25±34.40
HDL-C (mg/dl)	50.8 ± 14.4	47.88±12.87
TG (mg/dl)	142.72 ± 93.5	72.96±39.32
TC (mg/dl)	217.0 ± 43.5	172.01±38.47

TC: Total cholesterol; LDL-C: Low-density lipoprotein cholesterol; HDL-C; High-density lipoprotein cholesterol; TG: Triglycerides

Table 2

Biometric and quantitative data (mean±SD) of resequencing samples of 95 NHWs, and 95 African blacks

	NHWs (n=95)			African Blacks (n=95)		
	High HDL-C (n=47)	**Low HDL-C (n=48)**	**P-value***	**High HDL-C (n=48)**	**Low HDL-C (n=47)**	**P-value***
Sex (M/F)	24/23	24/24	1.00	24/24	23/24	1.00
Age (years)	55.45 ± 9.80	53.03 ± 10.54	0.25	41.29 ± 8.72	40.87 ± 7.12	0.80
BMI (kg/m^2)	23.17 ± 3.17	27.35 ± 3.90	1.2E-07	22.06 ± 4.70	23.91 ± 5.51	0.08
TC (mg/dl)	227.34 ± 51.76	208.81± 44.65	0.07	201±39.68	141.68 ± 31.03	2.4E-12
LDL-C (mg/dl)	126.84 ± 46.95	125.54 ± 54.97	0.90	112.55 ± 39.75	95.04 ± 28.28	0.02
HDL-C (mg/dl)	77.68 ± 13.32	31.81 ± 4.37	2.2E-16	76.05 ± 7.53	25.51 ± 5.66	2.2E-16
TG (mg/dl)	114.09 ± 60.88	240.21 ±153.22	1.7E-06	61.98 ± 19.85	95.79 ± 73.21	0.004

TC: Total cholesterol; LDL-C: Low-density lipoprotein cholesterol; HDL-C; High-density lipoprotein cholesterol; TG: Triglycerides;

*P-values were calculated using t-test. No covariates were included.

Table 3

CETP gene-based association test in NHWs and African blacks

	NHWs				African blacks			
Trait	**Number of SNVs***	**Test Statistics**	***P***	***b* Smallest *P***	**Number of SNVs***	**Test Statistics**	***P***	***b* Smallest *P***
HDL-C	100	322.1438	8.87E-04	7.41E-05 (rs247617)	131	470.565	9.00E-06	7.88E-10 (rs34065661)
LDL-C	100	81.92025	0.639	6.864E-03 (CTP13868)	131	150.8933	0.277	0.019 (rs80296794)
TG	100	52.73295	0.944	0.01562 (rs56208677)	131	104.4884	0.733	3.097E-03 (rs114908369)
TC	100	91.41172	0.538	2.05E-04(CTP13868)	131	181.023	0.117	3.057E-03 (rs114203109)

[a] P: gene-based,

[b] Smallest P detected for a single SNV

* Variants with missing phenotype data were not included

Table 4

Association results (*P*<0.05) of common *CETP* variants with HDL-C levels in NHWs (n=623) and African blacks (n=788)

				NHWs				African blacks			
† RefSNP ID	†† Chr16 Position	Locations	MAF	Beta	*P*	FDR	MAF	Beta	*P*	FDR	‡ RegulomeDB Scores
rs9989419	56951227	5' flanking	0.4	−0.026	0.014	0.067	-	-	-	-	1f
rs247617	56956804	5' flanking	0.347	0.043	7.41E-05	0.004	-	-	-	-	5
rs183130	56957451	5' flanking	0.347	0.04	1.91E-04	0.004	0.244	1.03	2.25E-06	7.37E-05	No data
rs12149545	56959249	5' flanking	0.33	0.042	1.66E-04	0.004	-	-	-	-	No data
rs12708967	56959299	5' flanking	0.205	-0.034	1.14E-02	0.065	-	-	-	-	No data
rs3764261	56959412	5' flanking	0.328	0.043	1.63E-04	0.004	0.323	0.62	0.002	0.02	No data
rs17231506	56960616	5' flanking	0.343	0.042	1.25E-04	0.004	-	-	-	-	6
rs4783961	56960982	5' flanking	-	-	-	-	0.454	0.64	0.001	0.011	4
rs1800775	56961324	5' flanking	0.489	-0.027	1.25E-02	0.004	0.395	−0.53	0.006	0.041	3a
rs17231520	56961915	5' flanking	-	-	-	-	0.09	1.99	1.86E-09	1.22E-07	5
rs34065661	56962023	Exon 1 (A15G)	-	-	-	-	0.088	2.07	7.88E-10	1.03E-07	5
rs3816117	56962246	Intron 1	0.488	−0.025	1.84E-02	0.065	0.394	−0.54	0.006	0.041	5
rs711752	56962299	Intron 1	0.443	0.033	2.19E-03	0.083	0.232	0.91	3.70E-05	0.001	5
rs708272	56962376	Intron 1	0.442	0.033	2.42E-03	0.031	0.236	0.88	6.33E-05	0.001	5
rs1864163	56963321	Intron 2	0.265	−0.035	3.28E-03	0.031	0.278	−0.7	0.001	0.013	5
rs7203984	56965346	Intron 2	0.206	−0.032	0.011	0.034	-	-	-	-	5
rs11508026	56965416	Intron 2	0.472	0.025	0.012	0.065	-	-	-	-	5
rs17231569	56965867	Intron 2	0.19	-0.031	0.019	0.065	-	-	-	-	5
rs142058276	56966072	Intron 2	-	-	-	-	0.082	1.66	2.06E-06	7.37E-05	No data
rs12720922	56966973	Intron 2	0.192	−0.03	0.022	0.091	-	-	-	-	5
rs12708969	56967672	Intron 2	0.439	0.028	0.009	0.065	-	-	-	-	No data
rs9924087	56968330	Intron 2	-	-	-	-	0.207	0.57	0.011	0.061	
rs9926440	56968751	Intron 2	0.302	−0.027	0.019	0.083	-	-	-	-	4
rs9939224	56968820	Intron 2	0.205	−0.034	0.008	0.065	-	-	-	-	4
rs11076174	56969234	Intron 2	-	-	-	-	0.132	−0.71	0.013	0.068	5

				NHWs				African blacks			
† RefSNP ID	†† Chr16 Position	Locations	MAF	Beta	*P*	FDR	MAF	Beta	*P*	FDR	‡ RegulomeDB Scores
rs891141	56969811	Intron 4	-	-	-	-	0.13	0.79	0.004	0.031	4
rs891143	56970068	Intron 5	-	-	-	-	0.09	1.29	1.13E-04	0.002	No data
rs7205804	56970977	Intron 5	0.453	0.031	0.004	0.039	0.097	−0.76	0.015	0.071	5
rs1532624	56971567	Intron 7	0.452	0.032	0.003	0.031	0.097	−0.77	0.014	0.071	5
rs11076175	56972466	Intron 8	0.184	−0.034	0.01	0.065	0.243	−0.51	0.02	0.086	No data
rs7499892	56972678	Intron 8	0.185	−0.033	0.011	0.065	-	-	-	-	No data
rs9930761	56973280	Intron 8	-	-	-	-	0.148	0.72	0.01	0.059	4
rs5883	56973441	Exon 9 (F287F)	-	-	-	-	0.155	0.65	0.017	0.075	4
rs11076176	56973534	Intron 9	-	-	-	-	0.403	−0.43	0.027	0.106	5
rs289718	56976020	Intron 10	0.304	0.023	0.043	0.133	0.438	−0.4	0.035	0.129	4
rs289719	56976029	Intron 10	0.3	0.023	0.043	0.133	0.493	0.41	0.033	0.124	5
rs1968905	56977036	Intron 10	-	-	-	-	0.36	0.63	0.002	0.02	6
rs12708980	56978467	Intron 11	-	-	-	-	0.342	−0.51	0.011	0.061	6
rs12720889	56978651	Intron 11	0.29	0.025	0.031	0.117	-	-	-	-	No data
rs7195984	56981551	Intron 12	-	-	-	-	0.169	0.72	0.005	0.035	4
rs1800774	56981633	Intron 12	-	-	-	-	0.217	−0.51	0.025	0.103	4
rs289740	56983038	Intron 14	-	-	-	-	0.117	0.87	0.003	0.031	4
rs1801706	56983750	3'UTR-Exon 16	-	-	-	-	0.176	0.57	0.021	0.088	4
rs289743	56983884	3'flanking	-	-	-	-	0.315	−0.39	0.0480.015	0.165	2b
rs66495554	56984724	3'flanking	-	-	-	-	0.193	−0.58		0.071	No data

† dbSNPbuild 141

†† RefSeq: hg19, NC_000016.10 MAF: Minor allele frequency FDR; false discovery rate

‡ RegulomeDB scores were generated by using http://regulome.stanford.edu/.

Scores represents; Category 1 (Likely to affect binding and linked to expression of a gene target): 1a- eQTL + TF binding + matched TF motif + matched DNase Footprint + DNase peak;1b- eQTL + TF binding + any motif + DNase Footprint + DNase peak;1c- eQTL + TF binding + matched TF motif + DNase peak;1d- eQTL + TF binding + any motif + DNase peak;1e- eQTL + TF binding + matched TF motif;1f- eQTL + TF binding / DNase peak; Category 2 (Likely to affect binding): 2a- TF binding + matched TF motif + matched DNase Footprint + DNase peak; 2b- TF binding + any motif + DNase Footprint + DNase peak; 2c- TF binding + matched TF motif + DNase peak; Category 3 (Less likely to affect binding) 3a- TF binding + any motif + DNase peak;3b- TF binding + matched TF motif; Category 4–6 (Minimal binding evidence): 4- TF binding + DNase peak; 5-TF binding or DNase peak; 6-Motif hit

Table 5

Summary results of rare variant association analysis (SKAT-O) with HDL-C in NHWs and African blacks

	NHWs					African blacks				
MAF threshold	**N.RV[a]**	**N.Sample_RV[b]**	**N.Sample_NoRV[c]**	**Stat**	***P***	**N.RV[a]**	**N.Sample_RV[b]**	**N.Sample_NoRV[c]**	**Stat**	***P***
Bin 1 (MAF <0.05)	35	312	311	1.10E+05	0.030	45	598	190	2.16E+05	0.077
Bin 2 (MAF <0.02)	29	147	476	5.94E+04	0.072	17	195	593	2.27E+05	0.121
Bin 3 (MAF <0.01)	26	103	520	6.93E+04	0.024	6	30	758	3.47E+04	0.009

Abbreviation: SKAT-O, optimal sequencing Kernel association test. Bin 1 contained all variants with MAF<0.05, Bin 2 contained all variants with MAF<0.02 and Bin 3 contained all variants with MAF<0.01.

[a] N.RV: Number of rare variants;

[b] N_Sample_RV: Number of individuals carrying the rare allele with defined MAF cut offs.

N.Sample_NoRV: Number of individuals who do not carry any rare alleles with defined MAF cut offs.