Resequencing Genomic DNA of Patients With Severe Hypertriglyceridemia (MIM 144650)

Jian Wang, Henian Cao, Matthew R. Ban, Brooke A. Kennedy, Siqi Zhu, Sonia Anand, Salim Yusuf, Rebecca L. Pollex, Robert A. Hegele

Objective—The genetic determinants of severe hypertriglyceridemia (HTG; MIM 144650) in adults are poorly defined. We therefore resequenced 3 candidate genes, namely LPL, APOC2, and APOA5, to search for accumulation of missense mutations in patients with severe HTG compared with normolipidemic subjects.

Methods and Results—We resequenced >2 million base pairs of genomic DNA from 110 nondiabetic patients with severe HTG and determined the prevalence of coding sequence variants compared with 472 age- and sex-matched normolipidemic controls. We found: (1) heterozygous mutations (LPL p.Q-12E >11X, p.D25H, p.W86R, p.G188E, p.I194T and p.P207L; APOC2 p.K19T and IVS2–30G>A) in 10.0% of severe HTG patients compared with 0.2% of controls (carrier odds ratio [OR] 52, 95% confidence interval [CI] 8.6 to 319); and (2) an association of the APOA5 p.S19W missense variant with severe HTG (carrier OR 5.5 95% CI 3.3 to 9.1). Furthermore, either rare mutations or the APOA5 p.S19W variant were found in 41.8% of HTG subjects compared with 8.9% of controls (carrier OR 7.4, 95% CI 4.5 to 12.0). Also, heterozygotes for rare mutations had a significantly reduced plasma triglyceride response to fibrate monotherapy.

Conclusions—Both common and rare DNA variants in candidate genes were found in a substantial proportion of severe HTG patients. The findings underscore the value of candidate gene resequencing to understand the genetic contribution in complex lipoprotein and metabolic disorders.

Key Words: complex trait ■ metabolism ■ atherosclerosis ■ pancreatitis ■ mutation

Hypertriglyceridemia (HTG) is a commonly encountered phenotype that is a defining component of the metabolic syndrome1 and is associated with numerous comorbidities, including increased coronary heart disease (CHD) risk.2 Furthermore, plasma triglyceride (TG) concentrations >10 mmol/L—a level that defines adult patients with Frederickson type 5 hyperlipoproteinemia (MIM 144650)—are associated with increased risk of acute pancreatitis.3,4 Plasma TG concentration >10 mmol/L is seen in ~1 in 600 adult North Americans.5 Although both genetic and lifestyle factors determine plasma TG concentration, the genetic component remains incompletely defined.6

Complex quantitative traits, such as plasma TG, do not conform to Mendelian inheritance patterns; instead their genetic basis represents the cumulative contribution of multiple DNA variants.7 A promising new strategy in human genetics to understand common complex traits is called the “missense accumulation approach”, which aims to detect enrichment of rare, deleterious missense DNA variants in cases taken from one extreme of the distribution of a quantitative trait versus a control group.7 The cumulative frequency of missense mutations rather than their individual frequencies is then compared between cases and controls.7 This method has proven to be successful for investigation of common disease traits that have a very heterogeneous spectrum of predisposing alleles.7 For instance, the missense accumulation approach has been successfully used to evaluate the MC4R gene6 and several other genes in obesity,7 and the tyrosine phosphatome in colorectal cancers.10

However, the most successful application of the missense accumulation strategy has been in lipoprotein metabolism, as evidenced by the pioneering work of Hobbs and Cohen.11-14 They have found enrichment of missense mutations in individuals at the extremes of several plasma lipoprotein traits, including: (1) increased missense mutations in LCAT, APOAI, and ABCA1 among individuals with depressed high-density lipoprotein (HDL) cholesterol13; (2) increased PCSK9 missense or nonsense mutations among individuals with depressed low-density lipoprotein (LDL) cholesterol14; (3) increased missense mutations in NPC1L1 in individuals with reduced sterol absorption and low plasma LDL cholesterol12; and (4) increased missense mutations in ANGPTL4 in indi-
individuals with depressed triglyceride (TG) and increased HDL cholesterol.11 In these studies, the statistical association of the accumulation of rare coding sequence variants implicated the gene as contributing to the traits under study, whereas no direct experimental evidence of dysfunction for the mutations was provided.11–14 Furthermore, most rare missense variants that accumulate in patients clustered at the extremes of a quantitative trait are dysfunctional.7

Homozygous mutations in candidate genes for plasma TG metabolism, namely LPL encoding the main plasma hydrolytic enzyme lipoprotein lipase (LPL), and APOC2 encoding its circulating cofactor apolipoprotein (apo) C-II, are found in patients with Frederickson type 1 hyperlipoproteinemia15–17 (MIM 238600), a disorder that affects ~1 in 1 million people.13,15 Also, homozygous nonsense mutations in APOA5 encoding apo A-V, a protein that promotes LPL activity,18 have been found in probands with late-onset chylomicronemia.19 Because the prevalence of coding sequence variants in adults with severe HTG is unknown, we resequenced a total of >2 million base pairs of genomic DNA from nondiabetic patients with severe HTG and used the missense accumulation approach to determine the association of variants in LPL, APOC2, and APOA5 with severe hypertriglyceridemia.

Methods

Subjects

We studied 110 nondiabetic patients of European geographic ancestry with severe HTG, defined as having fasting plasma TG >10 mmol/L documented on ≥2 occasions, from a single tertiary referral lipid clinic. Patients underwent a complete medical history and examination; basic clinical, biochemical, and demographic variables were collected. Normolipidemic nondiabetic adult controls were taken from the European subgroup of the Study of Health Assessment and Risk in Ethnic groups (SHARE), a survey of individuals with depressed triglyceride (TG) and increased HDL cholesterol.11 In these studies, the statistical association of the accumulation of rare coding sequence variants implicated the gene as contributing to the traits under study, whereas no direct experimental evidence of dysfunction for the mutations was provided.11–14 Furthermore, most rare missense variants that accumulate in patients clustered at the extremes of a quantitative trait are dysfunctional.7

Homozygous mutations in candidate genes for plasma TG metabolism, namely LPL encoding the main plasma hydrolytic enzyme lipoprotein lipase (LPL), and APOC2 encoding its circulating cofactor apolipoprotein (apo) C-II, are found in patients with Frederickson type 1 hyperlipoproteinemia15–17 (MIM 238600), a disorder that affects ~1 in 1 million people.13,15 Also, homozygous nonsense mutations in APOA5 encoding apo A-V, a protein that promotes LPL activity,18 have been found in probands with late-onset chylomicronemia.19 Because the prevalence of coding sequence variants in adults with severe HTG is unknown, we resequenced a total of >2 million base pairs of genomic DNA from nondiabetic patients with severe HTG and used the missense accumulation approach to determine the association of variants in LPL, APOC2, and APOA5 with severe hypertriglyceridemia.

DNA Analysis

DNA was extracted as described.21 Coding regions and intron-exon boundaries of LPL (10 exons), APOC2 (4 exons), and APOA5 (4 exons) were amplified, purified, and then directly sequenced in 5′- and 3′-directions in an ABI 3730 DNA Analyzer (Applied Biosystems) using reagents shown in supplemental Table I. DNA sequences were analyzed using Sequence Navigator software (Applied Biosystems). DNA variants were confirmed in an independent sample on another day. Screening of controls for sequence variants was performed using allele-specific methods such as restriction endonuclease analysis or a method called SNAPSHOT (Applied Biosystems), as summarized in supplemental Table II. Blinded between-day replicated genotypes of a random 5% of samples showed >99.9% concordance. DNA variants with a minor allele frequency (MAF) <1% in controls were analyzed separately from variants with MAF >1%.

Bioinformatic Studies

We used both the PANTHER (www.pantherdb.org)22,23 and PolyPhen24 algorithms to impute dysfunction of sequence variants. Predictions of dysfunction from both programs are well correlated with in vitro functional assessment.22,23 The scores from each program were grouped into 3 categories: “probably deleterious”, “possibly deleterious”, and “benign”. The majority of biochemically-

### Table 1. Baseline Attributes of Study Subjects

<table>
<thead>
<tr>
<th></th>
<th>Severe HTG</th>
<th>Controls</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>110</td>
<td>472</td>
<td></td>
</tr>
<tr>
<td>Percent female</td>
<td>32.1%</td>
<td>37.3%</td>
<td>NS (0.29)</td>
</tr>
<tr>
<td>Age, years</td>
<td>49.9±12.9</td>
<td>47.3±14.4</td>
<td>NS (0.10)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>29.4±4.2</td>
<td>27.8±4.4</td>
<td>NS (0.67)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>11.9±6.0</td>
<td>5.20±0.93</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>32.6±26.5</td>
<td>1.46±0.73</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/L</td>
<td>0.78±0.35</td>
<td>1.23±0.33</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data shown for quantitative variable are means±SD. HTG indicates hypertriglyceridemia; HDL, high-density lipoprotein; NS, not significant at nominal level of significance.

proven functional mutations have scores of either “probably” or “possibly” deleterious for both programs.24,23

### Results

#### Clinical and Biochemical Features

Baseline attributes of the study sample are shown in Table 1. 110 nondiabetic severe HTG cases were each matched with up to 4 controls based on age within 5 years and sex. By definition, severe HTG patients had markedly higher plasma TG and total cholesterol and significantly lower HDL cholesterol. Plasma TG concentration in severe HTG patients ranged from 10.1 to 180 mmol/L. In addition, 32/110 severe HTG patients (29.0%) had been hospitalized on ≥1 occasion with pancreatitis.

#### Rare Mutations in Candidate Genes

Mutations, defined as DNA sequence variants with MAF <1% in controls or known functional disease-causing mutations that were found in the genomic DNA of severe HTG patients, with their highest recorded plasma TG concentrations, are summarized in Table 2. In the severe HTG patients, we found 12 occurrences of heterozygous candidate gene mutations. Carriers were heterozygous for 1 of 9, mostly known disease-causing mutations: 6 in LPL, 2 in APOC2 and 1 in APOA5. For instance, in the homozygous state, LPL p.W86R, p.G188E, p.I194T, and p.P207L each cause HLP types 1 and 1 each is dysfunctional in vitro, with significantly impaired or absent hydrolytic capacity of the mutant gene product.26–29 Among the novel heterozygous mutations observed in this study, LPL p.Q-12E >11X was a frameshift mutation with a very prematurely truncated product, whereas LPL p.D25H was predicted to be deleterious in both PANTHER and PolyPhen. The known APOC2 p.K197T variant was previously associated with dyslipidemia.30,31 The novel APOC2 IVS2–30G>A variant potentially affects RNA splicing. APOA5 p.A315V32 was predicted to be possibly deleter-
rious; however, in the absence of more definitive demonstration of dysfunction, we treated the single carrier of this mutation as a noncarrier in subsequent analyses.

Common DNA Sequence Variants
By resequencing, we found 5 reported candidate gene single nucleotide polymorphisms (SNPs) with MAF <1% in controls: 3 in LPL, namely p.D9N, p.N291S, and p.S447X and 2 in APOA5, namely p.S19W and p.V153 (Table 3). The LPL SNPs were previously functionally assessed: p.D9N had compromised LDL uptake but not impaired hydrolysis, whereas p.N291S and p.S447X had 50% decreased and 30% increased hydrolytic capacity, respectively. APOA5 p.S19W is defectively secreted in vitro. The APOA5 p.S19W allele was significantly more prevalent in severe HTG cases compared with controls (Table 3).

Differences in Distribution of DNA Variants Between Cases and Controls
Frequencies of carriers of rare mutations in severe HTG patients and normotriglyceridemic controls are shown in Table 4. Heterozygous LPL mutations p.Q-12E-11X (once), p.D25H (once), p.W86R (once), p.G188E (twice), p.I194T (once), and p.P207L (once) were present cumulatively in 7/110 (6.4%) of severe HTG patients compared with 0/472 controls; the carrier odds ratio (OR) was infinite (P<0.00001). When heterozygotes for either APOC2 p.K19T or IVS2–30G>A were included, 10.0% of severe HTG patients compared with 0.2% of controls were carriers of mutations (carrier OR 52, 95% confidence interval [CI] 8.6 to 319; P<10^-7).

The APOA5 p.S19W loss-of-function allele was strongly associated with severe HTG: 34.6% of HTG subjects were carriers versus 8.8% of controls (OR 3.2, 95% CI 1.5 to 7.0; P=0.0017). The LPL p.S447X variant had a borderline associated with protection from severe HTG: 6.4% of HTG subjects were carriers versus 10.8% of controls (OR 0.44, 95% CI 0.20 to 0.99; P=0.043). To quantify the total genetic contribution of the most significantly associated variants, we determined carrier OR for subjects with 1 copy of either the heterozygous rare mutations or 1 copy of the APOA5 p.S19W allele. We found that 41.8% of HTG subjects were carriers compared with 8.9% of controls (OR 7.4, 95% CI 4.5 to 12.0; P<10^-11).

<table>
<thead>
<tr>
<th>Variant Name</th>
<th>New or Known</th>
<th>Predicted Dysfunction</th>
<th>Published Dysfunction</th>
<th>Severe HTG (n=110)</th>
<th>Controls (n=472)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene: LPL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.Q-12E &gt;11X</td>
<td>New</td>
<td>Frameshift with early truncation</td>
<td>None</td>
<td>1 (11.4)*</td>
<td>0</td>
</tr>
<tr>
<td>p.D25H</td>
<td>New</td>
<td>Probable</td>
<td>Probable</td>
<td>1 (33.4)</td>
<td>0</td>
</tr>
<tr>
<td>p.W86R</td>
<td>Known</td>
<td>Probable</td>
<td>Probable</td>
<td>1 (44.1)</td>
<td>0</td>
</tr>
<tr>
<td>p.G188E</td>
<td>Known</td>
<td>Possible</td>
<td>Possible</td>
<td>2 (17.7; 54.7)</td>
<td>0</td>
</tr>
<tr>
<td>p.I194T</td>
<td>Known</td>
<td>Possible</td>
<td>Probable</td>
<td>1 (35.7)</td>
<td>0</td>
</tr>
<tr>
<td>p.P207L</td>
<td>Known</td>
<td>Probable</td>
<td>Probable</td>
<td>1 (34.8)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Gene: APOC2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.K19T</td>
<td>Known</td>
<td>Possible</td>
<td>Probable</td>
<td>3 (44.2; 15.1; 22.6)</td>
<td>1 (2.0)</td>
</tr>
<tr>
<td>IVS2-30G&gt;A</td>
<td>New</td>
<td>Splicing</td>
<td>mutation</td>
<td>None</td>
<td>1 (11.4)</td>
</tr>
<tr>
<td><strong>Gene: APOA5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.A315V</td>
<td>Known</td>
<td>Possible</td>
<td>None</td>
<td>1 (41.8)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Maximum recorded plasma triglyceride concentration (in mmol/L) for the carrier(s) of the specified mutation. HTG indicates hypertriglyceridemia; LPL, gene encoding lipoprotein lipase; APOC2, gene encoding apolipoprotein C-II; APOA5, gene encoding apolipoprotein A-V; HLP, hyperlipoproteinemia; WT, wild-type gene product.
Response to Fibrate Therapy According to Genotype

To determine a possible between-genotype difference in plasma TG response to oral fibrate treatment, we performed an exploratory post hoc analysis in the subgroup of 53 nondiabetic HTG patients whose treatment consisted only of dietary counseling and usual doses of fenofibrate, gemfibrozil, or bezafibrate as monotherapy. In this subgroup (50.1 ± 12.9 years, 34% female), we recorded the maximal percent change from baseline plasma lipoproteins within 12 months of initiating treatment. The subgroup comprised 7, 18, and 28 HTG patients who had ≥1 copy of either the heterozygous rare mutations, ≥1 copy of the APOA5 p.S19W allele, and neither, respectively. We observed a significant difference in plasma TG response between genotypes: patients who had ≥1 copy of the rare mutations had a blunted maximal decrease in plasma TG compared with other subjects (Figure). We observed significant between-group differences in increased plasma HDL cholesterol but no difference in total cholesterol on treatment (Figure).

Discussion

By resequencing 3 candidate genes, we found an association of several genetic variants with severe HTG in Canadian patients of European ancestry. Specifically, we found that 41.8% of subjects with plasma TG >10 mmol/L have ≥1 copy of several rare coding sequence variants in candidate genes (LPL or APOC2) or ≥1 copy of the common APOA5 p.S19W allele, whereas this assortment of genetic variants is present in only 8.9% of controls (OR 7.4, 95% CI 4.5 to 12.0; P < 10−13). This is among the most substantial genetic contribution yet detected for a dyslipoproteinemia phenotype. We also observed that carriers of ≥1 copy of several rare mutations in LPL, APOC2, or APOA5 genes had a smaller decrease in plasma TG in response to oral fibrate treatment than subjects with other genotypes.

Previous studies of patients with extreme lipoprotein phenotypes showed that rare candidate gene mutations are present in a significant minority of cases. For instance among patients with low HDL, ~16% had candidate gene mutations compared with ~2% of controls.13 Our findings support a similar significant contribution of rare candidate gene mutations in a minority of patients with severe HTG: rare mutations were seen in 10.0% of severe HTG patients compared with 0.2% of controls were carriers of mutations (OR 52, 95% CI 8.6 to 319; P < 10−7). In addition, we also found a very strong association of severe HTG with the common dysfunctional APOA5 p.S19W variant: 34.6% of HTG subjects were carriers versus 8.8% of controls (OR 5.5, 95% CI 3.3 to 9.1; P < 10−7). Thus, the genetic component of this complex metabolic trait is comprised of both rare and common variants, which together account for a greater proportion of affected individuals (>40% in this sample) than rare mutations alone.

The present findings quantify the potential contribution of mutant LPL to type 5 hyperlipoproteinemia and solidify a key physiological role for apo A-V, which was only discovered 5 years ago.
years ago using bioinformatic analysis. The APOA5 p.S19W allele has been evaluated in several studies, some of which have shown modest associations with mildly elevated plasma TG. Recently, plasma apo A-V concentrations and p.S19W allele frequency were shown to be elevated in patients with relatively mild TG elevation. Among numerous SNPs at the APOA5 locus, p.S19W is unique because it: (1) alters the amino acid sequence and has proven dysfunction in vitro; (2) is relatively common, with an allele frequency of 7% to 11% in control samples of European ancestry; and (3) is the defining polymorphism of a unique haplotype associated with moderately elevated TG. Together, the data would indicate that APOA5 p.S19W might be a clinically useful risk marker of this extreme phenotype.

We selected LPL, APOC2, or APOA5 as candidate genes for association with type 5 hyperlipoproteinemia because homozygous mutations in each cause severe HTG with chylomicronemia and especially type 1 hyperlipoproteinemia (MIM 238600), a disorder whose genetic basis is well understood. Most LPL mutations that we found in severe HTG patients were already proven to be disease-causing in the homozygous state, with documented functional impairment. The novel mutations were by and large imputed to the homozygous state, with documented functional impairment. Finally, heterozygous relatives of probands homozygous for truncating mutations in APOA5 and severe HTG were variably found to have moderately elevated plasma TG. Thus, evidence from the pre- and post-genomic eras, including findings from this study, indicates that heterozygosity for rare dysfunctional coding sequence mutations is strongly associated with severe HTG.

Besides APOA5 p.S19W, among the other coding variants with MAF >1%, LPL p.D9N, and p.S447X were found to be associated with susceptibility and protection from HTG, respectively. We noted that the presence of the so-called “gain-of-function” LPL p.S447X variant was not “protective” for 7 carriers among the severe HTG patients. Such anecdotal cases may be important considering that p.S447X variant is being proposed as the central component of a gene therapy strategy designed to treat patients with severe HTG.

Thus, our findings are consistent with the emerging model that the cumulative contributions of multiple rare alleles with large genetic effects are found among individuals at the extremes of a complex genetic trait. However, in contrast to findings from studies of patients at extremes of very low HDL and LDL cholesterol in which no single variant had a frequency >5%, our findings indicate that a variety of low frequency mutations together with the common APOA5 p.S19W polymorphism, underlie an increased risk in a large segment of patients with extremely high plasma TG. We do not suggest that the heterozygous mutations identified here are directly causative because hyperlipoproteinemia type 5 is a complex trait with no single simple genetic cause and other factors, both genetic and nongenetic, are likely to be very important. We also showed a difference in plasma TG response to fibrates, with carriers of ≥1 copy of several rare coding sequence variants in either LPL, APOC2 or APOA5 having a significantly less favorable response compared with other subjects. The findings further confirm that the genetic contribution to severe HTG is complex and suggest that other genes may still have an important role to play.

Acknowledgments

Rebecca Provost, Rachel Rollings, and Valerie Orr each provided outstanding technical assistance.

Sources of Funding

Dr Hegele is a Career Investigator of the Heart and Stroke Foundation of Ontario and holds the Edith Schulich Vinet Canada Research Chair (Tier I) in Human Genetics and the Jacob J. Wolfe Distinguished Medical Research Chair. This work was supported by operating grants from the Canadian Institutes of Health Research (MT14030 and MOP-79533), the Heart and Stroke Foundation of Ontario, and Genome Canada through the Ontario Genomics Institute.

Disclosures

None.

References

Wang et al

Resequencing in Severe Hypertriglyceridemia

2455


Resequencing Genomic DNA of Patients With Severe Hypertriglyceridemia (MIM 144650)
Jian Wang, Henian Cao, Matthew R. Ban, Brooke A. Kennedy, Siqi Zhu, Sonia Anand, Salim Yusuf, Rebecca L. Pollex and Robert A. Hegele

Arterioscler Thromb Vasc Biol. 2007;27:2450-2455; originally published online August 23, 2007;
doi: 10.1161/ATVBAHA.107.150680

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/27/11/2450

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/