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Rare *PPARA* variants and extreme response to fenofibrate in the Genetics of Lipid Lowering Drugs and Diet Network Study

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Abstract

Objective—Fenofibrate, a peroxisome proliferator-activated receptor-alpha (PPARα) agonist, reduces triglyceride (TG) concentrations by 25–60%. Given significant inter-individual variation in TG response, we investigated the association of *PPARA* rare variants with treatment response in the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) study.

Methods—We calculated change in TG concentration (ΔTG) among 861 GOLDN participants treated with fenofibrate (160 mg/day) for 3 weeks. From the distribution of ΔTG adjusted for age and sex, the 150 highest and 150 lowest fenofibrate responders were selected from the tails of the distribution for *PPARA* resequencing. The resequencing strategy was based on VariantSEQr^{tm} technology for the amplification of exons and regulatory regions.

Results—We identified 73 variants with an average minor allele frequency (MAF) of 4.8% (range 0.2%–16%). We tested the association of rare variants located in a coding or regulatory region (MAF<1%, 13 variants) with treatment response group via an indicator variable (presence/absence of ≥1 rare variant) using general linear mixed models to allow for adjustment for family relationship. After adjusting for baseline fasting TG concentration carrying at least one rare variant was associated with low fenofibrate response (odds ratio=6.46; 95% CI 1.4–30.8). Carrier status was also associated with relative change in total cholesterol concentration (*P*=0.02), but not high density lipoprotein or low density lipoprotein concentration.

Conclusions—Rare, potentially functional variants in *PPARA* may play a role in TG response to fenofibrate, but future experimental studies will be necessary to replicate the findings and confirm functional effects.

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Keywords

clinical trials; lipids; DNA sequence analysis; PPARalpha

Introduction

Hypertriglyceridemia is considered an independent risk factor for cardiovascular disease (CVD), and is defined by the National Cholesterol Education Program (NCEP) as triglyceride (TG) concentration >150 mg/dL (1,2). It is a common condition that occurs in approximately one-third of US adults resulting from the hypocatabolism of triglyceride rich lipoproteins (TRLs) of dietary and/or hepatic origin (3,4). Failure to clear the lipolytic products of TRLs (remnant TRLs) to the liver efficiently is linked to CVD through endothelial dysfunction and upregulation of proinflammatory pathways (5,6). It has been estimated in the United States that about 1.3% of the population uses some form of triglyceride lowering therapy including fenofibrate, gemfibrozil, or niacin (3).

Peroxisome proliferator-activated receptors (PPARs) are members of a large family of ligand-inducible transcription factors (7). Fenofibrate is a synthetic ligand that binds to and activates the nuclear transcription factor peroxisome proliferator-activated receptor alpha (PPARα) in a process that ultimately modulates the expression of genes regulating lipid metabolism. It is best known for its triglyceride lowering effects though it also raises high density lipoprotein cholesterol concentration (HDL-C), lowers low density lipoprotein cholesterol concentration (LDL-C), and affects inflammatory biomarkers levels (8). There is significant inter-individual variation in response to fenofibrate (25–60%) (9). Findings from a number of studies have suggested that lipid response to drug therapy is under genetic influence (10–14). Given that PPARα is the molecular target for fenofibrate and variability in drug response exists, we investigated whether rare, potentially functional variations in the gene, *PPARA*, are associated with lipid lowering response to three-weeks of fenofibrate treatment in the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) study.

Methods

Study Population

The GOLDN study was designed to identify genes that determine response of lipids to two interventions, one to raise (ingestion of high-fat meal) and one to lower lipids levels (fenofibrate treatment). The GOLDN study has been previously described in Corella et al (15). Briefly, the study ascertained and recruited families from the NHLBI Family Heart Study at two centers, Minneapolis, MN and Salt Lake City, UT who were self-reported to be white. In each case, only families with at least two siblings were recruited and only participants who did not take lipid-lowering agents (pharmaceuticals or nutraceuticals) for at least 4 weeks prior to the initial visit were included. To meet the sample size goals and increase relative pairs, participants were offered the option of doing either part of the protocol, high-fat meal and/or fenofibrate treatment. A total of 861 GOLDN participants received open label, once daily 160 mg micronized fenofibrate for three weeks and were followed for treatment response. While a longer treatment duration may be optimal, a small randomized clinical trial of fenofibrate response observed the largest effect on lipid concentrations occurred between weeks two and three (16). The three week duration of treatment in GOLDN was chosen based on the hypothesis any pharmacogenetic response to the drug would occur early.

In the current study we calculated change in fasting TG concentration (ΔTG) over three weeks for the fenofibrate treatment arm of the study. We regressed age and sex on ΔTG and

estimated the predicted ΔTG using the regression procedure in SAS v. 9.2. From the predictedΔTG values, the 150 highest and 150 lowest fenofibrate responders were selected from the tails of the distribution for *PPARA* resequencing.

Resequencing

The human PPARα gene spans 83.7 kb on chromosome 22. The general structure of the gene is comprised of 8 exons with a 5′ untranslated region (UTR) encoded by exons 1–2 and part of exon 3; a coding region comprised by the remainder of exon 3, exons 4–7, and part of exon 8; and a 3′ UTR encoded by the remainder of exon 8 (7). Multiple transcripts have been described where alternate exons and promoters have been identified in the 5′ UTR region (17). In the current study, the resequencing strategy was based on the VariantSEQr platform for amplification of exons and regulatory regions using similar PCR conditions and sequencing with a single universal primer (Applied Biosystems, Foster City CA). Specifically, a total of 11.4 kb (exons, promoter, and intronic regions flanking the exons) were amplified from each of 300 genomic DNA samples, and sequenced on both strands with a capillary-based ABI 3730xl DNA Analyzer (BigDye Terminator cycle sequencing kits v3.1) as previously described (18). Sequence traces were assembled and aligned for variant calling using Lasergene SeqMan v8.1 (DNASTAR, Madison WI). The variants were oriented according to Human Genome Sequence Build 36 and dbSNP build 130 (NCBI). Each *PPARA* variant identified through the resequencing strategy was tested for Hardy Weinberg Equilibrium (HWE), and variants with more than 10% missing genotypes or HWE $P < 0.05$ were excluded from the analysis.

Statistical Methods

Differences by extreme responder group for continuous and categorical variables (including genotype) were determined by the t-test and chi-square test, respectively. The usual association tests for common variants are underpowered for detecting variants of lower frequency (19). Many methods for analyzing rare variants are based on differences in rare allele counts between groups but they assume subjects are unrelated (19–21). Our data consists of 300 people belonging to 136 families. We created an indicator variable for the presence or absence of at least one rare variant located in a regulatory or coding region (MAF<1%; N=13) meeting the QC criteria described above for each individual. We tested the association of the indicator variable with TG response status (high or low responder) adjusted for the fixed effect of baseline TG concentration and the random effect of family using the Glimmix procedure in SAS. Additionally, we examined percent change in HDL-C, LDL-C and total cholesterol (TC) concentration after fenofibrate treatment (((post-fenofibrate minus pre-fenofibrate concentration)/pre-fenofibrate concentration) *100) adjusting for fixed effects of baseline lipid concentration, age, sex, and a random effect of family using the Mixed procedure in SAS. The least square means statement within the mixed model procedure was used to estimate the adjusted mean change in lipid concentration by rare variant category. This study tested the association of one independent variable with three correlated phenotypes (TG response status and percent change in LDL and HDL). Therefore, we consider the true *P*-value for significance to lie between 0.05 and 0.05/3=0.01666~0.02 (the Bonferrori correction assuming all three phenotypes are uncorrelated). Given this argument $P \leq 0.02$ was considered significant in the current study.

Haploview was used to determine linkage disequilibrium (LD) between variants identified in *PPARA* in this study (22). The novelty of variants identified through the resequencing strategy was determined using dbSNP (23). Finally, the UCSC genome browser was used to query The Encylopedia of DNA elements (ENCODE) plus other bioinformatic resources for examining a potential role in regulation and/or methylation/acetylation of loci highlighted (24,25).

Results

A total of 73 variants in *PPARA* including 71 SNPs and two indels were identified through the targeted resequencing strategy (67% non-intronic). The average MAF was 0.048 (range 0.2%–16%) and 39 of the 73 variants were annotated in dbSNP (Supplemental Table 1). None of the common variants (MAF > 1%, N=47), including the frequently studied L162V variant (rs1800206), (7,26–28) were associated with responder status at the univariate level (*P*>0.05, Supplemental Table 1) and were not considered further in this report. Since our focus was on examining rare variants that were most likely to affect function, we selected SNPs located in exons, 5′ or 3′ UTRs, or within or adjacent to known functional regulatory elements (e.g., transcription factor binding sites (TFBS), intron/exon junctions), and meeting the QC criteria described for missing data and HWE. Of the remaining 26 rare variants, 13 SNPs met these qualifications (Supplemental Table 1). Only one of these 13 rare variants (variant 1, Table 1) was located in an intron and it was included in our analysis because it is near two putative conserved TFBS and it is in a region of chromatin modification (Supplemental Figure 1). Three of the final 13 variants that we analyzed were annotated in dbSNP (rs4253663, rs1042311, rs41332048) and the other 10 were considered novel. Rare variants were not expected to be in high LD with surrounding variants and the 13 variants were not in LD with each other or any other variant identified (29,30).

The high and low fenofibrate responders considered as part of this study were not statistically significantly different by age or sex as expected (Table 2), but were different by baseline lipid concentrations as well as response to fenofibrate lipid concentrations considered as part of this analysis. On average the high responders had higher lipid concentrations (or lower HDL-C) than the low responders at baseline. The groups were selected based upon differences in TG response to fenofibrate and results show fenofibrate's lipid modifying effects also included HDL-C, LDL-C and TC before adjustment. The distribution of carriers of at least 1 (of 26) rare variants was not different between the groups (*P*=0.23). On the other hand, there were significantly more carriers of at least 1 (of 13) coding or regulatory rare variants among the non-responder group (*P*=0.003).

After adjusting for baseline TG concentration and family relationship, carrying at least one of the 13 rare variants was associated with a decreased response to fenofibrate. The odds ratio for low response associated with carrying at least one of the 13 rare variants was 6.46 (95% CI 1.4–30.8; *P*=0.02). After adjustment, carrier status was also associated with percent change in TC after treatment, driven by the TG component, as carrier status was not associated with percent change in HDL-C or LDL-C (Table 3). Fenofibrate is also known to modulate inflammatory biomarker levels, however carrying at least one of the 13 rare variants described was not associated with interleukin-6, interleukin-2, c-reactive protein, monocyte chemotactic protein-1, or tumor necrosis factor alpha concentration response to treatment (data not shown).

Discussion

The PPARα agonist fenofibrate is an effective treatment for the reduction of triglyceride concentration, however, variation in response to the treatment is considerable and may be governed by genetic factors (9–12). Additionally, recent hypotheses suggest rare sequence variations in genes within pedigrees may contribute substantially to the heritability of common traits and that considering a "composite test of mutational load" (31) can be an effective manner of identifying functionally relevant loci (32). Studies have reported rare variations discovered through resequencing experiments in candidate loci to be associated with lipid levels (33–37). Additionally, rare variation in 3-hydroxyl-3-methylglutaryl-3 coenzyme A reductase (HMGCR), the direct enzymatic target of statins, has been associated

with a reduced LDL-C response to treatment (14). Here we show that rare variation in the PPARα gene, the molecular target of fenofibrate, is associated with reduced triglyceride treatment response in families from the GOLDN Study.

We have investigated potential functional effects of these loci on the action and/or expression of the PPARα protein using freely available bioinformatic resources accessible through the UCSC genome browser (24). Among the rare SNPs we analyzed, intronic variant 1 (Table 1) was included because it is near apparent regulatory functional elements. Specifically, it lies 35 base pairs upstream of exon two near two putative conserved TFBS and it is located in a region where chromatin modification has been indicated, denoted by increased mono and tri methylation of lysine 4 in histone 3 (H3K4me1 and H3K4me3 marks, respectively-see Supplemental Figure 1). H3K4me1 combined with H3K4me3 modification has been shown to predict promoter regions and help discriminate promoters from enhancers (38). The variant rs4253793 (variant 2 in Table 1) is located in the 5′ UTR, in the first half of exon 3. Of the two coding SNPs included in the analysis, variant 3 from Table 1 is a novel synonymous mutation (base pair location 44972983) located in exon 3 and variant 4 is an annotated (rs1042311) missense mutation (Alanine to Valine) located in exon 7. While functional effects of missense mutations are more clearly defined, evidence exists silent mutations can also have functional effects in the corresponding protein (39). The remaining nine of the 13 rare variants considered are located in the 3′ UTR of *PPARA*. The 3′ UTR region often contains sequence targets for binding and regulation of gene expression by microRNAs (miRNAs). In fact, Supplemental Figure 1 displays several putative miRNA binding sites in the 3′ UTR of the gene which these rare variants could disrupt. Future studies of regulatory elements surrounding confirmed risk variants are necessary to fully characterize functional effects of the variants described in this report.

There are several limitations to the current study that should be mentioned. Response to fenofibrate is complex and change in lipid concentration after fibrates has been reported to be dependent on baseline concentration (40–42). In our study the high responder group had significantly higher baseline lipid concentrations (or lower for HDL, see Table 2) in comparison to the low responder group which could confound our results.. For instance, we found baseline triglyceride concentration to be associated with the rare variant indicator variable (P=0.03) meaning baseline values, rather than the response to the drug, could be driving the observed genetic association. Alternatively, this genetic background may be associated with both baseline triglyceride levels and treatment response. Even though all our analyses were adjusted for baseline, future studies in the rest of GOLDN matched on baseline triglyceride concentration are necessary to rule out the potential for residual confounding. Finally, we note, that when collapsing rare variants across multiple markers the direction of effect of the individual variants on the outcome can enhance (if same direction) or quell the signal (if the variants confer both high and low risk) of the collapsed variant (19). Methods to analyze rare variants have been developed that account for directional effects, but they are not designed to accommodate correlated familial data such as in GOLDN (21). We found some evidence that grouping the variants by direction of effect resulted in stronger findings (data not shown) and we plan to use this preliminary information for future studies.

In conclusion, we resequenced the PPARα gene and identified 13 potentially functional rare variants that became the focus of this study. Having at least one of these 13 variants was associated with low TG response to 3 weeks of fenofibrate treatment in a group of extreme treatment responders from GOLDN, but not any other individual lipid or inflammatory biomarker response. Using bioinformatic resources, functional DNA elements were identified near the variants of interest offering some insight into how these variants could compositely affect PPARα function/expression leading to an altered treatment response.

This result needs to be replicated in the rest of GOLDN and external study populations, but supports the hypothesis that rare variation can contribute to the heritability of common traits including pharmacogenetic responses. Additionally, studies such as this one are more important than ever as the FDA recently ordered the label for fenofibric acid to be changed to indicate the drug has not been shown to reduce the risk of heart attack or stroke, but noted the clinical significance in at least one subgroup is unclear. Understanding rare variation in *PPARA* may help expedite the identification of patient subgroups who should receive fenofibrate on the basis of a likely optimal TG lowering response to fenofibrate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Thirteen *PPARA* rare variants (MAF<1%) identified through targeted resequencing and examined in the current study.

Variant	Base Pair	Allele	Loc[#]	Annotation	MAF[*]	Function	HWE *P*[†]
1	44926420	C>T	I1	-	0.8%	Intron 1	0.88
2	44972935	T>C	E3	rs4253793	0.7%	5′ UTR	0.90
3	44972983	A>G	E3	-	0.2%	Synonymous	0.97
4	45006444	C>T	E7	rs1042311	0.5%	Missense	0.93
5	45010179	G>A	E8	-	0.5%	3′ UTR	0.92
6	45011466	C>T	E8	rs41332048	0.7%	3′ UTR	0.90
7	45011665	C>A	E8	-	0.2%	3′ UTR	0.97
8	45011765	C>T	E8	-	0.2%	3′ UTR	0.97
9	45013342	C>T	E8	-	0.5%	3′ UTR	0.93
10	45013867	C>T	E8	-	0.2%	3′ UTR	0.98
11	45013894	C>T	E8	-	0.2%	3′ UTR	0.98
12	45017131	T>A	E8	-	0.4%	3′ UTR	0.95
13	45017149	A>G	E8	-	0.2%	3′ UTR	0.97

[#] Locationin gene (I=intron, E=exon)

[*] Minor Allele Frequency

[†] Hardy-Weinberg Equilibrium

Table 2

Characteristics of the study population by fenofibrate triglyceride (TG) response status

Variable	High Responder N=150	Low Responder N=150	*P**
Age	49.8	47.7	0.06
Sex (F)	66	65	0.90
Baseline TG mg/dL	270.6	96.2	<0.0001
Baseline HDL-C mg/dL	40.7	48.4	<0.0001
Baseline LDL-C mg/dL	131.0	121.6	0.01
Baseline TC mg/dL	215.6	185.1	<0.0001
ΔTG-C mg/dL	−148.8	9.2	<0.0001
ΔHDL-C mg/dL	4.4	0.28	<0.0001
ΔLDL-C mg/dL	−9.0	−20.3	0.0001
ΔTC mg/dL	−29.7	−21.9	0.0046
≥1 of 26 Rare Variants	23	31	0.23
≥1 of 13 Tested Rare Variants†	7	22	0.0034

* unadjusted

† rare variants located in exons, 5′ or 3′ UTRs, or within or adjacent to known functional regulatory elements (e.g., transcription factor binding sites (TFBS), intron/exon junctions)

Table 3

Mean non-triglyceride lipid response (95% CI) to fenofibrate by *PPARA* rare variant carrier status (at least 1 of 13 rare variants) adjusted for age, sex, baseline lipid concentration and family

Outcome*	Carrier N=29	Non-carrier N=271	*P*
ΔHDL-C %	6.7 (2, 11)	6.6 (5, 8)	0.95
ΔLDL-C %	−5.3 (−12, 2)	−10.9 (−13, −8)	0.14
ΔTC %	−8.2 (−12, −5)	−12.7 (−14, −12)	0.02

* outcome is percent change defined as (((post-fenofibrate minus pre-fenofibrate concentration)/pre-fenofibrate concentration) *100)