Published in final edited form as:
Arterioscler Thromb Vasc Biol. 2009 January ; 29(1): 147–152. doi:10.1161/ATVBAHA.108.178533.

Galanin preproprotein is associated with elevated plasma triglycerides

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Abstract

Objective—There is increasing physiological evidence in rodents connecting the neuropeptide galanin to triglyceride (TG) levels. We hypothesized that variation in the galanin preproprotein (*GAL*) gene may contribute to hypertriglyceridemia (HTG) in humans.

Methods and Results—We investigated *GAL* as a TG candidate gene by genotyping four tagSNPs in Dutch, Finnish and Mexican familial combined hyperlipidemia (FCHL) families as well as in Caucasian combined hyperlipidemia cases/controls (n=2,471). The common allele of rs2187331, residing in the promoter region of *GAL*, was significantly associated with HTG (p-value=0.00038). In an unascertained population sample of 4,463 Finnish males, the rare allele of rs2187331 was associated with higher TGs (p-value=0.0028−0.00016). We also observed an allele specific difference with rs2187331 in reporter gene expression and nuclear factor binding *in vitro*. Furthermore, we detected differential expression of many key lipid genes in adipose tissue based on rs2187331 genotypes.

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Disclosure
None of the authors have a financial interest related to this work. Dr. Tjerk W.A. de Bruin has been employed by GlaxoSmithKline. This work was not supported in full or part by GlaxoSmithKline.

Conclusions—The SNP rs2187331 is associated with HTG in FCHL and Caucasian combined hyperlipidemia cases/controls and influences TG levels in the population. Further studies are warranted to elucidate the allelic difference observed between FCHL and the general population. Functional evidence shows that rs2187331 has an allele specific cis-regulatory function and influences the expression of lipid related genes in adipose.

Elevated plasma lipid levels are key risk factors for cardiovascular disease. Recent genome-wide association studies (GWAS) have been able to account for 5−8% of the variation in the levels of low density lipoprotein (LDL), high density lipoprotein (HDL), and triglycerides (TGs)[1]. This suggests that there are other genetic variants affecting plasma lipid levels still to be discovered.

Familial combined hyperlipidemia (FCHL; MIM:144250) is the most common dyslipidemia observed in patients with coronary artery disease (CAD) and their relatives. FCHL is characterized by familial segregation of elevated plasma triglycerides (TGs), total cholesterol (TC), or both[2]. Another common characteristic of FCHL is postprandial lipemia, the accumulation of triglyceride-rich lipoprotein particles (chylomicron, very low-density lipoprotein (VLDL) and their remnants) in plasma approximately 6−10 hours after a fatty meal.

A recent study demonstrated a correlation between plasma TGs and transcript levels of the neuropeptide galanin preproprotein (*GAL*) in the paraventricular nucleus (PVN) of the hypothalamus in rats fed a high-fat diet[3]. Furthermore, injection of *GAL* peptide directly onto the PVN of rats caused a significant upregulation of lipoprotein lipase (*LPL*) expression in adipose tissue and a significant decrease in *LPL* expression of skeletal muscle[3, 4]. As many as one third of FCHL patients are estimated to exhibit decreased *LPL* activity[5], suggesting that perturbations in *LPL* may contribute to FCHL[6].

We hypothesized that *GAL* is a compelling functional candidate gene for predisposition to elevated TGs in individuals with FCHL. Coincidentally, *GAL* is located near the peak marker linked to TGs and FCHL in Dutch and British FCHL families on chromosome 11p[7, 8]. We selected tagSNPs capturing the common genetic variation of *GAL* and tested these SNPs for association with hypertriglyceridemia (HTG) in study samples ascertained for hyperlipidemia and with TG levels at the population level. We also tested for allelic effects on reporter gene expression, nuclear factor binding and downstream differential expression of genes including *LPL*. Our data suggest that variation in the *GAL* promoter region influences TG levels in humans and that the associated variant is likely to be functional.

Materials & Methods

Each subject involved in this study provided a written informed consent. The study design was approved by the ethics committees of the participating institutions.

Dutch, Finnish and Mexican FCHL families

A total of 565 individuals from 33 Dutch FCHL families were collected at the Utrecht Academic University Hospital[7]. A total of 724 individuals from 60 Finnish families were collected in the Helsinki and Turku University Hospitals[9]. A total of 806 individuals from 55 mexican FCHL families were collected at Instituto National de Ciencias Médicas Y Nutritión, Salvador Zubirán. HTG status was determined using the 90th age-sex specific population percentiles for TGs. Detailed information about the family collection and determination of the affection status can be found in the Supplementary Materials and Methods.

Caucasian combined hyperlipidemia case/controls

The Caucasian combined hyperlipidemia cases and controls were collected both at the Academic Hospital Maastricht and at the University of California San Francisco, as described previously[10, 11]. Combined hyperlipidemic status was specified as both TC and TG levels >90th age-sex specific population percentiles. The controls had TC and TG levels <75th age-sex specific population percentiles. Detailed information about the case/control definition can be found in the Supplementary Materials and Methods.

METSIM subjects

The METSIM study is an ongoing population sample of Finnish males. A total of 4,463 males were included in this study (Supplementary Table 1). Further information about sample collection and quantitative trait measurements can be found in the Supplementary Materials and Methods.

SNP selection, genotyping and sequencing

SNPs were selected to capture the common genetic variation in the *GAL* region (*GAL* coding region and 10Kbp up and downstream of the gene) using the CEPH HapMap Phase I genotype data and the haplotype tagging software LDSelect[12] with the binning threshold set at $r^2 \geq 0.85$ and the minor allele frequency (MAF) filter set to ≥0.1. We identified three tagSNPs in the region (rs3136541, rs3181041 and rs6591350), and one singleton SNP (rs2187331) that was not in significant linkage disequilibrium (LD) ($r^2 < 0.85$) with any other SNPs in the region. Genotyping and sequencing procedures are described in detail in the Supplementary Materials and Methods.

Statistical analyses

We utilized the Family Based Association Testing (FBAT) software package to test for association in families with HTG[13]. Logistic regression was used to test for association in the cases/controls using an additive model of inheritance in the statistical software package R. We performed a combined analysis of the family-based and case-control studies (n=2471) using the Z-method to combine the statistics for the 4 SNPs. Regression based analysis of the METSIM study utilized ANCOVA in R adjusting for age, BMI and smoking status (smoking or not). Power was calculated using QUANTO[14]. Additional details of the statistical analyses can be found in the Supplementary Materials and Methods.

Transient transfections and luciferase assay

A region of 564bp containing the SNP rs2187331 was amplified and cloned from genomic DNA template into the pGL3-Promoter vector (Promega). All experiments were carried out in HEK293T and HepG2 cells. The dual-luciferase reporter assay (Promega) was used according to manufacturer's protocol. Fourty-eight hours after transfection, cells in each well were lysed using 50μl passive lysis buffer and 15μl of the lysate was used for the assay. Detailed description of construct, transfection and experimental design can be found in the Supplementary Materials and Methods.

Electrophoretic mobility shift assay (EMSA)

The nuclear proteins were extracted from the HepG2 and PANC-1 cell lines by NE-PER nuclear extraction reagents (Pierce) and protein concentrations were quantified by the Bradford assay. Complementary 5′ biotinylated and unlabeled oligonucleotide probes representing both alleles of rs2187331 were obtained commercially (Operon Biotechnologies). Specifics of EMSA can be found in the Supplementary Materials and Methods.

Microarrays and qRT-PCR

We collected 49 fat biopsies from umbilical subcutaneous adipose tissue under local anesthesia from unrelated Mexican FCHL cases/controls. Clinical characteristics are given in Supplementary Table 2. Microarray hybridization and qRT-PCR experiments are described in the Supplementary Material and Methods. The CEL files were imported into R 2.6.0 using the justRMA function of the Affymetrix library in Bioconductor, which does background subtraction and quantile normalization. The quality of each probe for every individual was assessed using the MAS 5.0 software from Affymetrix. The normalized relative expression of each gene was used as the dependent variable in linear regression with rs2187331 genotypes (TT=0, TC=1, CC=2) and the FCHL status as the independent variables using the anova and lm functions from the stats package in R. Pathway enrichment was conducted using the DAVID software package[15]. Microarray data can be accessed in MIAME compliant format from GEO (GSE13506).

Results

Association of *GAL* SNPs with elevated TGs

We genotyped four tagSNPs (rs2187331, rs3136541, rs3181041, rs6591350) covering *GAL* and tested them for association with HTG in both the FCHL families and combined hyperlipidemia cases/controls (n=2471) (Table 1). We observed an association with HTG in the combined hyperlipidemia cases/controls for the common allele T of rs2187331 (p-value=0.0015). The FCHL families showed a trend in the same direction (p-value=0.063). Combining the association results from the combined hyperlipidemia cases/controls and the FCHL families increased the significance (p-value=0.00038, n=2,471). The 12 tests conducted would require a Bonferroni corrected p-value of ≤0.0042 to maintain an experiment-wide type I error rate of 0.05. The association detected in the combined analysis of rs2187331 (p-value=0.00038) is thus considered experiment-wide significant. We did not observe a sex-specific effect for rs2187331 in the combined analysis when analyzing males and females separately (male p-value=0.011, female p-value=0.014), and the same allele was associated in both sexes (male Z=−2.53, female Z=−2.45). We did not observe any association with TC in the families (p-value>0.1).

Functional evidence for rs2187331

The SNP rs2187331 is located 5.6Kbp upstream of the *GAL* transcriptional start and is not in significant LD (r^2>0.85) with any other SNPs in the region. Therefore, we hypothesized that rs2187331 may be the causal SNP and likely functions in a regulatory role. To test this hypothesis, we cloned a 564bp-region surrounding rs2187331 into a luciferase reporter gene vector and assayed for allele specific expression in the human HEK293T (embryonic kidney) cell line. This cell line was selected because GAL expression was detected in both kidney tissue and the HEK293T cell line (Supplementary Figure 1), and also because it is easy to transfect. We found that the region containing the SNP rs2187331 functions as a regulatory element, because the T allele had higher expression than the C allele (p-value=0.017, T:C fold-change=1.53) (Figure 1). This effect was also observed when the insert was cloned in the opposite orientation (data not shown). We also observed the same effect in the hepatic HepG2 cell line (data not shown). Next we assayed for allele specific nuclear-factor binding with 31-mer double-stranded oligos, representing the two rs2187331 alleles using EMSA with nuclear extract from PANC-1 (pancreatic) and HepG2 (hepatic) cell lines (Figure 2). GAL was found to be expressed in both pancreas and the PANC-1 cell line (Supplementary Figure 1). We observed a similar allele specific binding difference in both cell lines (Figure 2). These data demonstrate that the alleles of rs2187331 alter the rate of transcription and affect differential binding of nuclear-factors *in vitro*.

Genes differentially expressed based on rs2187331 genotypes

The expression of GAL was too low to be accurately quantified by qRT-PCR (C_T values > 30) in the Mexican FCHL fat biopsies. However, it is known that *LPL* transcript levels in adipose tissue are sensitive to *GAL* peptide levels in the paraventricular nucleus (PVN) of the hypothalamus in rats[3, 4]. We hypothesized that *LPL* and other downstream genes may be differentially expressed based on rs2187331 genotypes. We analyzed 49 Mexican FCHL fat biopsies for differential expression by regressing rs2187331 genotypes on the normalized gene expression and adjusting for case/control status. We identified 1,884 differentially expressed probe sets with a p-value <0.05 (corresponding to 1,667 genes). Importantly, we found LPL to be differentially expressed with two independent probes sets (p-values=0.0052−0.0063). Using qRT-PCR we validated the differential expression of *LPL* and hormone sensitive lipase (*LIPE*) (Supplementary Figure 2). Interestingly, higher expression in the TT genotype carriers was observed for 77% of the 1,884 probes identified as differentially expressed based on rs2187331 genotypes (p-value<0.05) (Supplementary Table 6), suggesting that the direction of differential expression is important. The set of differentially expressed genes was significantly enriched for GO terms related to lipid metabolism (Benjamini corrected p-value<0.05) (Table 2). A subset of interesting lipid genes that were differentially expressed are shown in Supplementary Table 3 (p-value<0.05). Importantly, the same pathways that were identified in the DAVID analyses were also identified when the analysis included FCHL status, sex, age, and BMI as covariates (data not shown). These data suggest that rs2187331 genotypes influence expression of multiple genes of lipid metabolism in adipose, a tissue known to be responsive to *GAL* levels in the hypothalamus.

Resequencing of *GAL* coding regions

We resequenced the coding regions of *GAL* in 13 unrelated Dutch FCHL family members who had elevated plasma TGs (>90th age-sex-percentiles), and who were homozygous for the T allele of rs2187331. In addition we resequenced ~250bp upstream and downstream of the rs2187331. We did not detect any novel sequence variants in significant LD, defined as r^2>0.85 that could explain the association with TG in the coding region of *GAL* (NM_015973.3). The variants identified through resequencing are presented in Supplementary Table 4, and the primers used for resequencing are listed in Supplementary Table 5.

Investigation of *GAL* SNPs at the population level

We tested for association between quantitative TGs and the two SNPs with a p-value <0.10 in the study samples ascertained for hyperlipidemia (rs2187331 and rs3181041) in an unascertained population-based sample of 4,463 males from Kuopio, Finland (Table 3). We observed an association between the rare C allele of rs2187331 and high levels of plasma TGs in the total study sample (p-value=0.0028−0.00016) (Table 3). We did not observe an association with LDLC or TC in the METSIM (p-values>0.1). In the non-statin users (n=3963), the p-value was borderline significant (p=0.026−0.019), and no association was observed in T2DM subjects (n=658) (Table 3). These findings are in line with the recent meta analysis of quantitative lipid phenotypes of type 2 diabetes (T2D) genome-wide association studies (GWAS) that did not identify rs2187331 as significantly associated with TGs (p-value=0.057) [1]. Nor were there any genome-wide significant signals within 500Kbp of GAL (minimum p-value = 0.0095 for TGs). This meta-analysis included diabetic cases and excluded individuals on lipid-lowering therapy, primarily statins.

For the analysis of diabetics, we calculated that we have 89% power to detect an association while maintaining a type I error rate of 0.05 with the given sample size (n=658) and an additive model. In this power calculation, we used the same effect size as observed in the total METSIM study for TGs. It is worth noting that there are only 16 subjects with combined hyperlipidemia when using the TC and TG 90th age-sex specific Finnish population percentiles in the non-

statin user group, preventing an actual replication analysis with rs2187331. To conclude, we observed an effect of rs2187331 on TGs in an unascertained study sample of Finnish males. This effect is confounded by lipid-lowering therapy and diabetes.

Discussion

We observed that the T allele of rs2187331 residing in a putative regulatory region upstream of *GAL* is experiment-wide significantly associated with HTG in the combined analysis of FCHL families and combined hyperlipidemia cases/controls. In a non-ascertained population sample, the C allele of rs2187331 was associated with higher levels of TGs. We also provide evidence for the functional relevance of rs2187331 by demonstrating allelic differences in reporter gene expression, nuclear factor binding and expression of lipid metabolism related genes, including *LPL*, a known downstream target of *GAL*. These data demonstrate an association to a clinically relevant trait, a plausible mechanism by which this SNP may functionally alter *GAL* expression, and allelic differential expression of genes that are likely to play a role in the increased susceptibility for HTG.

We observed that *LPL* and many other lipid metabolism related genes are differentially expressed based on rs2187331 genotypes. Furthermore, 77% of these genes show higher expression with the TT genotype. This is consistent with data in rats where *GAL* was found to regulate *LPL* expression both in adipose and muscle[3, 4]. Interestingly, not only genes from the anabolic fat storage pathway have higher expression with the TT genotype (*CD36*, *FATP1*, *LPL*, *VLDLR*), but also genes from the catabolic pathway (*ATGL*, *LIPE*, *MGLL*). The molecular mechanism(s) underlying the coregulation of these genes will need to be investigated in future studies.

We found the C allele of rs2187331 associated with higher levels of TGs in the population-based METSIM study, whereas in samples ascertained for hyperlipidemia the T allele was associated with HTG. Approximately 10% of the METSIM individuals were taking statins and only 16 of the non-statin users had combined hyperlipidemia. Also 15% of the METSIM study are diabetic. The sample sizes of the HTG (n=2,471) and population (n=4,463) studies make it less likely that either finding is by chance alone. Furthermore, the functional evidence supports a regulative, allele-specific role of rs2187331. Moreover, ultimately an individual's risk to develop a complex cardiovascular trait is a combination of susceptibility variants, environmental factors, behavior and chance. The combination of variants and environmental factors are likely to differ between subjects with HTG and subjects representing the unascertained general population, thus contributing to these types of allelic differences. The difference in direction of the association between the METSIM and hyperlipidemic study samples could be due to these confounding factors (i.e. statins and diabetes status), and will have to be further investigated in independent study samples with refined quantitative phenotypes and highly detailed life style variables. Extensive resequencing of the entire genomic region of GAL in different study populations is also warranted to further explore the possibility that differences in LD structure and/or several functional variants and their interactions with rs2187331 may contribute to the disparity in the association signal.

Originally it was observed that injection of nanomolar levels of GAL peptide onto the paraventricular nucleus (PVN) of the hypothalamus stimulates feeding[16]. Subsequent genetic evidence from GAL knockout mice suggests that GAL plays a role in fat intake and preference[17-19]. Interestingly, a positive correlation was also observed between plasma glucose and circulating levels of galanin peptide in healthy and type 2 diabetic individuals[20, 21]. However, the inhibition of insulin secretion identified in other mammals was not observed in humans given a bolus of either porcine or human galanin[22, 23]. Variation near the GAL

gene has previously been associated with alcoholism and anxiety[24], whereas obesity studies have not yielded significant findings for GAL[25, 26].

In normolipidemic individuals plasma TG levels are increased following the consumption of a high fat meal. In response to the elevated plasma TG levels, *GAL* expression is up-regulated in the PVN[3], which in turn signals the up-regulation of genes involved in the lipolysis of TGs from triglyceride rich lipoproteins (TRLs) and import of free fatty acids for storage in adipose tissue. A secondary effect of lipolysis of TGs from TRLs is a reduction in the level of plasma TGs due to a relocation of TGs from plasma into another tissue. We hypothesize that in response to postprandial elevated plasma TGs individuals with HTG carrying the C allele of rs2187331 will have decreased expression or reduced upregulation of *GAL* in the PVN, which modifies the upregulation of lipolysis and storage related genes in adipose tissue.

In conclusion, our data suggest that rs2187331 residing in a putative regulatory region upstream of *GAL* contributes to hypertriglyceridemia in FCHL, TG levels in the general population, and has an allele-specific cis-regulatory function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank the individuals that participated in this study. We thank E. Nikkola and M. Lupsakko for laboratory technical assistance.

Sources of Funding

This study was funded by National Institutes of Health (HL-28481, HL082762); American Heart Association (072523Y to A.H.-V., 0465005Y to B.E.A.); National Human Genome Research Institute (T32 HG02536 to C.L.P., and D.W.-V.); Academy of Finland (to M.K.); Clinical Research Institute HUCH Ltd (to M-R.T.); and Finnish Heart Foundation (to M-R.T.).

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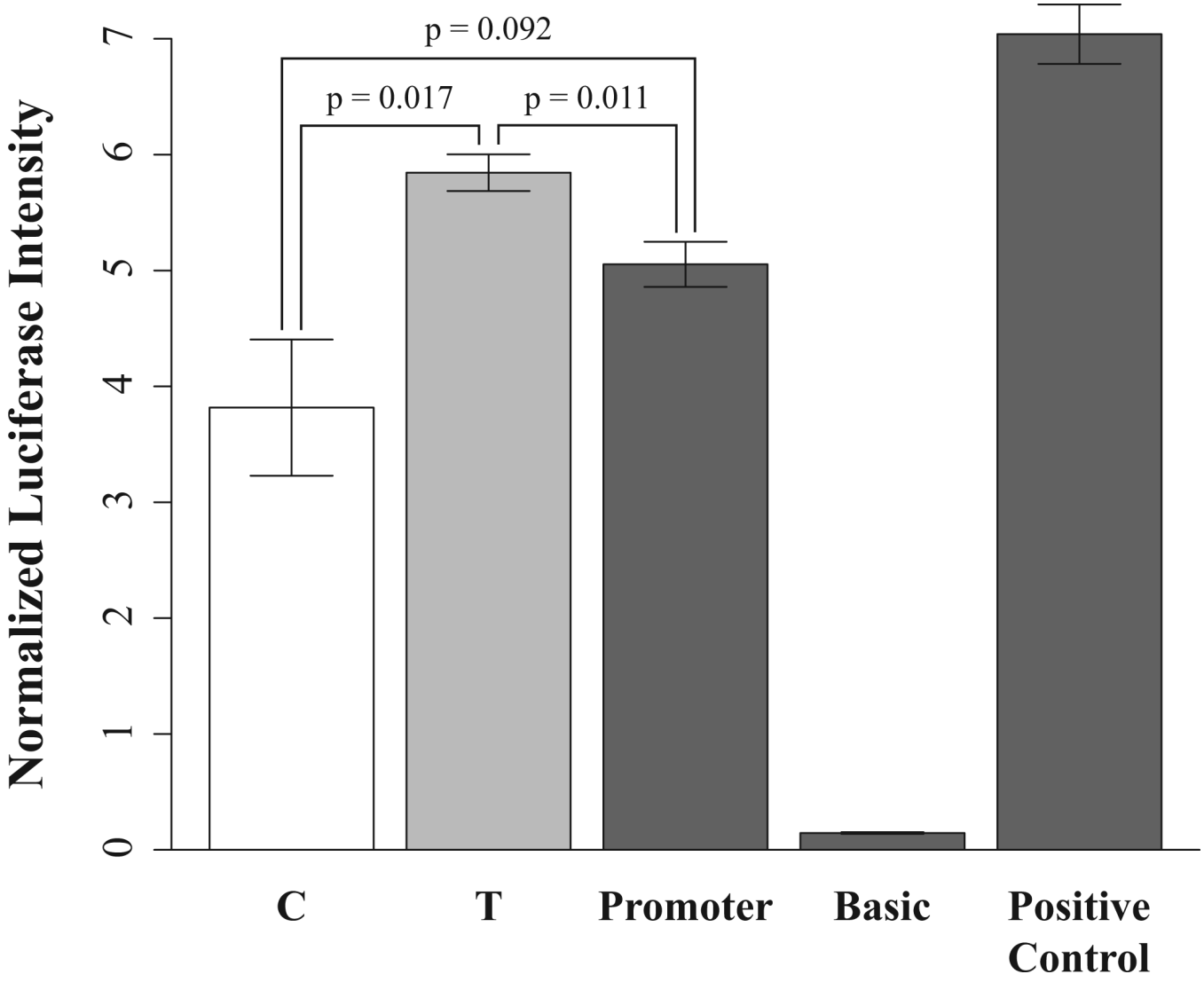

Figure 1.
Comparison of luciferase reporter gene expression between the two alleles of rs2187331 (C and T) in the pGL3-Promoter vector in the HEK293T cell line. Luciferase intensity of the C allele is significantly lower than the T allele (p-value=0.017). The T allele is also significantly higher than the empty pGL3-Promoter vector control (Promoter) (p-value=0.011), and the C allele shows a marginally lower expression than the Promoter vector (p-value=0.092). The pGL3 vector itself was included as a negative control (Basic), and the pGL3 vector with SV40 promoter and enhancer sequences was also included as a positive control (Positive Control).

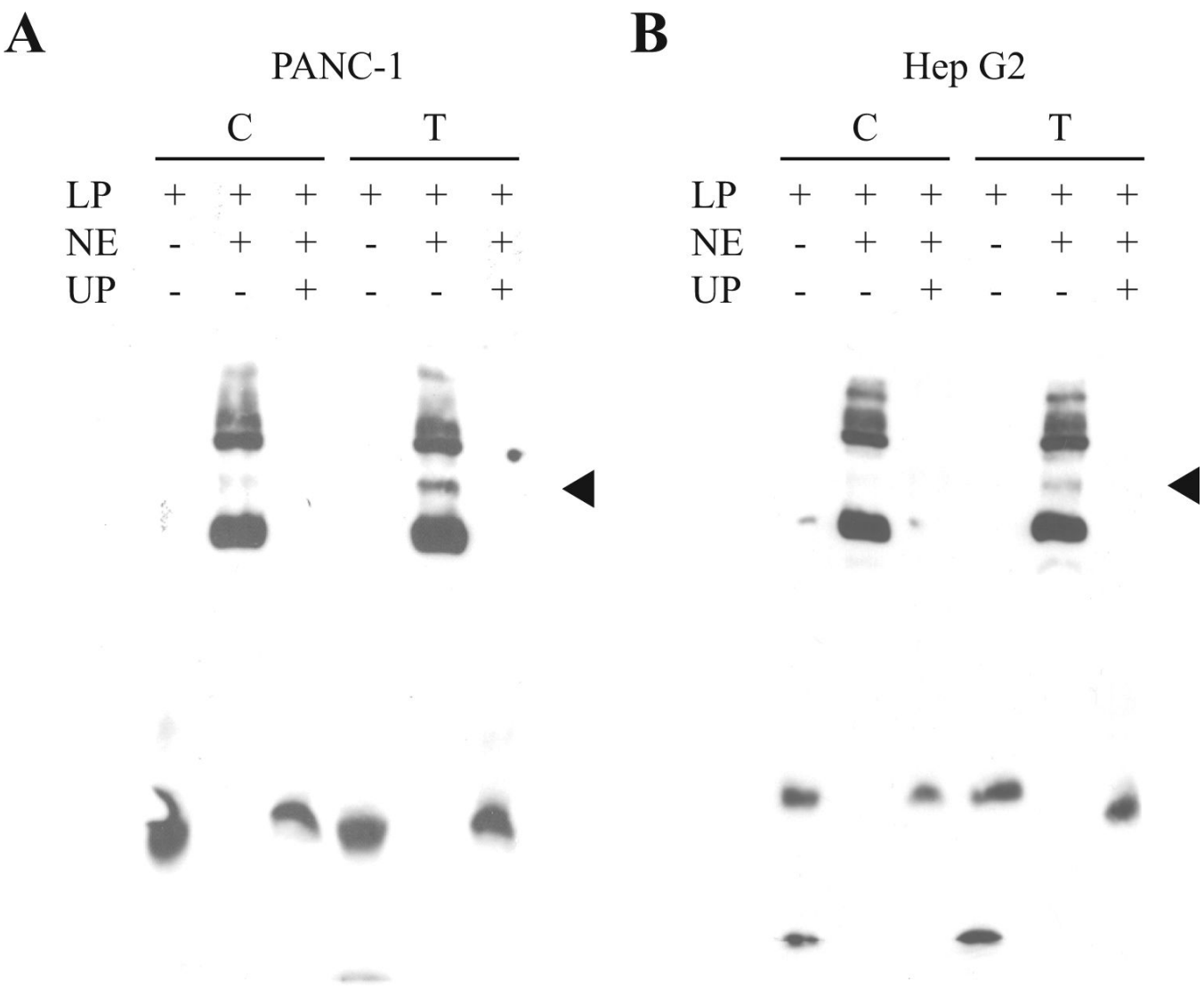

Figure 2.
EMSA showing the differential binding of nuclear factors to the C and T alleles of rs2187331 in a 31bp double stranded oligo. The observed allelic effect is indicated by the black arrowhead. Three different experimental conditions were tested for each allele: labeled probe (LP) as a negative control; LP and nuclear extract (NE); and LP, NE and unlabeled probe (UL) in excess to act as a competitor. (A) EMSA with PANC-1 nuclear extract. (B) EMSA with HepG2 nuclear extract.

Table 1

Association results of tagSNPs in the *GAL* region for HTG (TG≥90th age-sex specific population percentile) in the hyperlipidemic study samples.

SNP	Combined Hyperlipidemia Cases/ Controls (n=376)		FCHL Families (n=2,095)		Combined (n=2,471)		
	Z	p-value	Z	p-value	Z	p-value	Empiric p[A]
rs2187331	−3.17	0.0015	−1.86	0.063	−3.55	0.00038	0.00026
rs3136541	−0.34	ns	−0.42	ns	−0.54	ns	ns
rs3181041	−0.76	ns	−1.66	0.098	−1.71	0.087	0.086
rs6591350	−0.82	ns	−1.35	ns	−1.53	ns	ns

[A]Empiric p-values were calculated from 50,000 permutations. ns indicates p-values >0.1.

Table 2

Gene Ontology (GO) biological process term enrichment (Benjamini Corrected p-value<0.1) from genes the expression of which is dependent on rs2187331 genotypes.

GO Term Biological Process	p-value	Benjamini Corrected p-value
Generation of precursor metabolites and energy	4.8E-12	2.3E-08
Electron transport	4.8E-08	1.1E-04
Lipid metabolic process	9.8E-08	1.6E-04
Organic acid metabolic process	8.8E-07	1.0E-03
Carboxylic acid metabolic process	2.6E-06	2.5E-03
Cellular lipid metabolic process	5.1E-06	4.0E-03
Localization	1.5E-04	9.4E-02
Fatty acid metabolic process	1.6E-04	9.0E-02

Table 3

Association of rs2187331 and rs3181041 with logTGs in METSIM study.

SNP	Minor Allele	MAF	Mean TG by Genotype±SD mmol/L (n)[A]			Allelic p-value[B]	Allelic Effect Size±s.e.m.[C]	Genotypic p-value[B]
			11	12	22			
All (n=4,463)								
rs2187331	C	0.09	1.46±0.98 (3,658)	1.55±1.62 (766)	2.22±2.33 (38)	0.0028	0.108±0.036	0.00016
rs3181041	C	0.08	1.48±1.18 (3,742)	1.46±0.87 (700)	1.33±0.58 (21)	ns	0.006±0.039	ns
Non-statin users (n=3,963)								
rs2187331	C	0.09	1.44±0.98 (3,250)	1.49±0.94 (678)	2.00±2.30 (34)	0.026	0.086±0.038	0.019
rs3181041	C	0.08	1.46±1.02 (3,330)	1.43±0.83 (615)	1.30±0.56 (18)	ns	−0.017±0.041	ns
Statin users (n=497)								
rs2187331	C	0.1	1.56±0.97 (405)	1.99±4.01 (88)	4.09±1.99 (4)	ns	0.122±0.076	ns
rs3181041	C	0.09	1.64±2.05 (411)	1.71±1.11 (84)	1.63±0.93 (2)	ns	−0.002±0.081	ns
Non-diabetics (Fasting glucose<7mmol/L, n=3,802)								
rs2187331	C	0.09	1.38±0.91 (3,134)	1.46±1.64 (635)	2.34±2.48 (33)	0.0013	0.12±0.038	0.000012
rs3181041	C	0.08	1.40±1.13 (3,199)	1.38±0.85 (585)	1.33±0.60 (18)	ns	−0.006±0.041	ns
Diabetics (Fasting glucose≥7mmol/L, n=658)								
rs2187331	C	0.11	1.89±1.23 (522)	1.95±1.47 (131)	1.42±0.33 (5)	ns	−0.010±0.108	ns
rs3181041	C	0.09	1.90±1.35 (543)	1.88±0.88 (114)	1.37 (1)	ns	0.048±0.116	ns

ns indicates p-values >0.1.

[A] 1 is the major allele, and 2 is the minor allele.

[B] Analyses were conducted in R using linear regression with standardized $\log_{10}$ TG adjusting for the covariates age, BMI, smoking status (smoking or not). When n was <10 in the rare allele homozygote group, the heterozygotes and rare allele homozygotes were combined.

[C] Allelic effect size is given as the beta-coefficient (β), which represents the proportion of 1 standard deviation change.