Lipoprotein lipase gene polymorphism rs1059611 functionally influences serum lipid concentrations

Xingbo Mo\textsuperscript{a,1}, Xuehui Liu\textsuperscript{a,1}, Laiyuan Wang\textsuperscript{a}, Hongfan Li\textsuperscript{a}, Xiangfeng Lu\textsuperscript{a}, Jianfeng Huang\textsuperscript{a}, Jichun Chen\textsuperscript{a}, Jie Cao\textsuperscript{a}, Jianxin Li\textsuperscript{a}, Shufeng Chen\textsuperscript{a}, Yida Tang\textsuperscript{b}, Xiaozhong Peng\textsuperscript{c}, Dongfeng Gu\textsuperscript{a,}\textsuperscript{*}

\textsuperscript{a} State Key Laboratory of Cardiovascular Disease, Division of Population Genetics, Fuwai Hospital, National Center of Cardiovascular Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, 167 Beilishi Road, Beijing 100037, China
\textsuperscript{b} State Key Laboratory of Cardiovascular Disease, Department of Cardiology, Fuwai Hospital, National Center of Cardiovascular Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100037, China
\textsuperscript{c} State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100005, China

\textsuperscript{*} Corresponding author. Tel.: +86 10 68331752; fax: +86 10 88363812.
E-mail addresses: xingbomo@gmail.com (X. Mo), luxuehui209@yahoo.cn (X. Liu), wanglaiyuan@yahoo.cn.cn (L. Wang), ccms0965@gmail.com (H. Li), xiangfenglu@sina.com (X. Lu), jianhuang@sina.com (J. Huang), jchc70@sina.com (J. Chen), caoj315@sina.com (J. Cao), leelja@yahoo.com.cn (J. Li), shufengchen2001@yahoo.com.cn (S. Chen), yidatang@163.com (Y. Tang), pengxiaoaozhong@pumc.edu.cn (X. Peng), gudongfeng@vip.sina.com, gudf@yahoo.com (D. Gu).

Abstract

Objective: Dozens of single nucleotide polymorphisms (SNPs) in the lipoprotein lipase (LPL) gene have been reported to be associated with lipid concentrations. The aim of this study was to validate the association between rs1059611 in the LPL gene and serum lipid concentrations in the Chinese Han population and explore the biological relevance.

Methods: A total of 5664 participants were recruited and genotyped for the SNP. Gene expression levels of LPL in blood cells were evaluated by real-time PCR and western blotting analysis. The functional potential of the SNP was examined by luciferase reporter assay and electrophoretic mobility-shift assay (EMSA).

Results: We observed significant associations between rs1059611 and increased HDL-C ($P = 5.65 \times 10^{-5}$) and decreased TG concentrations ($P = 2.68 \times 10^{-7}$). We also found that participants with the C allele had higher mRNA expression level ($P = 0.0334$) and protein expression level ($P = 0.0641$) of LPL. The luciferase activity of the rs1059611 T construct was 0.69-fold of the rs1059611 C construct ($P = 0.0009$). The EMSA showed that the binding of the transcription factor(s) differed for the alleles of the SNP.

Conclusion: The results of our study demonstrated that rs1059611 was associated with HDL-C and TG concentrations in Chinese Han population and might have a functional effect on the transcription of LPL by differential binding of transcription factors.

© 2013 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Lipoprotein lipase (LPL) is a key enzyme in the metabolism of lipoproteins. It hydrolyzes plasma lipoprotein triglycerides (TG) into free fatty acids and glycerol, and converts very-low-density lipoprotein to low-density lipoprotein (LDL) \cite{1}. The LPL gene is located at 8p22 and comprises 10 exons spanning about 30 kb \cite{2,3}. Lipid concentrations are affected by both lifestyle factors, such as diet, obesity, and physical activity, and genetic factors \cite{4}. Several functional polymorphisms in LPL have been studied with regard to their associations with lipid profile and risk of coronary heart disease in candidate gene association studies \cite{5,6}. Variants in the 3′ region of the gene may play an important part in affecting gene transcription and expression \cite{7}. It was reported that a haplotype based on 19 single nucleotide polymorphisms (SNPs), of which 15 SNPs were located in the 3′-untranslation region (3′-UTR) of the LPL...
gene, was associated with increased LPL activity and multiple phenotypes related to the metabolic syndrome [8]. SNP rs1059611 located in the 3′-UTR of the LPL gene was in strong linkage disequilibrium (LD) with many lipid-associated SNPs in or near LPL identified by genome-wide association study (GWAS), such as rs328, and was reported to be associated with lipid levels in GWAS replication studies [9–11].

A functional SNP in non-coding regions would be most likely affecting gene regulation by altering the binding of some transcription factors. Computer-based analysis (i.e. TFSearch and TESS) showed that rs1059611 could alter the binding of a transcription factor YY1 (JASPAR ID: MA0095.1) at this site. YY1 is a ubiquitously distributed transcription factor belonging to the GLI-Kruppel class of zinc finger proteins. The protein is involved in repressing and activating a diverse number of promoters [12]. The algorithm miranda (http://www.mircrena.org/mircrena/home.do) also suggested that rs1059611 resided in a putative microRNA (miR-136) binding site. Besides, this polymorphism (among others) is able to modify gene expression but also affects activity through the LD with other SNPs in LPL gene. Whether rs1059611 is essentially a surrogate for a functional SNP that is so well characterized or a functional SNP needs to be tested.

The association between rs1059611 and lipid concentrations was less reported in the Chinese Han population. We conducted a study to validate the associations between rs1059611 and serum lipid concentrations and the biological relevance. We now report on the lipid associations and the findings of in vivo and in vitro experiments for rs1059611.

2. Materials and methods

2.1. Study populations

A group of 5664 participants were randomly selected from the International Collaborative Study of Cardiovascular Disease in Asia (InterASIA) [13] and judged to be free of coronary, cerebrovascular, renal diseases and other major chronic diseases by medical history. Standard questionnaire were used by trained interviewers to obtain information on demographic characteristics including age, sex, ethnicity, details of medical history, smoking and alcohol consumption. Blood pressure, weight and height were recorded. This study was approved by the local bioethics committee, and all subjects gave written informed consent.

2.2. Biochemical measurements

Overnight fasting blood samples were drawn by venipuncture to measure serum biochemical measurements including total cholesterol, High-density lipoprotein cholesterol (HDL-C), TG and glucose (GLU). Blood specimens were processed in the central clinical laboratory at the Department of Population Genetics at Fuwai Hospital of the Chinese Academy of Medical Sciences in Beijing. This laboratory participates in the Lipid Standardization Program of the US Centers for Disease Control and Prevention. Total cholesterol, HDL-C, TG and GLU were analyzed enzymatically on a Hitachi 7060 Clinical Analyzer (Hitachi High-Technologies Corp). The LDL-C concentrations were calculated by use of the Friedewald equation.

2.3. DNA extraction and genotyping

Genomic DNA was isolated from white blood cells according to a standard procedure using a DNA extraction kit (Tiangen Biotech, Beijing, China). SNP was genotyped using genomic DNA with the fluorogenic 5′-nucleic acid TaqMan allelic discrimination assay system (Applied Biosystems, Foster City, CA). The assays were performed under standard conditions on a 7900 HT Fast Real-Time PCR instrument. Probe and primer sequences can be found in Supplementary Table 1. Successful genotyping rate was over 98%. For quality control, 2% of the samples were randomly selected and genotyped in duplicate. A comparison of the duplicate results showed that the discrepancy was no more than 1%.

2.4. RNA extraction and real-time PCR

To analyze the mRNA level of the LPL, we separated peripheral blood mononuclear cells (PBMC) from fresh blood samples of 309 healthy individuals. Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA). After quantification, 1 µg of total RNA was reverse transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (Tiangen Biotech, Beijing, China) with oligo dT according to the manufacturer's instructions.

Real-time PCR was performed using the Applied Biosystems 7900 HT Fast Real-Time PCR System (Applied Biosystems, USA). Expression of all assays was measured in triplicates and average values of the triplicates were used for the analysis. Gene expression was quantified using the comparative Ct method, which standardized the Ct values to an internal housekeeping gene (ACTB) and calculated the relative expression values (ΔΔCt method).

2.5. Western blotting analysis

Proteins were extracted from PBMC in trizol lysis and soluted in 1% SDS. Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred by electroblotting to a nitrocellulose membrane. The membrane was blocked in 5% milk and then incubated with anti-LPL (sc32885, Santa Cruz, USA) and anti-ACTB (sc47778, Santa Cruz, USA) in 5% milk overnight at 4 °C, then washed and incubated with horseradish peroxidase-labeled second antibodies for 1 h at room temperature. Bands were visualized by the use of a super-western sensitivity chemiluminescence detection system (Pierce). Autoradiographs were quantitated by densitometry (Science Imaging System, Bio-Rad). ACTB was the internal control for protein normalization.

2.6. Construction of the luciferase reporter gene

The genomic sequences of LPL containing the site of rs1059611 (374-bp) were amplified by PCR from one individual homozygous for the major allele and one individual homozygous for the minor allele. PCR primers were presented in Supplementary Table 2. The PCR products were then purified and digested with the two designated restriction endonucleases (BamHI and Sall), and further sub-cloned into the firefly luciferase expressing pGL3-promoter vector (Promega) to create plasmid: pGL3-promoter-CC and pGL3-promoter-CT. Although there are SNPs in LD with rs1059611 in the region, the constructs have been sequenced to verify that the only ambiguity was the polymorphic site.

2.7. Cell cultures, transfections and luciferase assays

NIH3T3-L1 cells (mouse preadipocyte cells) were obtained from Dr. Peng's laboratory (Peking Union Medical College). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) at 37 °C in a humidified incubator containing 5% CO₂. Cells were plated at a density of 4 × 10⁴ cells per well in 24-well plates for transfection. Transfections were performed using Attractene transfection reagent (Qiagen, Valencia, CA) according to the
were combined with 15 peroxidase conjugate captured onto X-ray nylon membrane for 0.5 h at 380 mA, followed by cross-linking for gel in 0.5 reaction mixture was resolved on a non-denaturing 6% acrylamide gel-shift reaction (20 μl) a total of 50 fmol of protein-labeled probes were combined with 15 μg of nuclear extracts, 1 μg of poly (dl-dc), and 1 x binding buffer (10 mM Tris/HCl, 50 mM KCl and 1 mM dithiothreitol, pH 7.5). For competition assays, a 200-fold molar excess of unlabeled probe was pre-incubated for 5 min at 25 °C with nuclear extracts before the addition of the labeled probe. The reaction mixture was resolved on a non-denaturing 6% acrylamide gel in 0.5 x TBE buffer for 2.5 h at 100 V, and then transferred on to X. Mo et al. / Atherosclerosis 229 (2013) 511–516

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts from human visceral adipose tissue were prepared with Nuclear-Cytosol Extraction Kit (Applygen Technologies Inc. Beijing, China) according to the manufacturer’s protocol. Synthetic double-stranded oligonucleotide probes corresponding to the genomic sequence with either a C or T allele at the rs1059611 site (Supplementary Table 3) were labeled with biotin (Shanghai Sangon Biological Engineering Technology and Services). EMSAs were performed by using the LightShiftTM chemiluminescent EMSA kit (Pierce, Thermo Scientific, Loughborough, UK). For each gel-shift reaction (20 μl), a total of 50 fmol of protein-labeled probes were combined with 15 μg of nuclear extracts and 1 x binding buffer (10 mM Tris/HCl, 50 mM KCl and 1 mM dithiothreitol, pH 7.5). For competition assays, a 200-fold molar excess of unlabeled probe was pre-incubated for 5 min at 25 °C with nuclear extracts before the addition of the labeled probe. The reaction mixture was resolved on a non-denaturing 6% acrylamide gel in 0.5 x TBE buffer for 2.5 h at 100 V, and then transferred on to nylon membrane for 0.5 h at 380 mA, followed by cross-linking for 5 min with a UV cross-linker. Chemiluminescent detection of biotin-end-labeled DNA was performed with a strepavidin–horseradish peroxidase conjugate captured onto X-ray film and developed according to the manufacturers instructions.

2.9. Statistical analysis

Descriptive statistical analyses were performed with the SAS (Version 9.0) software package. The distributions of HDL-C and TG were evaluated by skewness and kurtosis statistics. TG values were log-transformed to approximate a normal distribution, and the log-transformed values were used in all association tests. Differences between categorical variables and Hardy–Weinberg equilibrium (HWE) were tested by χ² test. Continuous variables between different genotype groups were compared by using the independent student’s t-test. The SNP was analyzed as 0, 1 or 2 copies of the minor allele in an additive genetic model. Linear regression was used to test for association between the number of minor allele and the concentrations of HDL-C and TG. All models were adjusted for age, sex and body mass index (BMI). The trend of the mean LPL expression levels in different genotype groups was tested using simple linear regression test. In all analysis a P value less than 0.05 was considered statistically significant. In our study, we have 98% and 99% power to detect the associations between rs1059611 and HDL-C and TG concentrations under the additive model, respectively (estimated by Quanto; http://hydra.usc.edu/gxe/).

3. Results

3.1. Association between genotypes and lipid concentrations

Table 1 shows the general characteristics of the subjects included in this study. The minor allele frequency (MAF) for rs1059611 was 0.08 in our samples. No significant deviation from HWE was observed for this polymorphism (P = 0.525).

We investigated the associations between rs1059611 and lipid concentrations in the 5664 participants. Under an additive model, we observed significant associations between rs1059611 and increased HDL-C (Effect = 0.049(0.012), P = 5.65 x 10⁻⁵), and decreased TG concentrations (Effect = −0.090(0.018), P = 2.68 x 10⁻⁷). The mean HDL-C concentrations of individuals with TT, CT and CC genotypes were 1.36, 1.41 and 1.45 mmol/L, respectively. The corresponding mean TG concentrations were 1.51, 1.33 and 1.21 mmol/L. This SNP can explain 0.27% and 0.43% of variance in HDL-C and TG, respectively, after correcting for sex, age and BMI.

3.2. LPL mRNA and protein expression

Fig. 1 showed the result of gene expression analysis. We only have 3 CC homozygotes for rs1059611 due to its low frequency (Table 1). The standardized expression levels of the minor allele C carriers were higher than that of the major allele homozygotes. The expression levels increased as the copy of the C allele increased (P value for linear trend test was 0.0334). The CC homozygotes and CT heterozygotes had 1.41 and 1.23-fold higher expression levels than the TT homozygotes, respectively. P value for linear regression model adjusting for sex, age and BMI was 0.0979. This SNP can explain 0.79% of variance in LPL gene expression after correcting for sex, age and BMI.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics and genotype distributions of the study samples.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variables</td>
<td>Lipid association study</td>
</tr>
<tr>
<td></td>
<td>CC</td>
</tr>
<tr>
<td>N</td>
<td>35</td>
</tr>
<tr>
<td>Age</td>
<td>56.5 ± 9.8</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>138.3 ± 23.8</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>82.4 ± 11.1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.7 ± 3.85</td>
</tr>
<tr>
<td>GLU (mmol/L)</td>
<td>5.00 ± 1.20</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>2.95 ± 0.93</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.45 ± 0.32</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.21 ± 0.55</td>
</tr>
<tr>
<td>Male/female:</td>
<td>32/3</td>
</tr>
<tr>
<td>Male/female:</td>
<td>16/19</td>
</tr>
<tr>
<td>Male/female:</td>
<td>13/22</td>
</tr>
</tbody>
</table>

* Analysis of variance (ANOVA) for continuous variables, Chi-square test for categorical variables.
** Analysis of variance (ANOVA) for continuous variables, Fisher’s Exact Test for categorical variables.
We assessed protein expression of \(LPL\) by western blotting in PBMC from 3 subjects with CC, 7 with CT and 7 with TT genotype (Fig. 2). The mean protein level of the 7 CT samples is significantly higher than that of the 7 TT samples \((0.50 \pm 0.12 \text{ vs. } 0.33 \pm 0.10, \ P = 0.0167)\). The mean protein level of C allele carriers \((\text{CC} + \text{CT})\) seems higher than that of TT homozygotes \((0.45 \pm 0.17 \text{ vs. } 0.33 \pm 0.10, \ P = 0.0641)\).

3.3. Transcriptional activity and binding of nuclear proteins

Transfection of the reporter constructs with C or T allele of rs1059611 was carried out in NIH3T3-L1 cells, using a Renilla-containing vector to control for transfection efficiency. Fig. 3 shows the results. In NIH3T3-L1 cells, the luciferase activity of the rs1059611 T construct was 0.69-fold of the rs1059611 C construct \((P = 0.0009)\).

Using nuclear extracts from the human visceral adipose tissue cells and biotin-labeled probes corresponding to the C or T allele of rs1059611, we compared the binding ability of transcription factors between the two alleles. A band in the T allele but not in the C allele was observed (Fig. 4, lane 5 and lane 2). The shifted band could be completely abolished by 200 fold unlabeled probes (Fig. 4, lane 6). The experiments were repeated three times with similar results.

4. Discussion

In the present study, we analyzed the associations between rs1059611 and serum lipid concentrations in Chinese Han population. We found that rs1059611 was associated with decreased TG and increased HDL-C concentrations. The mRNA and protein expression levels of \(LPL\) gene in different genotype groups seemed to be discrepant. The functional analyses showed that expression levels from the reporter constructs and the binding of the transcription factor(s) differed between alleles for this SNP.

SNP rs1059611 was in strong LD with many GWAS-identified lipid associated SNPs. In GWAS replication studies, this SNP was selected and found to be associated with HDL-C and TG concentrations [9–11]. We successfully detected the associations in our samples, and the effects were consistent with previous studies in different populations.
Phil Kern demonstrated that rs1059611 was associated with decreased TG concentration in the SNP region were not detected. The SNP region contributes to a range of functional effects.

LD. Essentially, there is extensive LD at this locus, especially in the 3′-UTR.

On the other hand, in vitro expression of the LPL haplotypes defined by these SNPs has already been published. In experiments for which justification was provided to search for additional functional consequences of 3′-UTR SNPs above and beyond S447X, Dr. Phil Kern’s group recently showed that the LPL haplotype defined by rs328 and rs1059611 major and minor alleles had clear functional consequences, namely altered susceptibility to inhibition by adipocyte extract and altered susceptibility to translation inhibition associated with the complete haplotype constructed within the full-length 3.6 kb LPL mRNA [22]. It appears that the entire haplotype contributes to a range of functional effects.

The majority of genetic markers associated with traits or diseases are present in non-coding regions. A functional SNP in non-coding regions would be most likely affecting gene regulation by altering the binding of some transcription factors. Luciferase reporter assays and EMSA experiments were carried out in our study to investigate the functionality of rs1059611. A band in the T allele but not in the C allele was observed in EMSA. These results suggested that the binding elements might seem likely to be a repressor and allelic changes from T to C in rs1059611 might act as a functional enhancer in regulating gene expression, as the expression study showed that participants with the C allele had higher expression levels, and lead to higher HDL-C and lower TG concentrations in rs1059611 C allele carriers. The statistical evidence for rs1059611 on influencing gene expression may not be very convincing, however, according to previous reports, this polymorphism (among others) is able to modify gene expression but also affects activity through the LD with other SNPs in LPL gene.

Overall, we suggest that rs1059611 should be considered in future functional or mechanism studies and this SNP might be a regulatory SNP by altering DNA-binding properties and the genotypes seemed to have influence on the expression level of LPL. Further studies are needed to elucidate the underlying mechanisms.

**Funding**

This work was supported by National Basic Research Program of China (973 Plan) (Grant No. 2011CB503901 and 2012CB517501), National Natural Science Foundation of China (Grant No. 30930047).

**Conflict of interest statement**

None declared.

**Acknowledgments**

We would like to thank Ying Li, Liancheng Zhao, Donghua Liu, Jiping Chen, Fanghong Lu, Zhengong Liu, Caillang Yao, Chong Shen, Xiaodong Pu, Lin Yu, Xianping Wu, Dongsheng Hu, Dongshuang Guo, Chenlong Hou, Qingjie Meng, Jun Yang, Zili Yang and the many people who helped with the field and laboratory work. We appreciate Dr. Gang Hu with the great help of obtaining human visceral adipose tissue.

**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2013.05.005.

**References**


