A novel allele in the promoter of the hepatic lipase is associated with increased concentration of HDL-C and decreased promoter activity

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Abstract Hepatic lipase (HL) is a lipolytic enzyme involved in the metabolism of plasma lipoproteins, especially HDLs. Association of the polymorphisms in the promoter region of the *LIPC* gene to post-heparin plasma HL activity and the plasma HDL-C concentration has been investigated thoroughly, but to date little is known about this in the Chinese. In the present study, we analyzed the polymorphisms in the promoter region of *LIPC* gene in Chinese patients with coronary artery disease (CAD) using denaturing high performance liquid chromatography (DHPLC) and DNA sequencing. As the result, a novel single nucleotide polymorphism −586T-to-C was identified and no linkage of this variant with other polymorphisms in the promoter was found. Compared with the nonsymptomatic control subjects, excess of carriers of the −586T/C substitution were detected in the CAD patients (43% vs. 31%, $\chi^2 = 4.597$, degree of freedom = 2, $P = 0.032$).The –586C allele carriers in the CAD patients had a significantly higher HDL-C level than the noncarriers (1.13 ± 0.24 mmol/l vs. 0.91 ± 0.14 mmol/l, $P < 0.05$). To test the functionality of this substitution, luciferase-reporter assays was performed in HepG2 cells. Promoter activity of the −586C construct was decreased 2-fold than the −586T construct. Our studies suggest that a T-to-C substitution at −586 of the *LIPC* promoter is associated with a lowered HL activity and that this variation may contribute to the increased plasma HDL-C concentration in the Chinese.—Su, Z., S. Zhang, D. W. Nebert, L. Zhang, D. Huang, Y. Hou, L. Liao, and C. Xiao. **A novel allele in the promoter of the HL is associated with increased concentration of HDL-C and decreased promoter activity. *J. Lipid Res.* 2002.** 43: **1595–1601.**

Supplementary key words *LIPC* promoter • single nucleotide polymorphism • coronary artery disease • transient transfection • single nucleotide polymorphism

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Human hepatic lipase (HL, triacylglycerol, EC3.3.3.3) is a glycoprotein synthesized primarily in hepatocytes. Following secretion, the enzyme binds to the hepatic sinusoidal endothelial surface, where it hydrolyzes triglycerides and phospholipids in plasma lipoproteins (1–3). HL has been suggested to play a role in HDL metabolism. Evidence demonstrated that HDL-cholesterol (HDL-C) level was at least partly regulated by HL level and on this basis it had been thought that lowering HL would increase HDL-C (4). HL deficiency leads to elevation in HDL-C, increased levels of triglyceride in HDL and LDL, and impaired metabolism of post-prandial glyceride-rich lipoproteins (5–8), and all of these are considered to be risk factors for premature atherosclerosis. Although HL seems to be an important enzyme with multiple functions, the exact role of HL in lipoprotein metabolism has not yet been established.

The human *LIPC* gene has been assigned to chromosome 15q21 and spans over 35 kb with 9 exons encoding a cognate mRNA of 1.6 kb that is translated into a mature 476-amino acid protein (9–12). Several polymorphisms have now been described in the *LIPC* gene, including a number of mutations associated with the rare HL deficiency condition (5, 7, 8, 13–15). Recent studies demonstrated that polymorphisms in the promoter of the *LIPC* gene are related to variants in plasma HDL-C concentrations, and the associations between *LIPC* gene promoter variants and HL activity have been reported (16–24).

Abbreviations: CAD, coronary artery disease; DHPLC, denaturing high performance liquid chromatography; HL, hepatic lipase; HDL-C, high density lipoprotein cholesterol; SNP, single nucleotide polymorphism.

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There is a considerable body of evidence demonstrating that the rare alleles of some common promoter polymorphisms including −250G/A, −514C/T, −710T/C, and −763A/G are associated with marked decreased plasma HL activity and increased HDL-C levels. And some studies showed that these variants have a significant effect on the basal rate of transcription of the *LIPC* gene (25, 26); however, a recent study did not support this conclusion (27). We present evidence of a novel promoter single nucleotide polymorphism (SNP), which is associated with plasma HDL-C concentration and at the same time has significant effect on the transcription of *LIPC* gene.

MATERIALS AND METHODS

Subjects

One hundred sixty male patients (≤65-years-old) with coronary artery disease were from the West China Hospital, Sichuan University. All of them were examined by coronary angiography using the Judkins technique. Patients having any main coronary artery branch (left anterior descending, left circumflex artery, right coronary artery) with at least one stenosis of >60% were included in the study. Meanwhile, 150 unrelated age-matched male subjects selected via health screening at the same hospital, free of any clinical and biochemical signs of CAD, were used as controls for the study.

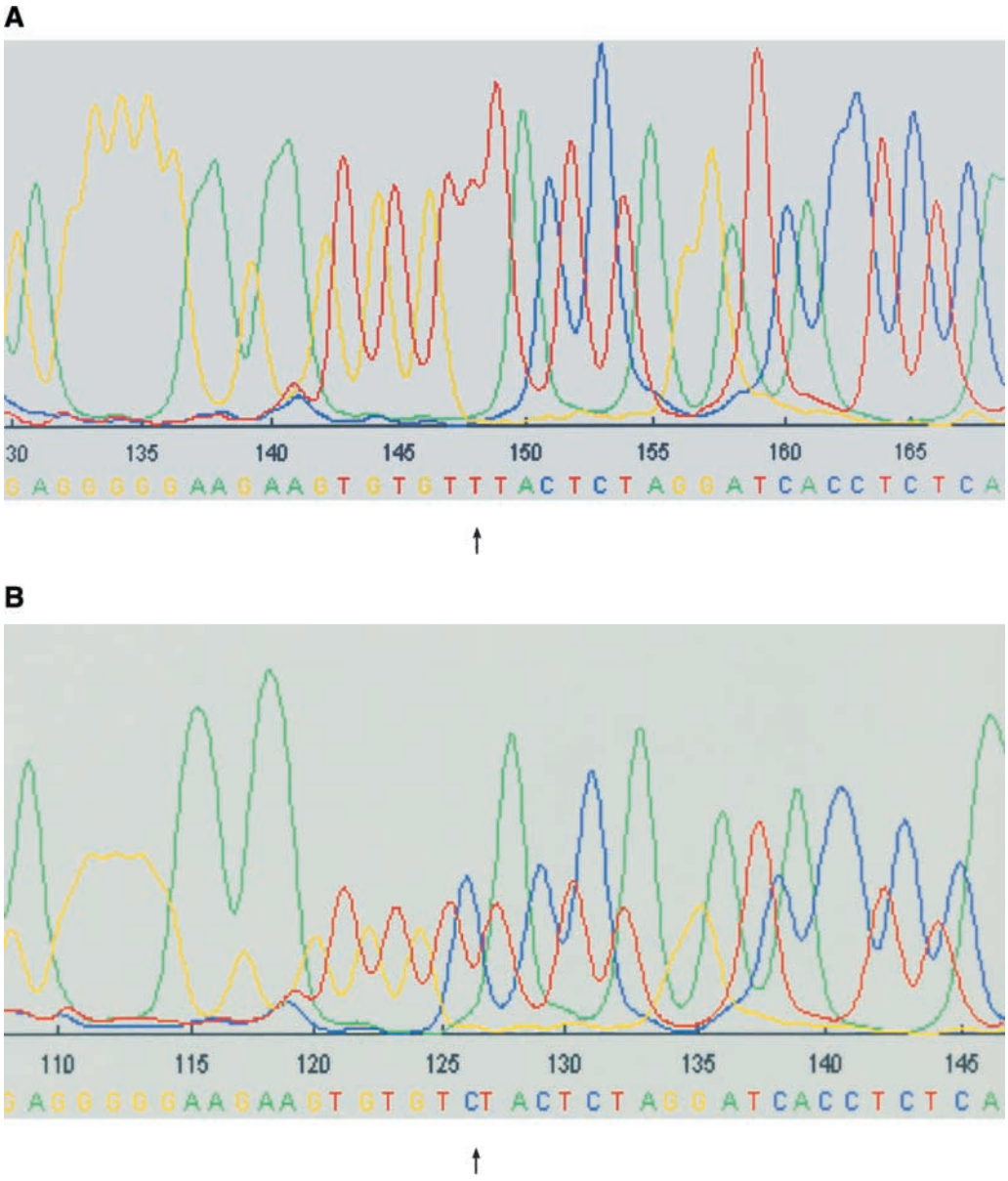

Fig. 1. Sequence analysis of SNP in the promoter region of *LIPC* gene. The arrow indicates the −586T/C. A: T allele (position 148) and B C allele (position 126).

TABLE 1. Genotype and allele frequencies of the variants in the *LIPC* promoter

Polymorphic Sites and Group	Genotype			Allele Frequency		*P*
−586 T/C	TT	TC	CC	T	C	
CAD patients	91	65	4	0.772	0.228	0.032
Controls	103	46	1	0.840	0.160	
−514 C/T	CC	CT	TT	C	T	
CAD patients	91	64	5	0.778	0.231	0.052
Controls	69	72	9	0.683	0.300	
−250 G/A	GG	GA	AA	G	A	
CAD patients	88	67	5	0.731	0.241	0.079
Controls	69	71	10	0.693	0.303	

The statistical significance of differences between control subjects and patients was tested by Pearson χ^2 analysis.

Measurement of lipids and lipoproteins

Blood samples were collected at baseline from patients and controls after an overnight fast. Plasma separated from cells by centrifugation at 500 *g* for 10 min at room temperature was used immediately for lipid and lipoprotein analysis. The levels of plasma cholesterol and triglyceride were determined with an enzymatic kit (Boehringer Mannheim) and calibrated with a serum calibrator. HDL-C was measured in the supernatant after precipitation of apoB-containing lipoproteins with a 4% sodium phosphotungstate solution after centrifugation. LDL-C was calculated by use of the Friedewald formula (28).

DNA preparation and PCR amplification

Genomic DNA was isolated from peripheral blood leukocytes using the "salting-out" procedure (29) and stored at 4°C. A 668 bp segment extending from nt +29 to −639 (30) was amplified by PCR with primers 5′-CAGTCCTCTACACAGCTGGAAC-3′ and 5′-CGGGGTCCAGGCTTTCTTGGT-3′. Each PCR amplification mixture contained 0.1 μg of genomic DNA, 40 pmol of each primer, 25 pmol dNTPs, and standard PCR buffer in a total volume of 50 μl. The reaction mixture was preheated at 94°C for 4 min. Subsequently, 0.4 units of Taq polymorase was added. The 30 cycles of PCR amplification was performed with a temperature profile consisting of denaturation for 45 s at 94°C, annealing for 30 s at 62°C, and extension for 30 s at 72°C. The reactions were carried out in a Perkin Elmer GeneAmp 9600 PCR System (Perkin Elmer).

Denaturing high performance liquid chromatography

Denaturing high performance liquid chromatography (DHPLC) screening for base variant was performed on an automated HPLC instrument (Hewlett Packard). The support for the stationary phase was made of a specially prepared wide-pore silica with a dense layer of an aliphatic organosilane packed into a 50 mm-X4.6-mmId dsDNA-analysis column (Hewlett Packard). The mobile phase was 0.1 mol/l trietylammonium acetate (PE BioSystems) buffer at pH 7.0 containing ethylenediamine tetraacetic acid (0.1 mmol/l).

PCR products were eluted with a linear acetonitrile gradient. The start and end points of the gradient were adjusted according to the size of the PCR products. The temperature required for successful resolution of heteroduplex molecules was predicted by the DHPLC algorithm available at http://insertion.stanford.edu/melt.html. In the present study, the appropriate temperature of analysis for amplicon was determined empirically by running it at different temperatures until a good resolution between homo- and heteroduplexes was obtained. The temperature of DHPLC for this amplified fragment is 63°C. The heteroduplex molecules are generally eluted ahead of homoduplex, therefore the appearance of additional peaks or shoulders during DHPLC was interpreted as indicative of a single base mismatch in heteroduplex DNA fragments and, therefore, reason for need of sequencing.

DNA sequencing and genotype assay

The location and chemical nature of the mismatch was confirmed by sequencing of the re-amplified product. The heterozygous and homozygous samples were cloned in T-Easy vector (Promega), then sequenced in both directions on the ALFexpress DNA automated sequencer, using the dye-terminator cycle thermal sequenase sequencing kit (USB company).

A T-to-C substitution at nucleotide −586, introducing a new *Acc*I restriction site, was confirmed by restriction analysis. Genotyping for the −250G/A and −514C/T polymorphisms was performed by digesting the 668 bp PCR fragment with *Dra*I and *Nla*III, respectively. Electrophoresis was performed on a 2% agarose gel containing ethidium bromide.

Generation of luciferase reporter constructs

For in vitro promoter studies, the 5′-flanking region of the *LIPC* gene was amplified with upstream primer 5′-TCATGGTACCCAGTCCTCTACACAGCTGGAAC-3′ and downstream primer 5′-TAGTCTCGAGCGGGGTCCAGGCTTTCTTGGT-3′, each of them contains a *Kpn*I and *Xho*I site introduced to the 5′ end (restriction sites underlined), respectively. The T allele and the C allele were amplified from individuals who differed in *LIPC* promoter structure, respectively.

The PCR products were digested with *Kpn*I and *Xho*I and purified by electrophoresis on a 1.5% low-melting-point agarose gel followed by magic PCR preps DNA purification system (Promega). The purified mutant and wild-type *LIPC* promoter sequences were cloned into promoterless pGL-3 enhancer vector (Promega). The vector containing either T allele or C allele was designated as pGL3-T or pGL3-C, respectively. Plasmid DNA was obtained by transforming the vector into JM109 cells and subsequent large-scale plasmid preparation using Qiagen plamid kit (Qiagen). Reporter constructs were sequenced prior to use in reporter assay.

TABLE 2. Estimate of pairwise haplotype frequencies and disequilibrium statistics

Polymorphic Sites and Subjects	Estimated Haplotype Frequency				D	D_{max} or D_{min}	D′	*P*
−250 and −586	GT	GC	AT	AC				
CAD patients	0.582	0.190	0.178	0.051	0.004	0.055	0.079	0.666
Controls	0.600	0.240	0.096	0.064	0.015	0.111	0.135	0.122
−514 and −586	CT	CC	TT	TC				
CAD patients	0.578	0.194	0.190	0.038	0.015	0.053	0.285	0.128
Controls	0.581	0.259	0.119	0.041	0.007	0.048	0.148	0.463

TABLE 3. Plasma lipids and lipoprotein in CAD patients with different genotypes

Lipid & Lipoprotein	TT	TC	CC	*P* Value		
				TT vs. TC	TT vs. CC	TC vs. CC
		mmol/l				
Cholesterol	5.94 ± 0.79	6.02 ± 0.67	6.16 ± 0.85	0.057	0.051	0.054
Triglyceride	1.67 ± 0.68	1.79 ± 0.81	1.67 ± 0.91	0.053	0.087	0.061
HDL-C	0.91 ± 0.34	0.98 ± 0.43	1.13 ± 0.24	0.042	0.011	0.023
LDL-C	4.27 ± 0.34	4.23 ± 0.15	4.30 ± 0.20	0.089	0.076	0.068

Cell culture and transient transfection

The human hepatoma cell line HepG2 was plated into 45 mm culture dishes at 20% confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (GibcoBRL, the Netherlands). In each experiment, three different luciferase reporter plasmids were transfected: *a*) pGL3-T; *b*) pGL3-C; and *c*) pGL3-control (Promega), which contains SV40 promoter and enhancer sequences. The DNA mixture for transfection was composed of test plasmid (0.75 μg) and pSV-β-galactosidase control vector (Promega; 0.04 μg) that served as internal control to normalize activities of luciferase. Transfection was carried out using the liposome method (Gene Therapy Systems, San Diego, CA). Luciferase activity was measured with a luminometer (Model TD-20/20; Promega), and the β-galactosidase activity was determined as described by the suppler (Promega). To correct for transfection efficiency, light units from the luciferase assay were divided by the absorbance reading from the β-galactosidase assay. The corrected *LIPC* promoter-driven luciferase activity is expressed as a percentage of the pGL3-control SV40 promoter-driven luciferase activity that served as the positive control in every transfection experiment. Luciferase activity was expressed as relative luciferase units. The promoterless pGL3-basic vector (Promega) lacking promoter and enhancer was used as a negative control in each of the transfection experiment. All constructs were tested in triplicate in three independent transfection experiments.

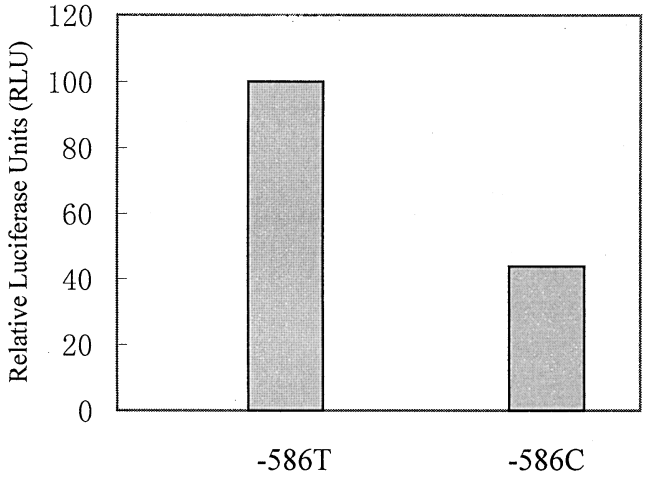

Fig. 2. Effect of the −586 T or C allele on *LIPC* promoter activity in HepG2 cells. Activity of *LIPC* promoter with −586C decreased 56% as compared with −586T. Data were normalized to β-galactosidase activity and are expressed as a percentage of the corrected activity of pGL3-control. (means of three independent experiments).

Statistical analysis

The assumption of Hardy-Weinberg equilibrium was tested by means of gene counting and χ^2 analysis. Haplotype frequencies for pairs of alleles, as well as χ^2 values for allele associations, were estimated by the Estimating Haplotype-Frequencies software program (31); LD coefficients $D' = D/D_{max}$ were calculated. The lipid phenotypic data among different genotypes were statistically analyzed using the Student's *t*-test.

RESULTS

A novel polymorphism T-to-C (HL-586) in the *LIPC* promoter

Screening for base variant of the 668 bp region in the 5′ flanking of the *LIPC* gene with DHPLC in CAD patients and controls revealed that there is a variation in some samples. As is known, any mismatched base pair in a heteroduplex molecule is generally eluted ahead of the homoduplex, resulting in one additional DHPLC peak (data not shown). The character of varied base was then identified by sequence analysis. As the result, a new base variation, namely −586T-to-C transition was discovered (**Fig. 1**).

Allele frequencies and the linkage disequilibrium of the *LIPC* promoter

To determine the prevalence of the −586T/C, −514C/T, and −250G/A polymorphisms, we screened them in all the 160 CAD patients and 150 controls. The genotype distribution and allele frequencies are listed in **Table 1**. No deviation from Hardy-Weinberg equilibrium was noted in both CAD and control groups. As the result, excess of carriers of the −586T/C substitution were detected in the CAD patients compared with the nonsymptomatic control subjects (43% vs. 31%, $\chi^2 = 4.597$, df = 2, $P = 0.032$). The prevalence of the –586C allele was significantly higher in the CAD patients than in control subjects ($\chi^2 = 4.575$, df = 1, $P = 0.032$). The extent of D in pairwise combinations of alleles at the *LIPC* promoter locus was estimated by means of maximum likelihood from the frequency of diploid genotypes in the CAD and control groups. Haplotype frequencies and the disequilibrium statistics D, D′, and D_{max} or D_{min} are given in **Table 2**. It is clear that the disequilibrium values for −586/−514 and −586/−250 pairs do not differ significantly from zero. So the −586 variant is a novel mutation.

Association between T-to-C substitution and plasma lipids

Studies on the relation between T-to-C (HL-586) and plasma lipid showed that neither cholesterol and triglyceride nor LDL-C was significantly different between subjects with or without this gene variant. However, HDL-C levels did differ among different genotypes($P = 0.011$). The subjects homozygous for the the C allele (HL-586) had the highest HDL-C values (1.13 ± 0.24 mmol/l), and subjects homozygous for the −586T allele had the lowest (0.91 ± 0.34 mmol/l), while the heterozygote had the intermediate value (0.98 ± 0.43 mmol/l) (**Table 3**).

Effects of −586 T/C polymorphism on promoter activity

To examine the potential effects of the −586T/C polymorphism on the rate of transcription of the *LIPC* gene in vitro, a 668-bp promoter fragment of *LIPC* gene (nt +29 to −639) carrying either T or C allele was inserted upstream of the luciferase gene in the pGL3 promoterless enhancer plasmid vector. The activity of *LIPC* T/C promoter-luciferase reporter gene constructs were assessed in transient transfection assay in HepG2 human hepatoma cells. Triplicate experiments were performed using DNA from different plasmid preparations. The luciferase light units in these assay ranged between 13,000 to 16,000 for the −586T construct and from 7,200 to 8,900 for the −586C construct. As shown in **Fig. 2**, significantly lower luciferase activities were observed for the pGL3-C construct as compared with the pGL3-T construct (a 56% decrease; $P < 0.001$). These results indicate that −586T/C polymorphism has a major influence on the basal transcription rate of the *LIPC* gene.

DISCUSSION

In the present study, a novel base variation (−586T/C) in the *LIPC* promoter region was found by DHPLC and DNA sequencing. This polymorphism was present in about 43% of patients with angiographically established coronary artery disease and in about 31% of nonsymptomatic control subjects. The T-to-C allele was significantly more frequent in the patient with CAD than in the control subjects.

There is considerable evidence that HL activity is an important determinant of plasma HDL-C concentrations. Clinical studies have consistently found an inverse relationship between HL activity measured in post heparin plasma and plasma HDL-C concentrations (22–37); and drugs, such as anabolic steroids, that increase HL activity cause a proportional reduction in plasma levels of HDL-C (38). Association studies showed that the −586T/C variation may account for the variation in plasma HDL-C concentration, at least in the tested Chinese. Since we did not measure the HL activity in the present study, we can only speculate that the −586 polymorphism may affect the activity of this enzyme and thereby influence the plasma HDL-C. Given the well established inverse relationship between HL activity and HDL-C concentrations, however, it seems very likely that the −586C is associated with low HL activity by directly affecting HL expression or through linkage disequilibrium with another polymorphism that directly decreases the enzyme activity. Since no linkage of the −586T/C variant with other polymorphisms in the first 668 bp of the *LIPC* promoter was found, it suggests that the base substitution may lead to a lowered HL expression. To determine whether *LIPC* allele containing −586C does influence the transcription rate of *LIPC* gene, the transient transfection studies in HepG2 cells were conducted. The results from the study strongly suggested that the T/C (HL-586) base substitution did have a significant contribution to the basal rate of transcription of the *LIPC* gene. This observation provided new strong evidence that the single nucleotide polymorphism in HL gene may be one of major factors in determination of plasma HDL-C levels.

The promoter sequence variant −514T in the HL gene has been shown to be significantly associated with low post-heparin HL activity (18, 21–24). Some studies have also found that the −514T variant is associated with elevation in plasma HDL-C (17–21, 23). It has generally been assumed that these associations are due to the change in rate of transcription of the *LIPC* gene because of the promoter polymorphisms. However, recent studies in vitro showed contradictory results (25–27). We tested for associations of the same *LIPC* −514T with plasma lipoprotein traits in Chinese CAD patients and normal controls. The *LIPC* −514T allele frequencies in these two groups were 0.231 and 0.300, respectively. No significant association was found between *LIPC* −514T and plasma HDL-C after adjusting for covariates including gender and body mass index, although the plasma HL activity was not available for analyses. There was no consistent relationship between the population mean plasma HDL-C concentration and the population *LIPC* −514T frequency. Our findings are consistent with the results from the Canadian populations (39), suggesting that the common promoter variation in *LIPC*, which has been reported to be associated with variation in post heparin HL activity and HDL triglyceride concentration, is not always associated with variation in plasma HDL-C concentration, possibly due to yet unspecified environmental or genetic factors.

Little is known about how the expression of the *LIPC* gene is regulated. The promoter region of the human *LIPC* gene has been characterized, and possible regulatory elements were identified by searching for consensus sequences. Transfection studies have indicated that multiple elements in the proximal promoter influence the transcription of the *LIPC* gene (30, 40, 41), but none of these elements have been identified and/or analyzed yet in detail.

In summary, we show here that a novel variant in the *LIPC* promoter is functional. The molecular mechanism and the transcription factors involved remain to be established.

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