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Single-nucleotide polymorphisms in the lipoprotein lipase gene associated with coronary heart disease in Chinese

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Abstract

Coronary heart disease is a complex disease reflecting the interaction of multiple genes with the environment (e.g. diet, life style). Lipoprotein lipase (LPL) plays an important role in lipid metabolism and the pathogenesis of coronary atherosclerosis. Recent associations between single-nucleotide polymorphisms in the *LPL* gene and heart disease have been reported, but little is known in Chinese. The *LPL* gene spans >26 kb, with an mRNA of 3549 bp. In the present study, we screened 5155 bp (565 bp of 5′ flanking region, nine exons and donor- and acceptor-splice sites, and some intronic bases) in 160 Chinese patients with confirmed coronary heart disease and 150 age- and gender-matched controls. Thirteen of the sixteen single-nucleotide polymorphisms that we found have not been previously reported. In males, significant ($P<0.05$) differences between the coronary heart disease patients and controls were found for five single-nucleotide polymorphisms: −421G>A (5′ flanking region); +13,577C>A (intron 2); +16,052G>A, R192Q (exon 5); +16,173C>G and +16,177T>C (intron 5). In females, significant differences between the patients with coronary heart disease and controls were found for only the −421G>A and +16,052G>A (R192Q) mutations. Among the coronary heart disease males, significant ($P<0.05$) associations were found between the low-HDL high-triglyceride (LHDL/HTG) phenotype and the non-LHDL/HTG trait for the 5′ flanking-421G, the intron 2+13,577C, and the exon 5+16,052G mutations, with odds–ratios (ORs)[confidence intervals] of 3.90[1.12–13.66], 3.38[1.22–9.40], and 3.22[1.04–10.01], respectively; no corresponding associations were found in females. There were 69, 51, 57 and 41 unphased haplotype patterns in male coronary heart disease, male control, female coronary heart disease and female control groups, respectively; the computer program PM-Plus found the heterogeneity model by far the best fit ($P<0.0001$ in males, >0.01 in females). These data show that some single-nucleotide polymorphisms in the *LPL* gene among Chinese are associated with abnormal lipid and lipoprotein profiles and predisposition to coronary heart disease, a genetically heterogeneous complex disease, and that they are gender-specific.

Keywords: Single-nucleotide polymorphism; Coronary heart disease; Chinese population study; Dyslipidemia; Lipoprotein lipase gene; High-density lipoprotein; Triglyceride

1. Introduction

Coronary heart disease is one of the most important cardiovascular disorders and a major cause of death in many countries. Epidemiological studies over the past 50 years have revealed numerous risk factors for coronary heart disease (Lusis, 2000). Data from the Prospective Cardiovascular Munster (PRO-CAM) study indicate that the relative abundance of the various plasma lipoproteins appears to be of primary importance in the pathogenesis of this disease; this seems reasonable, because elevated levels of atherogenic lipoproteins are a prerequisite for most forms of coronary heart disease (reviewed in Cullen et al., 1998; Cullen and Assmann, 1999).

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Lipoprotein lipase (LPL) plays a pivotal role in lipoprotein metabolism by catalyzing the hydrolysis of triglycerides of very-low-density lipoprotein (VLDL) and chylomicros, thereby delivering fatty acids to various tissues (Auwerx et al., 1992). Because of its central role in the regulation of lipid metabolism, LPL activity presumably has an important influence on the development of coronary heart disease and appears to be linked to the low high-density lipoprotein/hypertriglyceridemia (LHDL/HTG) phenotype (Funke and Assmann, 1995; Fisher et al., 1997; Moennig et al., 2000). Therefore, differences in expression of the *LPL* gene are likely to be an important factor affecting interindividual risk of atherosclerosis (Murthy et al., 1996).

The *LPL* gene contains 10 exons (Wion et al., 1987) and spans more than 26 kb on chromosome 8p22 (Sparkes et al., 1987). To date, 88 DNA sequence variants in the human *LPL* gene have been described. Specific homozygous mutations in the *LPL* gene have been demonstrated in subjects with type I hyperlipoproteinemia, a rare autosomal recessive disease biochemically characterized by strikingly diminished HDL-cholesterol ratios and very high-triglyceride concentrations (Bruzell, 1995). While the frequency of heterozygous LPL deficiency is as high as 3% to 7% (Benlian et al., 1996), fasting triglyceride levels in heterozygotes can be either normal or only moderately increased (Bijvoet et al., 1996). The phenotypic expression of heterozygous LPL deficiency has been extensively studied in cell culture (Wilson et al., 1990; Miesenbock et al., 1993; Minnich et al., 1995; Bijvoet et al., 1996; Holzl et al., 2000).

There are three fundamental types of DNA sequence variants. Insertions and deletions can occur for a single, or small number of, nucleotides or for several hundred or thousands of bases, which can include an entire gene or more. Insertions and deletions of repetitive sequences (e.g. short tandem repeats, variable number tandem repeats, *Alu* I) comprise a second type. Single-nucleotide polymorphisms are nucleotide substitutions and comprise probably >90% of all DNA sequence variants. There was a recent estimation of ~ 11 million polymorphic single-nucleotide polymorphisms (i.e. $q \geq 0.01$, as compared with rare single-nucleotide polymorphisms, $q < 0.01$) and the number of rare single-nucleotide polymorphisms ($q < 0.01$) will be much greater, among all humans on this planet (Kruglyak and Nickerson, 2000). It appears likely in the near future that haplotype patterns of single-nucleotide polymorphisms, in dozens or hundreds of genes, will be used to predict interindividual risk of complex diseases (Brookes, 1999; Kruglyak, 1999; Gray et al., 2000), as well as clinical pharmacogenetic disorders (Gelbert and Gregg, 1997; Bond et al., 1998; Nebert, 1999, 2000a,b).

The frequencies of particular single-nucleotide polymorphisms in the *LPL* gene vary widely between different ethnic groups. For example, in some Caucasian populations, the N291S is the most common mutation (Pimstone et al., 1996; Wittekoek et al., 1998), but in a French–Canadian population, the G188E, P207L and D250N variants are most abundant (Normand et al., 1992; Minnich et al., 1995). Furthermore, in other Caucasian groups, the G188E is widely present, whereas P207L and D250N are rarely found (Hayden et al., 1993). The frequency of a −93T>G promoter variant was found in 76.4% of South-African Blacks, but in only 1.7% of Caucasians (Ehrenborg et al., 1997).

In 71 unrelated individuals of European, African and Finnish descent, a contiguous portion (9.7 kb) of the human *LPL* gene was resequenced—exons 4 through 9, including five introns and portions of introns 3 and 9. Eighty-eight variable sites (79 single-nucleotide polymorphisms; 9 insertions and deletions) were found; 81 of these were in the introns, 7 in exons, and haplotype patterns of these single-nucleotide polymorphisms were described (Clark et al., 1998; Nickerson et al., 1998). This information was then used for identifying recombinational and mutational hotspots within this portion of the *LPL* gene (Templeton et al., 2000).

No data in a Chinese population have yet been reported. It seems likely that diminished LPL activity might lead to decreased HDL, increased triglycerides and, ultimately, premature atherosclerosis (Henderson et al., 1999). Only a few clinical studies have shown this connection (Thorn et al., 1990; Peacock et al., 1992), however, and these findings have sometimes been contradictory.

The aim of the present study was to screen for DNA sequence variants in the *LPL* gene in Chinese population—and attempt to determine genotype–phenotype associations. We specifically tested whether any of these mutations could be correlated with the LHDL/HTG phenotype and, hence, with the pathogenesis of coronary heart disease. To this end, we looked for single-nucleotide polymorphisms in the *LPL* gene from 160 patients with coronary heart disease and 150 controls, using a combination of polymerase chain reaction (PCR), denaturing high-performance liquid chromatography (DHPLC), and DNA sequencing. We then searched for possible gender-specific associations of individual single-nucleotide polymorphisms with the biochemical and clinical manifestations of coronary heart disease.

2. Materials and methods

2.1. Subjects

One-hundred sixty unrelated patients with coronary heart disease, all from the West China Hospital (Sichuan University), were selected by coronary angiography using the Judkins technique. Individuals having any major-coronary-artery branch (left anterior descending, left circumflex artery, right coronary artery) with at least one stenosis of >60% qualified as a "coronary heart disease patient" for the study. In addition, 150 unrelated age- and gender-matched subjects, selected via health-screening at the same hospital and free of any clinical or biochemical signs of coronary

heart disease, were used as controls for the study. All 310 subjects were from the same gene pool, and little admixture is present in this population. None of the 160 patients enrolled in this study was taking hypolipidemic drugs prior to coronary angiography or measurements of their lipid profiles. This study was approved by the Internal Review Board of the West China Hospital, Sichuan University, and signed informed-consent forms were obtained from all 310 subjects.

2.2. Measurement of lipids, lipoproteins and apolipoproteins

After an overnight fast, baseline blood samples were collected from all coronary heart disease patients and controls. Plasma was separated from the blood cells by centrifugation at 500 × *g* for 10 min at room temperature, and 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. After precipitation of apoB-containing lipoproteins with a 4% sodium phosphotungstate solution, HDL-cholesterol was measured in the supernatant fraction following centrifugation. LDL-cholesterol was calculated using the Friedewald Formula (Friedewald et al., 1972). Levels of the apolipoproteins apoA1 and apoB were determined by the immunonephelometric assay (Behring Nephelometer, Behringwerke, Germany).

2.3. DNA preparation and PCR amplification

Genomic DNA was isolated from peripheral blood leukocytes, using the "salting-out" procedure (Miller et al., 1988), and then stored at 4 °C. Fragments containing the 5′ flanking region or individual exon of the *LPL* gene-including all intron–exon boundaries (except the intron 9 splice-acceptor site) were amplified by PCR. Our design of all oligonucleotide primers (Table 1) for PCR was based on the cDNA sequence (Wion et al., 1987), GenBank accession No. AF050163, and NT_008081 information. The central regions of most introns were not sequenced. A total of 5155 bp was sequenced and included: 565 bp of 5′ flanking region; the 276-bp exon 1; 36 bp of the 5′ end and 85 bp of the 3′ end of intron 1; the 165-bp exon 2; 41 bp of the 5′ end and 65 bp of the 3′ end of intron 2; the 180-bp exon 3; 54 bp of the 5′ end and 75 bp of the 3′ end of intron 3; the 102-bp exon 4; 53 bp of the 5′ end and 394 bp of the 3′ end of intron 4; the 234-bp exon 5; 132 bp of the 5′ end and 67 bp of the 3′ end of intron 5; the 243-bp exon 6; 127 bp of the 5′ end and 65 bp of the 3′ end of intron 6; 642 bp in the middle of intron 6; the 121-bp exon 7; 179 bp of the 5′ end and 93 bp of the 3′ end of intron 7; the 183-bp exon 8; 354 bp of the 5′ end and 111 bp of the 3′ end of intron 8; the 105-bp exon 9; and 161 bp of the 5′ end and 247 bp of the 3′ end of intron 9. We chose not to sequence exon 10 (1940 bp), because the translation stop codon is located near the end of exon 9.

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 unit of Taq polymerase was added. The 30 cycles of PCR amplification were performed with a temperature profile consisting of denaturation for 45 s at 94 °C, annealing for 30 s at temperatures between 55 and 61 °C, and extension for 30 s at 72 °C. The reactions were carried out in a Perkin-Elmer GeneAmp 9600 PCR System (Perkin-Elmer, Norwalk, CT).

2.4. Denaturing high-performance liquid chromatography (DHPLC)

DHPLC screening for single-nucleotide polymorphisms was performed on an automated HPLC instrument (Hewlett Packard, Palo Alto, CA). 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 × 4.6-mm Id dsDNA-analysis column (Hewlett Packard). The mobile phase was 0.1 mol/l triethylammonium acetate (PE BioSystems, Foster City, CA) buffer at pH 7.0 containing ethylenediaminetetraacetic acid (0.1 mmol/l).

PCR products were eluted with a linear acetonitrile gradient of 1.8% per min at a flow-rate of 0.8 ml/min,

Table 1
Primers for PCR amplification of the *LPL* gene and length of the fragments amplified

Region amplified	5′ Primer sequence (5′-3′)	3′ Primer sequence (5′-3′)	Length of fragment (bp)
5′ Flank	GCTGATCCATCTTGCCAATGT	GCAGCTTTCCCTTGAGGAGGAG	565
Exon 1	TTCCAGTCACATAAGCAGCCTTG	AACTTCCTTCTTCTCATCCTCAG	396
Exon 2	CAACCCTCCAGTTAACCTCA	AACAATTGGGCAGGCCAGTC	271
Exon 3	TCTATGACAAGTCCTAGGTGGG	GAAAGAACAGCCGGTTTTCTGG	309
Exon 4	TTGGCAGAACTGTAAGCACCT	TGTGTATGCCACACCTTTGCC	549
Exon 5	ACCATGACTGTAGCCTAGGAG	TAGGACATTGGGTCAATAAGG	433
Exon 6	TGCACAGGACTATATCCTTGG	TGATGCAGTGAGCATGATGAA	435
Intron 6	TTGCCAGGAGTTTGTTTCACAC	GTTGTGAACTTCTGATAACAAT	641
Exon 7	TCTAGGTATGAACACTGTGCA	TCTAGGCATCGCTCTCTGCTT	393
Exon 8	AGAGAATCGTATGTGTGCTAT	ACAAGGTTTCCTAAAGCTCTC	649
Exon 9	ATTCTGATGTGGCCTGAGTGT	AATGTGTGCCTGGGTTGAAGG	514

and the start- and end-points of the gradient were adjusted according to the size of the PCR products. Generally, it took 8 min per analysis, including column regeneration and re-equilibration to the starting condition. 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 each amplificon was determined empirically by running it at different temperatures until a good resolution between homo- and heteroduplexes was obtained. The DHPLC temperatures for the 11 amplificons of the *LPL* gene listed in Table 1 were 63, 57, 59, 58, 62, 59, 61, 58, 57, 63 and 60 °C, respectively. The heteroduplex molecules are generally eluted ahead of the homoduplex molecules; therefore, the appearance of additional peaks or shoulders during DHPLC was interpreted as indicative of a single-base mismatch in heteroduplex DNA fragments and, thus, reason for need of sequencing.

2.5. DNA sequencing

The location and chemical nature of the mismatch were confirmed by sequencing of the re-amplified product. Heterozygous and homozygous samples were cloned into the T-Easy vector (Promage), then sequenced in both directions on the "ALFexpress DNA" automated sequencer, using the dye-terminator cycle Thermal Sequenase sequencing kit (USB).

2.6. Statistical analysis

The phenotypic data of the dyslipidemic vs. other coronary heart disease patients were adjusted for age and gender, and were statistically analyzed using Student's *t*-test. Chi-square tests were performed to compare overall allelic and genotype frequency of each SNP site in male and female patients separately and between coronary heart disease subjects and controls as a whole. The adjusted odds−ratios (ORs) for the coronary heart disease trait and for the LHDL/HTG phenotype were calculated using log regression analysis.

2.7. Haplotype analysis

Multiple-marker haplotype association analysis, based on haplotype frequencies from the 16 polymorphic markers, was carried out with a utility program that provides the required estimate of haplotypes (EH) format (Terwilliger and Ott, 1994). The EH program uses a model-free, nonparametric test for homogeneity in allele/haplotype frequencies between cases and controls and a permutation test for allele/haplotype association (Zhao et al., 1999, 2000); this is suitable for complex diseases with an unknown genetic contribution and/or with a large number of possible haplotypic combinations relative to the sample size. In theory, haplotype analysis of the single-nucleotide polymorphisms found in the present study could be estimated by the inference method of Clark (1990), except that the computer capacity and the time required for all 16 single-nucleotide polymorphisms in 620 chromosomes would be very large. Furthermore, at least one subject homozygous for a variant SNP is required for this method, and we found none in our population of 310 individuals.

Unphased haplotype analysis of all 16 single-nucleotide polymorphisms from the male subjects and from the female subjects was therefore estimated separately by the PM-Plus program (Zhao et al., 1999). PM-Plus (permutation and model-free analysis) uses different disease specifications and outputs five possible statistically different results: chi-square statistics for a possible user-specified model, recessive model, dominant model, model-free analysis, and heterogeneity model. For haplotype association analysis, we therefore used the model-free test, the nonparametric test for homogeneity in allele/haplotype frequencies between gender-specific cases and controls, and the permutation test for inferring allele/haplotype associations. This is the best approach for analysis of a complex disease with an unknown genetic model and/or with a large

Table 2
Demographics and lipid profiles of coronary heart disease patients and controls (means ± S.D.)[a]

Trait	Patients		Control		*P*	
	Male ($n=89$)	Female ($n=71$)	Male ($n=82$)	Female ($n=68$)	M vs. M	F vs. F
Age	52.3 ± 10.8	47.6 ± 8.72	50.3 ± 9.23	49.3 ± 8.41	NS[b]	NS[b]
BMI	24.17 ± 1.31	23.79 ± 0.97	23.92 ± 1.43	24.02 ± 0.83	NS	NS
Plasma cholesterol	5.30 ± 0.27	5.57 ± 0.24	4.88 ± 0.65	4.62 ± 0.24	<0.01	<0.01
Plasma triglycerides	1.73 ± 1.19	1.42 ± 0.74	1.21 ± 1.14	1.18 ± 0.59	<0.01	<0.01
LDL-cholesterol	3.57 ± 1.10	3.13 ± 1.01	2.75 ± 0.52	2.41 ± 0.35	<0.01	<0.01
HDL-cholesterol	0.94 ± 0.83	1.79 ± 0.92	1.58 ± 0.66	1.67 ± 0.32	<0.01	NS
ApoA-1	1.14 ± 0.35	1.05 ± 0.58	1.38 ± 0.22	1.32 ± 0.61	NS	NS
ApoB	1.11 ± 0.24	1.11 ± 0.18	1.12 ± 0.12	0.97 ± 0.19	NS	NS

[a] The mean values of lipid levels are given for 160 patients and 150 controls. Statistical analysis was performed using Student *t*-test. All levels are given in mmol/l except body mass index (BMI) which is given in kg/m^2 and apolipoprotein levels which are given in g/l.
[b] NS = no significant difference ($P>0.05$).

Table 3
Serum lipid and apolipoprotein levels (means ± S.D.) in coronary heart disease patients with or without the LHDL/HTG phenotype[a]

Trait	Patients with LHDL/HTG ($n=72$)		Patients without LHDL/HTG ($n=88$)		P	
	Male ($n=43$)	Female ($n=29$)	Male ($n=46$)	Female ($n=42$)	M vs. M	F vs. F
Age (year)	53.5 ± 7.9	52.1 ± 8.4	51.7 ± 8.3	48.8 ± 7.6	NS[b]	NS[b]
BMI (kg/m^2)	23.96 ± 1.26	23.64 ± 052	24.15 ± 1.38	23.94 ± 0.86	NS[b]	NS[b]
TC (mmol/l)	4.97 ± 0.62	5.09 ± 0.89	5.13 ± 0.77	5.17 ± 0.46	<0.01	<0.01
TG (mmol/l)	3.14 ± 1.13	3.21 ± 1.65	1.24 ± 1.07	1.36 ± 0.97	<0.01	<0.01
HDL-C (mmol/l)	0.86 ± 0.23	0.94 ± 0.18	1.27 ± 0.54	1.42 ± 0.37	<0.01	<0.01
LDL-C (mmol/l)	2.68 ± 0.15	2.69 ± 0.04	2.87 ± 0.25	2.73 ± 0.35	NS[b]	NS[b]

[a] The LHDL/HTG phenotype, characterized by an LDL-cholesterol/HDL-cholesterol ratio of >5, triglycerides >200 mg/dl, and HDL-cholesterol <35 mg/ml, is the commonly accepted clinical criteria for this trait. BMI, body mass index; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. Statistical analysis was performed using Student's t-test.

[b] NS = no significant difference ($P>0.05$).

number of possible haplotype combinations relative to the sample size.

3. Results

3.1. Phenotype of patients

Table 2 lists the demographics and lipid profiles (phenotypes) of the 310 subjects studied. Values for the plasma cholesterol, plasma triglycerides, and LDL-cholesterol were considerably higher in both male and female coronary heart disease patients than controls. HDL-cholesterol was considerably lower in male but not in female coronary heart disease patients, compared with that in controls. Since LPL activity is believed to have an effect on these plasma lipid levels, it seemed reasonable to look for single-nucleotide polymorphisms in the *LPL* gene in this population.

Abnormal postprandial lipid metabolism, determined best after an overnight fast, is generally thought to be diagnostic of the LHDL/HTG phenotype. Of the 160 coronary heart disease patients investigated (Table 3), we determined that 72 subjects fulfilled the commonly accepted clinical criteria for the LHDL/HTG phenotype (Moennig et al., 2000): triglycerides >2.25 mmol/l (200 mg/dl), HDL-cholesterol <0.902 mmol/l (35 mg/ml), and LDL-cholesterol-to-HDL-cholesterol ratio ≥5. Adjusting for the body mass index, we found that the data for each of these groups remained unaltered.

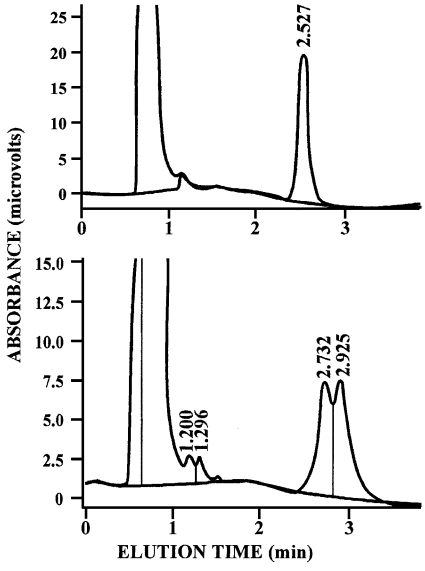

Fig. 1. DHPLC analysis of amplified fragments of the *LPL* gene. A single peak (top) represents a homoduplex molecule detected in a homozygous subject. A double peak (bottom) represents a heteroduplex molecule found in a heterozygous subject. The abscissa denotes time (in minutes). The ordinant measures absorbancy (in microvolts). The first large deflection in both chromatographic runs is due to residual dNTPs and primers from the PCR reaction.

3.2. Genotype of the 620 chromosomes

First, we screened for single-nucleotide polymorphisms with DHPLC (Fig. 1) in all of the first nine exons, all splice-donor and splice-acceptor sites and some intronic bases, and 565 bp of the 5′ flanking region (total of 5155 bp) of the *LPL* gene in the 160 coronary heart disease patients and 150 controls. Second, we then confirmed each candidate single-nucleotide polymorphisms by sequence analysis. In total,

Table 4
Characteristics of detected single-nucleotide polymorphisms in the *LPL* gene in patients with coronary heart disease and controls

Region	Sequence	Position
5′ Flank	gtgcgg[G/A]gtgagt	−421
Intron 2	tgtcat[C/A]atcttc	+13,557
Exon 4	ataaga[A/G]agtcaa	+15,222
Exon 5	ctggtc[G/A]aagcat	+16,052
Exon 5	gaagct[A/G]tccgcg	+16,141
Intron 5	atgtga[C/G]tcttat	+16,173
Intron 5	gactct[T/C]atcctt	+16,177
Intron 6	cactgc[A/G]tcacat	+19,208
Intron 6	ttctgt[C/A]cattgg	+19,228
Intron 6	ggtgat[T/C]cttaga	+20,444
Intron 6	ccagtg[G/A]ttccat	+22,190
Intron 7	atccca[T/C]tcactc	+22,432
Exon 8	gagtaa[A/G]agcagg	+24,123
Intron 8	cttcac[T/C]ttagac	+24,173
Intron 8	ggaata[T/C]caaaac	+25,055
Intron 9	tgccct[G/T]actcat	+25,380

we found 16 single-nucleotide polymorphisms in the portions of the gene that were screened (Table 4). Three of them (+16,173C>G and +16,177T>C in intron 5; +19,228C>A in intron 6) have been reported before; the other 13 have not. We found four coding single-nucleotide polymorphisms, and all four represent nonsynonymous mutations (K148R, R192Q, I222V and K407R).

3.3. Comparison of the single-nucleotide polymorphisms in coronary heart disease patients and controls

Gender is likely to have a significant effect on coronary heart disease history, so the association of single-nucleotide polymorphisms in the *LPL* gene with coronary heart disease was analyzed separately in males and in females. The genotype and allelic distribution of all 16 single-nucleotide polymorphisms between the coronary heart disease patients and controls are presented in Table 5. In males, we found significant differences in five single-nucleotide polymorphisms: −421G>A (5′ flanking region); +13,577C>A (intron 2); +16,052G>A, R192Q (exon 5); +16,173C>G and +16,177T>C (intron 5), whereas only the mutations −421G>A and +16,052G>A (R192Q) were significantly different in females. In the combined 310 total, we found all 16 single-nucleotide polymorphisms to be either homozygous for the consensus (“wild-type”) base or heterozygous for this consensus plus mutated base; no homozygotes for the variant bases were seen (Table 5). Although the allelic frequencies of all these single-nucleotide polymorphisms qualify as polymorphic variants ($q \geq 0.01$) rather than rare variants ($q < 0.01$), the likelihood of finding a homozygote for the polymorphic variant would be very low in a population of 310 subjects (e.g. the +16,173C>G mutation is the most frequent at 0.109, meaning the likelihood of finding a +16,173G/G homozygote would be $(0.109)2 = 0.0119$ or ~1 in 100 patients; not finding one homozygote in 310 patients has a P value by chi-square analysis of >0.05). These findings are not that different from the study of 401 patients by Moennig et al. (2000), who found only four homozygotes for the variant bases in the LPL gene. It is also conceivable that one or another of the variant bases (Table 5), when homozygous, might be lethal (in utero or, later in life, prematurely lethal). We also studied the Hardy–Weinberg distribution for all 16 single-nucleotide polymorphisms (data not shown) and determined that all were in accordance with Hardy–Weinberg expectations in both the coronary heart disease patients and controls.

3.4. Haplotype analysis of the single-nucleotide polymorphisms in the LPL gene

The +16,173C>G and +16,177T>C mutations, since they are only four bases apart, can obviously be sequenced simultaneously, and haplotype analysis thus easily done. Surprisingly, Table 6 shows that these two single-nucleotide polymorphisms sites were usually, but not always, in linkage disequilibrium. By showing the data of these two single-nucleotide polymorphisms separately for the coronary heart disease patients and controls, there are theoretically ($3^2 = 9$) nine possible genotype combinations; however, only four combinations were found, and the +16,173C/16,177T fol-

Table 5
Distribution of single-nucleotide polymorphisms (SNPs) frequencies in the *LPL* gene of patients with coronary heart disease and controls

Single-nucleotide polymorphisms		Patients ($n = 160$)				Controls ($n = 150$)				P values					
Region	SNP	Male		Female		Male		Female		Genotype-wise			Allele-wise		
		Wt	Het	Wt	Het	Wt	Het	Wt	Het	M vs. M	F vs. F	P vs. C	M vs. M	F vs. F	P vs. C
Promoter	−421g>a	76	13	62	9	78	4	66	2	0.034	0.034	0.003	0.039	0.037	0.003
Intron 2	13,577c>a	70	19	60	11	75	7	64	4	0.02	0.068	0.003	0.026	0.076	0.004
Exon 4	K148R	73	16	58	13	67	15	59	9	0.957	0.413	0.619	0.96	0.433	0.636
Exon 5	R192Q	74	15	59	12	79	3	67	1	0.005	0.002	0.000	0.006	0.002	0.000
Exon 5	I222V	81	8	61	10	78	4	63	5	0.293	0.201	0.101	0.302	0.214	0.109
Intron 5	16,173c>g	70	19	55	16	74	8	56	12	0.038	0.473	0.049	0.047	0.498	0.062
Intron 5	16,177t>c	76	13	54	17	78	4	57	11	0.034	0.254	0.029	0.039	0.282	0.036
Intron 6	19,208a>g	73	16	59	12	73	9	61	7	0.195	0.257	0.085	0.214	0.275	0.098
Intron 6	19,228c>a	83	6	62	9	77	5	60	8	0.864	0.87	0.828	0.866	0.874	0.832
Intron 6	20,444t>c	74	15	57	14	71	11	59	9	0.531	0.304	0.248	0.549	0.327	0.269
Intron 6	22,190t>c	79	10	59	12	73	9	56	12	0.957	0.907	0.949	0.958	0.912	0.951
Intron 7	22,432t>c	76	13	62	9	72	10	59	9	0.644	0.922	0.778	0.656	0.925	0.786
Exon 8	K407R	76	13	62	9	73	9	63	5	0.479	0.297	0.225	0.494	0.297	0.240
Intro 8	24,173t>c	84	5	64	7	76	6	59	9	0.651	0.533	0.435	0.656	0.546	0.446
Intron 8	25,055a>c	83	6	64	7	77	5	60	8	0.864	0.717	0.863	0.866	0.725	0.866
Intron 9	25,380g>t	78	11	61	10	73	9	58	10	0.778	0.917	0.904	0.785	0.920	0.908

In the first column of each group, the numbers of consensus (wild-type) (wt) individuals and heterozygous (Het) are given. No homozygous mutant variants were found in this study. In the last two columns, the P values of the genotype and allelic distributions are shown for the male patients vs. male controls (M vs. M), female patients vs. female controls (F vs. F), and the heart patients vs. controls with genders combined (P vs. C). Single-nucleotide polymorphisms with an amino-acid change are shown with the residue number for the mature LPL protein. For noncoding single-nucleotide polymorphisms, the number of the base is given, relative to +1 from the first base of the transcription start-site.

Table 6
Frequency of genotype combinations between +16,173C>G and +16,177T>C in patients with coronary heart disease and controls

Genotype combinations		Patients ($n=160$)		Controls ($n=150$)	
+16,173	+16,177	Male	Female	Male	Female
CC	TT	66	53	74	54
CC	TC	4	2	1	1
CG	TT	10	1	5	2
CG	TC	9	15	2	11

lowed by the +16,173G/16,177C are the two most common haplotypes. Intriguingly, the frequency of the +16,173G/16,177C haplotype was significantly higher in the male coronary heart disease patients than in the male controls ($x^2=4.90$; $P=0.027$), with an OR of 5.04[1.21–20.92].

Performing unphased haplotype analysis of all 16 single-nucleotide polymorphisms separately by the PM-Plus program, we found 69, 51, 57 and 41 haplotype patterns for the male coronary heart disease, male control, female coronary heart disease and female control groups, respectively (data not shown). Chi-square analysis was not significant ($P>0.05$) for the recessive model, dominant model, or model-free analysis. We did not include any user-specified model. Chi-square analysis for the heterogeneity model ($x^2=86.68$), on the other hand, was by far the best fit; the empirical P value for the heterogeneity model in males was <0.0001, and in females >0.01. These P values comprise the proportions of random permutations that would produce the same or higher chi-square statistics than those obtained from the real data.

3.5. Comparison of single-nucleotide polymorphisms in coronary heart disease patients with or without dyslipidemia

As described above, 72 of the 160 coronary heart disease patients fulfilled the criteria for the LHDL/HTG phenotype (Table 3). Between these two groups, we therefore looked for significantly different distribution frequencies of the five single-nucleotide polymorphisms in males and the two single-nucleotide polymorphisms in females, which had previously been found to differ significantly (Table 5). In male subjects (Table 7), a significant association was found between the LHDL/HTG phenotype and the non-LDHL/HTG trait for the −412G>A in the 5′ flanking region, the +13,577C>A in intron 2, and the +16,052G>A (causing an R192Q mutation) in exon 5 ($x^2=4.57$, 5.45 and 4.08, respectively; $P<0.05$ for all three), with ORs of 3.90[1.12–13.66], 3.38[1.22–9.40], and 3.22[1.04–10.01], respectively. No significant associations of the LHDL/HTG vs. non-LHDL/HTG phenotype for any of these single-nucleotide polymorphisms were uncovered in females. Comparing all coronary heart disease patients with all controls, we conclude that the intron 2 and exon 5 mutations are significant.

4. Discussion

Polymorphisms in the *LPL* gene have been associated with clinically abnormal lipid profiles and increased risk of coronary heart disease and atherosclerosis (Bruzell, 1995; Murthy et al., 1996) These data are similar to that for several other genes involved in human lipid metabolism. The result of our population study of the *LPL* gene in 310 Chinese, and the possible association of certain single-nucleotide polymorphisms with coronary heart disease and the LHDL/HTG phenotype provide further evidence for the importance of this gene and its role in coronary heart disease and dyslipidemia.

We were successful in assessing such a huge number of DNA bases in this large population size in a reasonably short period of time because of DHPLC, a highly sensitive and automated method that detects DNA variations with high sensitivity and efficiency (Underhill et al., 1997). By using DHPLC first, and then following this with DNA resequencing of the appropriate fragments, we identified 16 single-nucleotide polymorphisms in the *LPL* gene in the present study. Searching the NCBI database for single-nucleotide polymorphisms, we discovered that 13 of these 16 single-nucleotide polymorphisms had not previously been reported, including all four nonsynonymous coding single-nucleotide polymorphisms.

We found transitions to be more prevalent (11 of 16, 69%) than transversions (5 of 16, 31%) of the variant sites,

Table 7
Association of single-nucleotide polymorphisms (SNPs) of the *LPL* gene with dyslipidemia in the 160 patients with coronary heart disease

Single-nucleotide polymorphisms		With low-HDL high-triglyceride phenotype				Without low-HDL high-triglyceride phenotype				*P* values					
										Genotype-wise			Allele-wise		
Region	SNP	Male		Female		Male		Female		M vs. M	F vs. F	P vs. C	M vs. M	F vs. F	P vs. C
		Wt	Het	Wt	Het	Wt	Het	Wt	Het						
Promoter	−421g>a	33	10	28	1	43	3	34	8	0.026	0.052	0.612	0.032	0.061	0.625
Intron 2	13,577c>a	29	14	24	5	41	5	36	6	0.013	0.735	0.025	0.019	0.746	0.034
Exon 5	R192Q	32	11	21	8	42	4	38	4	0.033	0.046	0.004	0.043	0.057	0.006
Intron 5	16,173c>g	34	9	25	4	36	10	30	12	0.926	0.143	0.290	0.930	0.171	0.322
Intron 5	16,177t>c	35	8	23	6	41	5	31	11	0.302	0.593	0.839	0.322	0.620	0.847

Abbreviations and the format of this table are identical to that in previous tables. Significant P values are underlined.

which is consistent with other studies of human sequence variation (Wang et al., 1998). We found three new nonsynonymous coding single-nucleotide polymorphisms. It should be emphasized that single-nucleotide polymorphisms other than nonsynonymous coding single-nucleotide polymorphisms (i.e. those located inside, or 5′- or 3′-ward of the translated region) might affect activity of the gene product and tissue- and cell type-specific gene expression and therefore be important.

Because the association between certain single-nucleotide polymorphisms and patients with coronary heart disease differed between males and females, this implies a possible modulation by hormonal or other gender-specific factors (Schwertz and Penckofer, 2001). Our comparison of single-nucleotide polymorphisms in the *LPL* gene between coronary heart disease and control subjects suggests that in males, the mutations −421G>A in the 5′ flanking region, +13,577C>A in intron 2, +16,052G>A (R192Q) in exon 5, and +16,173C>G and 16,177T>C in intron 5 are in some manner associated with the complex coronary heart disease, whereas only the mutations −421G>A and the +16,052G>A (R192Q) showed this association in females. This finding indicates that the +13,577C>A, +16,173C>G and 16,177T>C mutations might have a context-dependent effect, i.e. they are much more likely to exert an effect on LPL activity under specific conditions in males.

Most of the actions in the 5′ flank of the *LPL* gene appear to be located in the first ~500 bp upstream of the transcription start-site (Previato et al., 1991), which is why we included sequence analysis in this region. When 730 bases upstream of the *LPL* transcriptional start-site were screened, several single-nucleotide polymorphisms were identified within known DNA motifs having putative functions: the TATA box at nucleotide −27, two CCAAT motifs at −65 and −506, homologous binding sites for C/EBP at −68 and −509, a glucocorticoid-responsive element at −644, a cyclic AMP-responsive element at −306, and three octanucleotide motifs at −580, −186, and −46 bp. In stably transfected preadipocyte cells, *LPL* promoter constructs extending at least 468 bp 5′-ward of the transcriptional start-site were shown to be necessary—if induction of a reporter gene was to accompany adipocyte differentiation; however, maximal effects required at least 1402 bp of the *LPL* promoter (Enerback et al., 1992). In the present study, one of the single-nucleotide polymorphisms (−421G>A)—associated with increased risk of coronary heart disease and dyslipidemia—is located within the interaction region (from −591 to −288 bp) between DNA that binds members of the nuclear factor-1-like (NF1-like) transcriptional regulatory family and other *cis*-acting elements of the *LPL* gene (Schoonjans et al., 1993). Therefore, the −421G>A mutation in the present study may affect expression of the *LPL* gene via regulation of the interaction of these *cis*- and *trans*-acting elements.

Both lowered HDL-cholesterol and elevated plasma triglycerides are likely to contribute to coronary heart disease risk (Bainton et al., 1992). In the present study, the significant association between the LHDL/HTG phenotype and the +13,577C>A polymorphism in intron 2 may not play a direct role in LPL function. Rather, this single-nucleotide polymorphism might be in linkage disequilibrium with other single-nucleotide polymorphic sites that alter the systemic (paracrine) and/or local (autocrine) cell type-specific expression of the *LPL* gene in response to diet and other environmental factors. Alternatively, this intron 2 single-nucleotide polymorphisms could be in linkage disequilibrium with some mutations that directly affect LPL activity. Similar concepts have been postulated for the C>T transition in intron 6 (Humphries et al., 1998) and the T>G transversion in intron 8 (Jemaa et al., 1995). It should also be noted that some of the intronic single-nucleotide polymorphisms reported in the present study are in the immediate vicinity of coding regions, and thus might play a role in regulation of gene expression and splicing.

The +16,052G>A (R192Q) mutation in exon 5 was significantly associated with the LHDL/HTG phenotype (Table 7). Is this substitution of glutamine for arginine at position 192 likely to be critical for enzyme function? Paulweber et al. (1991) investigated the probable conformation in the vicinity of residue 188 of the mature LPL protein. In the consensus (“wild-type”) enzyme, the most likely backbone conformation is a tight beta-turn formed by residues 189–192 (S–P–G–R), which is flanked by accompanying structures of functional importance. Introduction of the (larger) glutamine residue (C=O instead of arginine’s C=N) may result in unfavorable interactions of the negative charge with neighboring groups. Hence, the R192Q mutation may destabilize the conformation of the beta-turn. Evolutionary analysis of lipase genes in various species shows that the residue 188–192 region is highly conserved (reviewed in Wang et al., 1992). In particular, the amino-acid sequence surrounding Arg-192 is highly homologous in several mammals whose LPL sequences are known, as arginine is the only residue utilized at the analogous position in these species (Wang et al., 1992). We therefore conclude that the R192Q substitution is very likely to affect LPL enzymic function.

In summary, our results suggest that genetic variation at the *LPL* locus is involved in the determination of lipid levels and lipoprotein profiles and predisposition to the complex coronary heart disease. Whether any of the 13 new single-nucleotide polymorphisms found in this Asian population will be found in Caucasian, African or other ethnic populations remains to be determined. Further studies are needed to elucidate the biological mechanisms of the effects that these variants might cause.

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