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Lipoprotein lipase gene is linked and associated with hypertension in Taiwan young-onset hypertension genetic study

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Summary

Hypertriglyceridemia has been extensively associated with hypertension. However, the mechanism behind it is poorly understood. A positive linkage signal between Lipoprotein lipase (LPL) and young-onset hypertension has been identified by us as the strongest among 18 candidate genes. Here we report our fine mapping works with seven microsatellite markers flanking *LPL*, sequencing results for its promoter and exons, and an extended association study with the identified single nucleotide polymorphisms(SNP). First, using data from 213 individuals in 59 nuclear families of young-onset hypertension, multipoint analysis revealed a NPL score of 3.02 for the LPL (GZ-14/GZ-15) marker in intron 6. LPL marker ($p < 10^{-12}$) and the haplotypes containing its allele 1 ($p < 0.0001$) were also significantly associated with young hypertension by transmission disequilibrium test. In-depth sequencing revealed no mutation in promoter and exon regions, except two cSNP: 7754C → A (C/A: 0.91/0.09), a silent mutation in exon 8 and S447X (C/G: 0.92/0.08), a stop codon mutation in exon 9. Other 11 cSNPs documented in NCBI GenBank are absent in our sample. Constructed from the above 2 cSNPs, haplotype AC showed a moderate TDT association with elevated triglyceride ($p = 0.02$) and with hypertension and elevated triglyceride combined ($p = 0.06$). Again, in an extended case-control study, a significant association was found between S447X and patients with persistent hypertension and elevated triglyceride ($p = 0.02$). We conclude that *LPL* variants may play a causal role in the development of hypertension in Taiwan Han Chinese. The moderate association with SNP haplotype suggests that other regulatory *LPL* variant may exist.

Introduction

Hypertension is a major risk factor for cardiovascular diseases and a serious global public health problem. Approximately 1 billion people in the world suffer from this condition [1]. However, efforts to unravel the common pathological genes of this complex trait in human have not been productive [1], probably due to a complex interplay between genetic and environmental factors.

Lipoprotein lipase (LPL) is a key enzyme involved in the metabolism of triglyceride-rich particles. The level of triglyceride (TG) has been extensively associated with either blood pressure or hypertension [2–3]. However, the mechanism

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behind this connection is not well understood. If a genetic connection between this major triglyceride metabolism gene and hypertension can be established, the importance of hypertriglyceridemia in the development of hypertension would be underscored.

In recent years, efforts have been made to study the relations between hypertension and *LPL* variants. The known variants of *LPL*, for example, D9N, N291S, and S447X, etc. have been extensively investigated in association with hypertension, hypertriglyceridemia, and other lipid profiles [4–9]. Previous studies on the *LPL* Ser447X on exon 9 have demonstrated its effect not only on reducing plasma triglyceride level, increasing HDL-C, but also on decreasing blood pressure and protecting against coronary artery disease [10]. Using linkage approach, one Taiwanese study discovered that *LPL* was linked to systolic blood pressure (SBP) as a quantitative trait in diabetic families [11]. Another study in mainland Chinese also found linkage and association between *LPL* and BP, using several different types of markers [12–15]. But, linkage studies in non-Chinese populations are mostly negative [16].

Association or linkage study connecting one candidate gene and one phenotype cannot demonstrate the relative importance of a candidate gene in an ethnic population. Our preliminary report on multiple candidate genes [17], using the affected sibling-pair method, has demonstrated a positive linkage signal between a marker flanking *LPL* (D8S1145, $p=0.0284$) and young-onset hypertension. Here we reported the follow-up fine mapping processes, using seven microsatellite markers around or in the *LPL* gene, in a two-stage linkage approach. We found that the degree of this linkage and association with *LPL* is the strongest among 18 hypertension candidate genes including angiotensin converting enzyme we previously reported elsewhere [18]. Furthermore, we sequenced the promoter and coding regions of *LPL* in order to find new and common SNPs (Single Nucleotide Polymorphisms) which may explain the blood pressure variaton. Finally, an extended community-based association study was carried out to compare the genotype of the identified SNP between normal subjects and patients with persistent hypertension and elevated triglyceride.

Materials and methods

Family-based study

Patients and families for linkage and association analysis

Hypertension was defined by JNC VI criteria [19]. Recruited were non-obese (Body Mass Index <30 Kg/M^2) and non-diabetic essential hypertensive patients aged 40 or younger, and their siblings and parents via a hypertension clinic at Taipei Veterans General Hospital, numbering 213 individuals from 59 nuclear families which contribute 81 young-onset hypertension patients [17]. Among them, there were 25 pairs of affected (young-onset hypertension) siblings from 18 families and 42 complete trios (two parents and an affected offspring) and 39 partial trios (a single parent and an affected offspring). The protocol of this study was approved by the Human Investigation Committee of the Institute of Biomedical Sciences, Academia Sinica. The informed consents were obtained from all participants.

Genotyping microsatellite markers

Genomic DNA was isolated from peripheral lymphocytes using the phenol/chloroform extraction method. Selected from the Marshfield genetic map were seven polymorphic microsatellite markers flanking *LPL* on *8p22*: D8S1731 (31.73 cM), D8S261 (37.04 cM), D8S1145 (37.04 cM), GZ-14/GZ-15 (GDB: 177387 or LPL marker, 39.25 cM), D8S322 (41.55 cM), D8S1116 (42.85 cM), and D8S382 (51.15 cM). Among them, only the LPL marker (or named GZ-14/GZ-15) is inside *LPL* on its intron 6. The polymerase chain reaction (PCR) protocol for microsatellite markers was performed as previously reported [17]. Fragment analysis was performed using an ABI 377 DNA sequencer and analyzed by GeneScan version 3.0 and genotyper version 3.0 software. The allele calling was conducted independently by two readers and cross checked. Genotypes of all familial members were verified for Mendelian segregation.

Sequencing the promoter and coding regions of LPL

Since our fine mapping results from the microsatellite markers showed significant linkage and association with the LPL marker in intron 6; we sequenced 571 bp of the promoter region and exons 1–9 using DNA from 41 subjects who were

among young-onset hypertension families contributing most to the positive NPL score, in order to identify new and/or common cSNP. Information on primer sequence is provided in Table 3.

The polymerase chain reaction was performed on a thermocycler (AG-9600 thermal station; Biotronics Corp., USA) using 5 μl of 10× AmpliTaq PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphates (dNTPs), 0.4 μM of each primer, 80–100 ng genomic DNA, and 0.5 μl of AmpliTaq.gold (5 U/μl) (Perkin–Elmer Centus) in 50-μl reaction. The amplified products were purified with a spin cartridge (Life Technologies, Taiwan, ROC) and were sequenced using ABI 377 sequencer (Foster City, Calif.) and analyzed by Sequencher version 2.1.1.

In addition to the identified cSNPs via sequencing, we also genotyped another 11 known *LPL* cSNPs in NCBI GenBank, using the MassARRAY SNP genotyping system (SEQUENOM, Inc., San Diego, CA), for 267 subjects from 66 young-onset hypertension families, including 57 original young-onset hypertension families with sufficient DNA and additional nine families with elevated triglyceride combined with hypertension from the second phase of the study. The polymerase chain reaction was performed on a thermocycler (GeneAmp 9700, Perkin Elmer, Foster City, CA.) using 10 ng genomic DNA in 20 μl reaction volume. The PCR products were extended using extension primers designed using Spectro-Designer software (SEQUENOM, Inc.).

Statistical analysis

The multipoint linkage analysis and transmission disequilibrium tests (TDT) were performed to identify regions highly linked to young-onset hypertension. The former used "GENEHUNTER" program [20] to analyze microsatellite data from 25 pairs of affected sib-pair. Conventional TDT developed by Spielman et al. [21] was carried out, using data from 42 complete trios and 39 partial trios. In addition, sib TDT (S-TDT) [22] was also performed, using information from 25 hypertensive patients and 21 normotensive sibs in 17 families. Then a Z score was obtained by combining information from the conventional TDT and the S-TDT. A Bonferroni procedure was carried out to adjust for multiple comparisons. Haplotype TDT using the "TRANSMIT" program [23] was carried out either for microsatellite markers or for SNPs haplotype analysis.

An extended case-control study

In order to confirm the relationship of the *LPL* genotype and phenotype; 92 patients with persistent (diagnosed in three consecutive occasions over 6 years) hypertension and elevated triglyceride (fasting plasma triglyceride ≥150 mg/dl), and 92 age- and sex-matched normal controls were selected from a community-based study: the CardioVascular Disease risk Factors Two-township Study (CVDFACTS) [24]. CVDFACTS is a longitudinal study on CVD risk factor evolution and on incidence of cardiovascular diseases in two Taiwanese townships, Chu-Dung (a Hakka community) and Pu-Tzu (a Fukienese community). The potential cSNP (S447X) was genotyped for the study subjects. The statistical testing between patients and controls for the phenotypic characteristics and for genotypic distribution was performed by Student's *t*-test and Fisher's exact test, respectively.

Results

Linkage and association between microsatellite markers and young-onset hypertension in the family study

Multipoint analysis showed linkage between young-onset hypertension and the LPL (GZ-14/GZ-15) marker located on intron 6 of the *LPL* gene, with a NPL score of 3.02 (Table 1). The NPL scores for other markers were much lower. In the NPL analysis, there were families showing positive (19 families; 32%) or no contributions (40 families; 68%) to the NPL score for LPL marker. Overall speaking, there was a positive finding. In addition, a very significant association was demonstrated by the Spielman's S-TDT for either LPL marker or the nearby D8S322. Allele 1 of LPL marker ($p = 1.5 \times 10^{-13}$) and allele 2 of D8S322 ($p = 3.8 \times 10^{-8}$) were significantly higher in their transmission to young-onset hypertension offspring compared to other alleles (Table 1).

TDT association of LPL haplotypes with young-onset hypertension in the family study

For haplotype TDT, the haplotypes created by two or three markers around *LPL* were included

Table 1. Results of Spielman's sib TDT and multipoint linkage analysis.

STRP marker	Position (cM)	N[a]	Allele[b]	TDT			S-TDT	Combined scores		Multipoint linkage analysis
				Trans[c]	Untrans[c]	χ^2	Z score	Combined Z score	*p*-value	NPL[d]
D8S1145	37.04	56	5	28	15	3.9	0.5	2.04	0.04	0.34
			3	8	17	3.2	0	1.33	0.18	
LPL	39.25	55	1	60	3	51.6	1.7	7.39	1.5×10^{-13}*	3.02
			3	1	38	35.1	2.0	6.23	4.6×10^{-10}*	
			2	4	24	14.3	1.0	3.05	0.002	
D8S322	41.55	49	2	38	4	27.5	1.8	5.50	3.8×10^{-8}*	1.38
			4	11	37	14.1	−0.2	3.49	0.0005	
D8S1116	42.85	47	1	4	9	1.9	1.0	1.68	0.09	1.19
D8S382	51.15	45	3	8	16	2.7	0.6	1.75	0.08	0.27

[a]N, the number of informative trios.
[b]D8S1145, Allele 3 = 281 bp, Allele 5 = 289 bp, LPL, Allele 1 = 123 bp, Allele 2 = 127 bp, Allele 3 = 131 bp; D8S322: Allele 2 = 218 bp, Allele 4 = 226 bp.
[c] Trans, Number of times that the designated allele was transmitted to affected offspring, when others were not.
Untrans, Number of times that the designated allele was not transmitted to affected offspring, when others were.
[d]NPL, Non-parametric LOD score for multipoint linkage analysis.
*$p < 10^{-5}$ This significance level was set, considering Bonferroni adjustment for multiple markers and alleles.

in the analyses (Table 2). The haplotype 5-1 of D8S1145-LPL was significantly higher in its transmission to offspring with young-onset hypertension ($p = 0.0001$), so were other haplotypes such as haplotype 6-1 of D8S1145-LPL, haplotype 1-2 of LPL-D8S322, and haplotype 1-4 of LPL-D8S322. Similar positive associations were observed for 3-marker haplotypes when allele 1 of the LPL marker is present. The haplotype 5-1-2 of D8S1145-LPL-D8S322 was the most significant one among all 3-marker haplotypes ($p = 4.4 \times 10^{-5}$).

Sequencing results and the association between the cSNP and phenotypes

After sequencing 5′ flanking region and nine exons of *LPL* gene for the 41 subjects as described in the methods, we confirmed two known cSNPs in our population: a silent mutation (7754C → A; Thr^{361} → Thr) [25] in exon 8 and S447X (C → G; Ser^{447} → stop codon) in exon 9 with their minor allele frequency of 9% and 8%, respectively (Table 3). No new cSNPs was discovered. We have also genotyped all samples for 11

Table 2. Results of haplotype TDT using TRANSMIT program.

Haplotype	D8S1145[a]	LPL[b]	D8S322[c]	OBS	EXP	χ^2	*p*-value
2 markers	5	1		29.1	18.9	14.89	0.0001
	6	1		20.5	14.0	8.47	0.0036
		1	2	52.3	29.1	41.67	1.1×10^{-10}
		1	4	59.3	45.8	10.95	0.0009
3 markers	4	1	2	8.0	4.6	5.26	0.022
	5	1	2	17.0	8.7	16.71	4.4×10^{-5}
	3	1	4	6.0	3.0	5.88	0.015

[a]Allele 3 = 281 bp, 4 = 285 bp, 5 = 289 bp, 6 = 293 bp.
[b]Allele 1 = 123 bp.
[c]Allele 2 = 218 bp, 4 = 226 bp.
OBS, the observed number of the designated haplotype transmitted to affected offsprings; EXP, expected number of transmission.

Table 3. SNP screening of the LPL promoter and exons and summary of the identified SNPs.

Location	Primer sequences	Amplified products	Position in NCBI GenBank NT_{030737}		Polymorphisms[a] (allele frequency)
			Start	End	
Promoter	5′ GATCCATCTTGCCAATGTTA 3′ 5′ TAGAAGTGGGCAGCTTTC 3′	571	7607114	7607684	–
Exon 1	5′ CCTTGCAGCTCCTCCAGAGGG 3′ 5′ AGGGGAGTTTGCGCGCAAAA 3′	276	7607685	7607960	–
Exon 2	5′ CTCATATCCAATTTTTCCTT 3′ 5′ CTCTTCCCCAAAGAGCCTCC 3′	161	7616612	7616772	–
Exon 3	5′ AAGCTTGTGTCATCATCTTC 3′ 5′ ATAAGTCTCCTTCTCCCAGT 3′	180	7620201	7620380	–
Exon 4	5′ TTTTGGCAGAACTGTAAGCA 3′ 5′ GACAGTCTTTTCACCTCTTA 3′	112	7621742	7621853	–
Exon 5	5′ TGTTCCTGCTTTTTTCCCTT 3′ 5′ TAATTCGCTTCTAAATAATA 3′	234	7622552	7622785	–
Exon 6	5′ GCCGAGATACAATCTTGGTG 3′ 5′ GCATGATGAAATAGGACTCC 3′	243	7624273	7624515	–
Exon 7	5′ CATGTTCGAATTTCCTCCCC 3′ 5′ ATGACCGCCCCCCTGTGCTA 3′	121	7627692	7627812	–
Exon 8	5′ CCAAATTTATTGCTTTTTTG 3′ 5′ AAGGAAGAAAAATACATTTA 3′	183	7629333	7629515	7754C → A (C/A:0.91/0.09)[b]
Exon 9	5′ TATTCACATCCATTTTCTTC 3′ 5′ GTCAGCTTTAGCCCAGAATG 3′	105	7630547	7630651	S447X (C/G: 0.92/0.08)[b]

[a]The DNA samples used for sequencing were from 41 subjects of the young-onset hypertension families.
[b]The samples used for determining allele frequency were 267 subjects from 66 families (see Methods).
–, No polymorphisms were discovered.

known *LPL* cSNPs from NCBI GenBank, but none were found in our study population with more than 200 founder chromosomes.

The TDT for each the two identified cSNPs was not statistically significant. Haplotype TDT analysis revealed three haplotypes, i.e., C–C, A–C, and C–G in the order of 7754C → A and S447X. The frequencies of three haplotypes were 81.1%, 10.1%, and 8.8%, respectively. The haplotype A–C was significantly higher in its transmission to offsprings with elevated triglyceride ($p=0.019$). There was a similar trend of greater transmission observed for the phenotype of hypertension combined with elevated triglyceride ($p=0.063$).

Genotype distribution of S447X between cases and controls in an extended community-based association study

Comparing 92 cases with persistent hypertension and elevated triglyceride and 92 matched normal controls, we found that the distributions of CC, CG, and GG genotypes of S447X were significantly different between cases and controls ($p=0.02$) (Table 4). The proportion with GG or CG genotypes was 9.8% and 25.3% in cases and controls, respectively. G allele of S447X occurred more frequently in control group than in case group.

Discussion

We carried out a gene mapping study for young-onset hypertension with 18 hypertension candidate genes involving lipid metabolism, insulin resistance, sodium homeostasis, and signal transduction, etc. [17]. Although our preliminary results points to four potential genes, only angiotension converting enzyme (*DCP1*) [18] and *LPL* (the present study) sustained their genetic effects in our fine mapping endeavors. We demonstrated in Taiwan Han Chinese that the LPL marker located in the intron 6 of *LPL* gene was positively linked

Table 4. The characteristics and the genotype frequency of S447X between patients with persistent hypertension and elevated fasting triglyceride and normal controls.

Characteristics[a] and S447X genotype		Patients	Normal controls	*p*-value[b]
Male (%)		60 (65.2%)	60 (65.2%)	1.0
Female (%)		32 (34.8%)	32 (34.8%)	
Age (year)		46.8 ± 7.3	47.7 ± 7.2	0.4219
Triglyceride[c] (mg/dl)		241.3 ± 98.2	79.2 ± 19.2	<0.0001
S447X	CC	83 (90.2%)	68 (74.7%)	0.0219
(Exon 9)	CG	8 (8.7%)	21 (23.1%)	
	GG	1 (1.1%)	2 (2.2%)	

[a]Presented as number (%) or mean ± SD.
[b]*t*-test for continuous variables and Fisher's exact test for proportions.
[c]The mean of triglyceride level averaged from values obtained from three occasions over 6 years.

with young-onset hypertension. This finding was further supported by TDT either via single microsatellite marker or the haplotype analyses. The degree of linkage and association for *LPL* is much greater than that of *DCP1* [18] in our young-onset hypertension study, indicating the relative importance of *LPL* in the development of hypertension in Taiwan Han Chinese. On the other hand, only 32% of the family demonstrated a positive linkage score for LPL marker, suggesting the existence of genetic heterogeneity of hypertension.

Elevated mean fasting plasma triglyceride level has been previously observed in our studied patients. The young hypertension patients had a significantly higher mean of fasting plasma triglyceride (41 mg/dl higher for men, $p=0.017$; 33 mg/dl for women, $p=0.04$) than their matched controls with regard to age, sex, residential area and BMI level [26]. Thus, the positive linkage result on *LPL* coincides with the phenotypic trait of our patients. This clustering phenomenon between high blood pressure and high plasma triglyceride is now known as a part of metabolic syndrome in which central obesity, low HDL-C, and hyperglycemia, and insulin resistance are also present. A recent study [27] has shown linkage between *LPL* haplotype and glucose infusion rate, a direct physiologic measurement of insulin sensitivity in Mexican Americans. LPL has also been associated with obesity [28, 29]. Whether LPL plays a central role in the etiology of metabolic syndrome awaits further insights.

The positive finding between *LPL* and hypertension in our young hypertension study adds to several association and linkage studies in Chinese [11–15], but is in contrary with those carried out in Caucasians [16]. Therefore, we would suggest that *LPL* may play a race-specific role in the development of hypertension, either via its genetic variations or due to differential gene-environmental interactions. Our sequencing results also substantiate the phenomenon of population diversity in *LPL* gene. We did not find any SNP in the promoter and exon regions except the two known ones in exon 8 and in exon 9. And none of the other 11 SNPs documented by NCBI GenBank were found in our population, indicating that *LPL* coding region may be more conserved in Taiwan Han Chinese than those previously reported in other populations.

Haplotype A–C constructed from the two cSNPs was associated with elevated plasma triglyceride; however, it was not associated with young-onset hypertension per se. The level of the significance is also far weaker than that obtained for the LPL marker in intron 6 and young-onset hypertension. We suspect that there probably exists other genetic variant near or in the intron 6. Further studies are required to unravel the new *LPL* genetic variants if any.

We found, in the present study, not only haplotype A–C moderately associated with elevated triglyceride and with hypertension combined with elevated triglyceride in the family study, but also S447X associated with persistent hypertension and elevated triglyceride in a extended community-based case-control study. However, how *LPL* variant may involve in the development of hypertension is poorly understood. Lipotoxicity could be a possible pathophysiological hypothesis.

Elevated plasma lipids could induce hyperinsulinemia and contribute to development of hypertension and other metabolic syndrome-related comorbidities [28, 29]. On the other hand, cell culture studies showed that high level of glucose and saturated fatty acids could stimulate LPL expression of macrophage in the arterial wall [30, 31]. It has been hypothesized that LPL overexpression may be associated with atherosclerosis and diabetes. On the contrary, an inhibitory effect of high fat-diet on plasma and adipocyte LPL has also been documented in rats [32], so has the impairment of insulin-stimulated LPL activity been shown by such a diet in adipose tissue of the normal subjects [33]. In-depth functional studies are needed to elucidate the mechanism underlying the relations between *LPL* and hypertension.

Overall, our result suggests *LPL* is linked and associated with young-onset hypertension. The G allele of S447X may have a protective effect on the development of hypertension and hypertriglyceridemia. Further works are needed to confirm the findings in other samples or populations, to unravel the genetic variant if different from S447X, and to unfold the mechanism via which *LPL* contributes to the development of hypertension.

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