Polymorphism of the Gene Encoding Lipoprotein Lipase in Thai Primary Hyperlipoproteinemias

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Abstract

Lipoprotein lipase (LPL) plays a central role in the clearance of very low density lipoprotein (VLDL) and chylomicrons from the circulation. It also affects the maturation of high density lipoprotein (HDL) and low density lipoprotein (LDL). LPL is an important candidate gene in determining the risk factor in metabolic disorders including primary hyperlipidemia. Our study is the first report from Thailand on the characterization of two common DNA polymorphisms, i.e *Pvu* II and *Hind* III at introns 6 and 8, respectively of the LPL gene in 94 Thai dyslipidemic subjects compared to 32 normolipidemic subjects using PCR-RFLP. It was observed that the frequencies of the cut and uncut alleles of *Pvu* II were 0.67 and 0.33 in normolipidemic subjects. Such frequencies were 0.64 and 0.36 in hyperlipidemic subjects. Additionally, the frequencies of the cut and uncut alleles of *Hind* III were found to be 0.73 and 0.27 in normolipidemic subjects. They were 0.85 and 0.15 in hyperlipidemic subjects. The allele frequencies of the *Hind* III but not *Pvu* II polymorphism in hyperlipidemic subjects were significantly different from normolipidemic subjects ($p<0.05$). The relation between these polymorphisms and lipid traits was not statistically significant ($p>0.05$).

Key word : Lipoprotein Lipase, DNA Polymorphism

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Lipoprotein lipase (LPL) is a critical enzyme in the catabolism and transport of lipids (1,2). Its primary function is the hydrolysis of core triglcerides of circulating chylomicrons and VLDL, thus, delivering free fatty acids to tissues for oxidation in the heart and other tissues or for storage

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in adipose tissue. Catabolism of VLDL by LPL results in a triglyceride-depleted particle, which after further lipolysis becomes IDL and LDL. Furthermore, LPL acitivity affects the maturation of HDL particles, which in turn may take up tissue cholesterol for transport to be modified at the liver. Individuals genetically deficient in LPL activity exhibit extreme postprandial hypertriglyceridemia[3]. Because of its intimate involvement in lipid metabolism, the LPL gene is considered to be an important candidate gene in determining the risk factor in metabolic disorder e.g. atherosclerosis, obesity and coronary heart disease etc[4].

DNA polymorphism is a useful marker to analyze disorders with genetic backgrounds, eventhough the genetic cause of the disease has not been elucidated. A number of DNA polymorphisms have been used to examine their possible linkage with a hereditary predisposition to common polygenic disorders such as dislipidemia and diabetes mellitus [3,5,6]. Polymorphism at the LPL gene locus have previously been detected with the Southern blot hybridization[1,7,8]. This conventional procedure requires considerable time, labour and skill as well as radiolabelled LPL gene probes. Therefore, the technique is inappropriate for a large scale study. Recently, Polymerase Chain Reaction (PCR)-Restriction Fragment Length Polymorphism (PCR-RFLP) has been applied to the molecular study of the LPL gene and various DNA polymorphisms have been found to be associated with dyslipidemia[9-11]. To study the impact of the LPL polymorphism on primary hyperlipidemia in Thai subjects, *Pvu* II and *Hind* III polymorphisms at the introns 6 and 8 of the LPL gene, respectively, will thus, be observed using PCR-RFLP.

MATERIAL AND METHOD

Subjects

One-hundred and twenty-six experimental subjects were divided into 32 normolipidemic and 94 primary hyperlipidemic subjects. Normal controls were obtained from healthy unrelated individuals who visited The Health Screening Clinic, Department of Preventive Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University. They consisted of 10 males (age 34-64 years, mean age 43.8 ± 14.2 years) and 22 females (age 23-58 years, mean age 41.3 ± 10.1 years). Their cholesterol and triglyceride levels were ≤200 mg/dL and ≤200 mg/dL, respectively. Primary hyperlipidemic subjects were recruited from 36 males (age 35-81 years, mean age 52.8 ± 11.3 years) and 58 females (age 27-75 years, mean age 56 ± 10.8 years) who attended the Lipid Clinic, Department of Preventive Medicine, Faculty of Medicine Siriraj Hospital. They were diagnosed on the basis of at least three fasting pretreatment lipid measurements with cholesterol and triglyceride levels that were >200 mg/dL and >200 mg/dL, respectively. All subjects were informed consent for participation in the study.

Collection of blood, lipid analyses and DNA isolation

Fasting venous blood samples (10 mL) were collected into tubes containing EDTA. Plasma was collected from each sample for determination of the levels of total cholesterol, triglyceride and HDL-cholesterol (HDL-C) which were examined by Department of Clinical Chemistry, Faculty of Medical Technology, Mahidol University using the enzymatic methods the kits used were from Boeringer Mannheim and Hoffman La Roche for determination of total cholesterol and triglyceride & HDL-C, respectively). An automated analyzer (Hitachi 917) was used to determine all lipid concentrations. the LDL-cholesterol (LDL-C) level was calculated using the method described by Friedewald *et al*[12]. Leukocyte DNA was extracted by guanidine-HCl method[13]. The DNA was then redissolved in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.6) and stored at –20°C until used.

Oligonucleotide primers

DNA primers for PCR were synthesized by the BioService Unit, BIOTEC Center, NSTDA. The primer sequences for the study of polymorphisms in introns 6 and 8 were obtained from Gotoda *et al*[9]. The primer sets for the study of *Pvu* II polymorphism in intron 6 were Pv1 ; 5'-GCTTAA TTCTCAATTCAATG-3' and Pv2 ; 5'-CTTTAGAC TCTTGTCCAGGT-3'. In addition, Hd1; 5'-TGAA GCTCAAATGGAAGAGT-3' and Hd2 ; 5'-TACAA GCAAATGACTAAA-3' were used to study the *Hind* III polymorphism in intron 8.

Amplification of genomic DNA

Amplification was performed in a Perkin Elmer 2400 thermal cycler. The 20 mL PCR reaction mixture contained 0.2-0.5 μg gDNA, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, dTTP

(Pharmacia), 0.3 μM of each primer and 2.5 units of *Taq* DNA polymerase (Pharmacia). Blanks containing no added DNA were run with each set of amplification. The PCR cycles were slightly modified from that described by Gotoda *et al*[9]. They were 5 min denaturation at 94°C, followed by 35 cycles of 1 min at 94°C, 2 min at 50-52°C and 3 min at 72°C, followed by a 7 min extension at 72°C. The annealing temperatures used for the amplification of introns 6 and 8 were 52°C and 50°C respectively.

Restriction endonuclease digestion and electrophoretic analyses

The PCR-amplified DNA was directly used for digestion with restriction enzymes *Pvu* II or *Hind* III (New England BioLabs). The final volume of 20 μL reaction contained 7 μL of the amplified DNA, 10 units of the enzymes and an incubation buffer recommended by the manufacturer's instructions. The reaction mixture was incubated overnight at 37°C. The digested products were analyzed by electrophoresis on a 2.5-3 per cent agarose gel in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3) at 100 volts for 1 h. DNA was visualized on a UV-transilluminator after staining with ethidium bromide. The 100 bp ladder (Pharmacia) was used as a reference molecular weight marker.

Statistical analysis

Haplotype frequencies of the *Pvu* II and *Hind* III polymorphic restriction sites of the LPL gene were calculated from gene counting. Chi-square (X^2)-test was used to determine the statistical significance between the allele frequencies and those two polymorphisms. To investigate the relationship between such polymorphisms and lipid concentrations, either Kruskall-Wallis or Mann-Whitney U tests were applied. All calculations were completed using Statview release 5.0.

RESULTS

Lipid analysis

Plasma levels of total cholesterol, triglyceride, LDL-C and HDL-C of the normolipidemic and hyperlipidemic subjects are summarized in Table 1. Mean ages were 42.13 ± 11.42 and 54.74 ± 11.04 years for the normolipidemias and hyperlipidemias, respectively.

Pvu II Polymorphism of the LPL gene in intron 6

Intron 6 of the LPL gene was amplified by PCR and subsequently digested with *Pvu* II. This polymorphism was caused by a C → T transition within the *Pvu* II site of this intron. The C/C (or +/+) alleles generated the 150 and 39 bp upon digestion with *Pvu* II. On the contrary, the T/T (or -/-) alleles were not digested by the enzyme and generated a 189 bp fragment. In addition, the heterozygous pattern contained these 3 DNA fragments (Fig. 1). Using PCR-RFLP, a polymorphism of the LPL gene in the intron 6 among the normolipidemic and hyperlipidemic subjects was characterized and the allele frequencies are shown in Table 2. It was demonstrated that the frequencies of the C (cut)- and T (uncut)-alleles were 0.67 and 0.33, respectively for the normolipidemic subjects and 0.64 and 0.36, respectively for the hyperlipidemic subjects. The population exhibited this genotype frequencies was consistent with those expected under Hardy-Weinberg equilibrium condition. Additionally, the frequencies of the cut and uncut alleles of the pooled subjects were 0.65 and 0.35, respectively (Table 2).

Hind III polymorphism of the LPL gene in intron 8

A polymorphism of the LPL gene at intron 8 was also characterized by PCR-RFLP. This polymorphism was caused by a T → G transversion within the *Hind* III site of this intron. The T/T (or +/+) alleles generated the 600 and 115 bp upon digestion with the enzyme. On the other hand, the G/G (or -/-) alleles were not digested by the enzyme resulting in a 715 bp DNA fragment. All of

Table 1. Mean age and lipid profiles of the Thai normolipidemic and hyperlipidemic subjects

	Normolipidemias* (n =32)	Hyperlipidemias* (n = 94)
Age (years)	42.13 ± 11.42	54.74 ± 11.04
Cholesterol (mg/dL)	180.38 ± 22.60	281.43 ± 48.55
Triglyceride (mg/dL)	87.56 ± 37.16	255.55 ± 231.36
LDL-C (mg/dL)	102.49 ± 30.91	182.75 ± 59.40
HDL-C (mg/dL)	60.38 ± 17.42	49.81 ± 26.88

* Mean ± S.D.

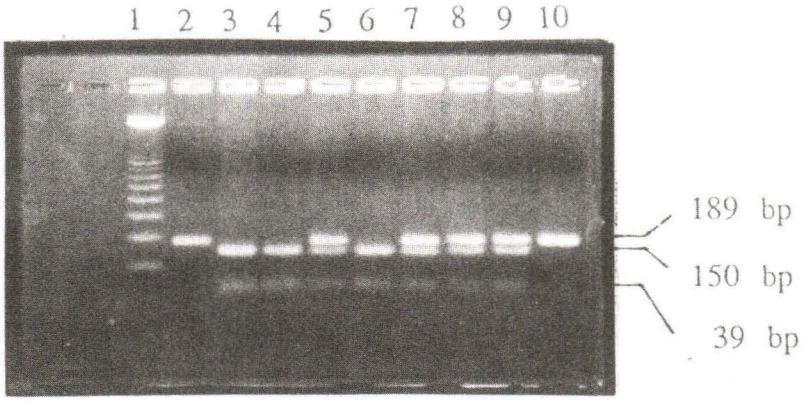

Fig. 1. 3% agarose gel electrophoretic pattern of the *Pvu* II-digested PCR-amplified intron 6 of the LPL gene. Lane 1 was 100 bp DNA molecular weight marker. Lane 2 was the undigested PCR-amplified DNA. Lanes 3-10 were examples of the *Pvu* II-digested DNA (Lanes 3, 4 and 6 were +/+, 5 and 7-9 were +/-, 10 was -/- polymorphisms).

Table 2. Allele frequencies of the LPL DNA polymorphisms in the introns 6 and 8 of the normolipidemic and hyperlipidemic subjects.

Polymorphism	Normolipidemias (n = 32)	Hyperlipidemias n = 94)	Pooled samples (n = 126)
***Pvu* II**			
Cut	0.67	0.64	0.65
Uncut	0.33	0.36	0.35
***Hin*d III**			
Cut	0.73	0.85	0.82
Uncut	0.27	0.15	0.18

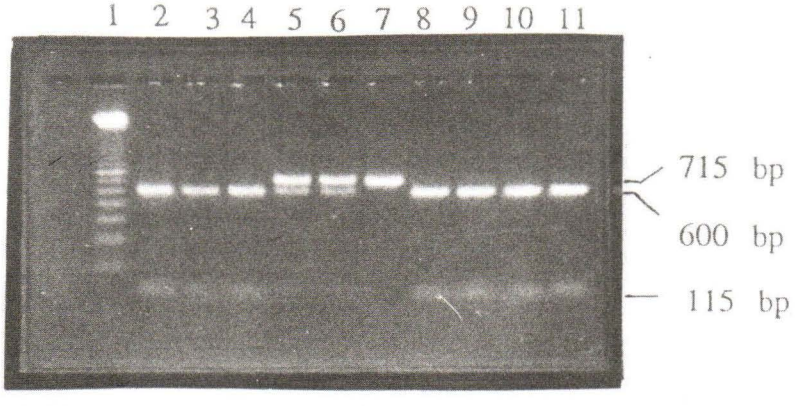

Fig. 2. 2.5% agarose gel electrophoretic pattern of the *Hin*d III-digested PCR-amplified intron 8 of the LPL gene. Lane 1 was 100 bp DNA molecular weight marker. Lanes 2-11 were examples of the *Hin*d III-digested DNA (2-4 and 8-11 were +/+, 5-6 were +/- and 7 was -/- polymorphisms).

these 3 DNA fragments were observed in the heterozygous pattern (Fig. 2). A polymorphism of the LPL gene at intron 8 among the normolipidemic and hyperlipidemic subjects was characterized and the allele frequencies are shown in Table 2. It was observed that the frequencies of the cut and uncut alleles were 0.73 and 0.27, respectively for the normolipidemic and 0.85 and 0.15, respectively for the hyperlipidemic subjects. The population exhibited this genotype frequencies was also consistent with those expected under Hardy-Weinberg equilibrium condition. Furthermore, the frequencies of the cut and uncut alleles of the pooled subjects were 0.82 and 0.18, respectively (Table 2).

In addition, the lipid profile of the normolipidemias and hyperlipidemias are classified according to the different genotypes of LPL (Table 3).

DISCUSSION

Genetic and environmental risks e.g diet and lifestyle are found to influence dyslipidemia which is a complicated multifactorial disorder and can be secondary to various diseases e.g obesity, diabetes mellitus, renal diseases, coronary heart disease etc[4].

Dyslipidemia is also common in Thailand. However, this metabolic disorder has never been studied at the molecular level. Our study is the first report of polymorphism of the gene encoding LPL in Thai primary hyperlipidemias. Two polymorphisms of the LPL gene i.e *Pvu* II and *Hind* III at introns 6 and 8, respectively, were screened for using PCR-RFLP. For *Hind* III polymorphism, our results revealed that the frequencies of the cut and uncut alleles were 0.73 and 0.27, respectively in the normolipidemic subjects. Such frequencies were 0.85 and 0.15 respectively, for the hyperlipidemic subjects. The allele frequencies of the hyperlipidemic subjects were significantly different from the normolipidemic subjects (X^2 = 4.649, p = 0.03).

For *Pvu* II polymorphism, the frequencies of the cut and uncut alleles were 0.67 and 0.33, respectively in the normolipidemic subjects. Such frequencies were 0.64 and 0.36 respectively, for the hyperlipidemic subjects. These frequencies of the hyperlipidemic subjects were, in contrast to the *Hind* III polymorphism, not substantially different from the normolipidemic subjects (X^2 = 0.188, p=0.664).

Allele frequencies of the *Hind* III and *Pvu* II polymorphism of the LPL gene in Thai subjects were compared with those reported for other populations (Table 4). It was observed that the allele frequencies of both *Hind* III and *Pvu* II polymorphisms of the Thai subjects were significantly different from Mediterranian (Australia), US Whites and Blacks and Japanese populations ($p<0.05$). It is suggested that these two polymorphisms of the LPL gene might be influenced by ethnicity. However, the allele frequencies of these polymorphisms in the Thai subjects were significantly different from the normolipidemic Japanese (Table 4). These allele frequencies in the Thais were calculated from the pooled subjects, hence, were enriched by those with primary hyperlipidemia and, thus, may not be entirely representative of the Thai population.

From our study, the *Hind* III but not the *Pvu* II polymorphism of the LPL gene was significantly associated with hyperlipidemia. However, neither of these polymorphisms exhibited a significant association with the plasma lipid traits (Kruskall-Wallis or Mann Whitney U tests, $p>0.05$). From other reports, *Hind* III RFLP was found to be associated with variation in HDL-C level, hypertriglyceridemia, premature coronary atherosclerosis, coronary artery disease and myocardial infarction[7,14,15-19]. In addition, this polymorphism was found to be significantly correlated with cholesterol and LDL-C levels but not triglyceride and HDL-C levels in 342 female Caucasians[20].

The *Pvu* II polymorphism was not found to be significantly associated with cholesterol, triglyceride, LDL-C or HDL-C levels[7,14]. On the contrary, this polymorphism was found to be significantly correlated to cord plasma lipid traits in male newborns in Singapore[21], coronary artery disease (CAD) severity and with type II diabetes in CAD patients[22].

In conclusion, we have characterized polymorphisms of the LPL gene and found that the allele frequencies of the *Hind* III but not *Pvu* II polymorphism are substantially associated with dyslipidemia which can lead to more severe diseases e.g atherosclerosis and CAD. The fact that dyslipidemia has an inherited component implies that there must be some underlying genetic variants not necessarily sufficient to produce the disease but which will provide a predisposition to it when the appropriate environmental conditions are encountered. Thus, understanding the genetic basis of dys-

Table 3. Mean age and lipid profiles of the normolipidemic and hyperlipidemic subjects for the *Pvu* II and *Hind* III genotypes of the LPL gene.

Pvu II Polymorphism	Normolipidemic subjects (n = 32)			Hyperlipidemic subjects (n = 94)		
	+/+ (n = 16)	+/- (n = 11)	-/- (n = 5)	+/+ (n = 42)	+/- (n = 37)	-/- (n = 15)
Age (years)	40.27 ± 12.38	42.00 ± 11.37	48.00 ± 7.91	54.46 ± 11.12	54.78 ± 11.32	55.40 ± 10.83
Cholesterol (mg/dL)	177.69 ± 22.56	180.46 ± 22.96	188.80 ± 24.78	271.33 ± 45.61	294.43 ± 52.28	277.60 ± 42.06
Triglycerides (mg/dL)	79.00 ± 34.67	88.36 ± 24.47	113.20 ± 59.85	280.79 ± 301.89	222.97 ± 151.22	265.27 ± 158.33
LDL-C (mg/dL)	97.54 ± 30.62	103.37 ± 34.15	116.36 ± 25.17	170.53 ± 57.46	193.06 ± 63.28	192.15 ± 50.87
HDL-C (mg/dL)	64.34 ± 18.59	59.41 ± 16.88	49.80 ± 11.78	52.39 ± 36.44	50.24 ± 15.99	40.94 ± 10.49
Hind III Polymorphism	Normolipidemic subjects (n = 32)			Hyperlipidemic subjects (n = 94)		
	+/+ (n = 16)	+/- (n = 11)	-/- (n = 5)	+/+ (n = 42)	+/- (n = 37)	-/- (n = 1)
Age (years)	39.76 ± 11.86	42.18 ± 10.16	55.33 ± 2.52	54.25 ± 11.63	55.89 ± 9.80	56
Cholesterol (mg/dL)	179.50 ± 22.11	181.55 ± 21.98	181.33 ± 36.47	275.98 ± 43.24	294.96 ± 59.08	275
Triglycerides (mg/dL)	79.89 ± 32.78	86.45 ± 29.81	137.67 ± 59.37	261.45 ± 259.98	241.59 ± 148.98	243
LDL-C (mg/dL)	97.97 ± 29.32	112.25 ± 29.71	93.80 ± 47.66	175.87 ± 58.83	200.33 ± 59.48	180
HDL-C (mg/dL)	65.56 ± 18.01	52.00 ± 14.57	60.00 ± 16.09	50.16 ± 30.56	49.04 ± 15.14	47

Table 4. Distribution of the *Pvu* II and *Hind* III alleles of the LPL gene in selected populations.

Population	*Pvu* II		*Hind* III		Reference number
	Cut	Uncut	Cut	Uncut	
Thai (n = 126)	0.65	0.35	0.82	0.18	This study
Mediterranean (USA)[a]	0.58	0.42	0.76	0.24	1
Mediterranean (Australia) (n = 144)	0.52	0.48	0.67	0.33	14
US Whites[b]	0.54	0.45	0.73	0.27	1
US Blacks[c]	0.22	0.78	0.68	0.32	1
Caucasians (n = 20)	ND[d]	ND[d]	0.75	0.25	10
Caucasians (US) (n = 15)	0.56	0.44	ND[d]	ND[d]	11
Japanese (n = 70)	0.76	0.24	0.73	0.27	9

[a]134 and 132 samples were studied for *Pvu* II and *Hind* III polymorphisms, respectively.
[b]164 and 189 samples were studied for *Pvu* II and *Hind* III polymorphisms, respectively.
[c]55 and 78 samples were studied for *Pvu* II and *Hind* III polymorphisms, respectively.
[d]Not done.

lipidemia might shed some light on prevention or productive treatment of primary or secondary dyslipidemia and other related diseases.

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ความหลากหลายของยีนที่สร้างเอนไซม์ไลโปโปรตีนไลเปสในคนไทยที่มีภาวะไขมันสูง ปฐมภูมิ

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ไลโปโปรตีนไลเปสเป็นเอนไซม์สำคัญที่ทำหน้าที่กำจัด VLDL และ chylomicrons จากกระแสเลือด นอกจากนี้ยังช่วยในการสร้าง HDL และ LDL พบว่าความหลากหลายของยีนที่ทำหน้าที่ในการสร้างเอนไซม์นี้ จะใช้บ่งถึงปัจจัยเสี่ยงต่อการเกิดความผิดปกติของโรคต่างๆหลายชนิดรวมทั้งการเกิดภาวะไขมันสูงได้ รายงานนี้จะเป็นรายงานการวิจัยฉบับแรกของไทยที่กล่าวถึงความหลากหลายของยีนที่สร้างเอนไซม์ไลโปโปรตีนไลเปสในคนไทยที่มีภาวะไขมันสูง เปรียบเทียบกับคนปกติ อนึ่งความหลากหลายของยีน LPL ที่พบได้บ่อย คือ *Pvu* II และ *Hind* III polymorphism ที่บริเวณ introns 6 และ 8 ของยีนดังกล่าว ในที่นี้จะทำการศึกษาโดยใช้วิธี PCR-RFLP ซึ่งจะต้องเพิ่มจำนวนของยีน LPL ที่บริเวณ introns 6 และ 8 ด้วยวิธี PCR ก่อน จากนั้นจึงนำไปย่อยด้วยเอนไซม์ *Pvu* II หรือ *Hind* III แล้วจึงคำนวณความถี่ของ allele ที่ถูกย่อยและไม่ถูกย่อยด้วยเอนไซม์ทั้ง 2 พบว่าความถี่ของ allele ที่ถูกย่อย และไม่ถูกย่อยด้วยเอนไซม์ *Pvu* II จะเป็น 0.67 กับ 0.33 ในคนปกติ และ 0.64 กับ 0.36 ในผู้ที่มีภาวะไขมันสูง ส่วนความถี่ของ allele ที่ถูกย่อย และไม่ถูกย่อยด้วยเอนไซม์ *Hind* III จะเป็น 0.73 กับ 0.27 ในคนปกติ และ 0.85 กับ 0.15 ในผู้ที่มีภาวะไขมันสูง ซึ่งความถี่ของยีนที่ได้จาก *Hind* III polymorphism ที่ intron 8 ของยีน LPL ในผู้ที่มีภาวะไขมันสูงนี้ จะพบว่ามีความแตกต่างจากคนปกติอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) อย่างไรก็ตามจากการศึกษานี้ จะไม่พบความสัมพันธ์ของความหลากหลายของยีน LPL ที่ introns ทั้ง 2 กับระดับของไขมันทั้ง 4 ชนิด คือ โคเลสเตอรอลรวม, ไตรกลีเซอร์ไรด์, HDL-cholesterol และ LDL-cholesterol

คำสำคัญ : ไลโปโปรตีนไลเปส, ความหลากหลายของยีน

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