Thr-6Pro missense mutation in human lysosomal acid lipase (LAL) gene in patients with familial hypercholesterolemia in Korea

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Lysosomal acid lipase (LAL) plays a central role in the intracellular degradation of neutral lipids derived from plasma lipoproteins. In this study, we investigated the missense mutation within exon 2 of human LAL gene changing of codon -6 of prepeptide from threonine to proline. The Thr-6Pro mutation was detected by the HaeIII restriction fragment length polymorphism (RFLP) and single-strand conformation polymorphism (SSCP). We analyzed the mutation in subjects with 221 unrelated randomly selected control samples and 86 patients with familial hypercholesterolemia (FH) in Korea. We observed that mutation is present with high frequency in Korea compared to other populations studied previously. The frequency of PP homozygote in the FH group was observed considerably higher than that of control. However, there was no significant difference of genotype frequency between two groups. These results, together with the fact that plasma lipids and lipoproteins levels between genotypes showed no statistical difference, suggest that the Thr-6Pro mutation in the LAL gene may have no association with the increased risk of FH development.

Keywords: Familial hypercholesterolemia, HaeIII RFLP, lysosomal acid lipase, Thr-6Pro mutation

INTRODUCTION

Human lysosomal acid lipase (LAL; E.C. 3.1.1.13), otherwise known as acid cholesteryl ester hydrolase, serves a central role in the intracellular metabolism of neutral lipids that have been internalized via receptor-mediated endocytosis of lipoprotein particles. The enzyme hydrolyzes cholesteryl esters and triacylglycerols, releasing cholesterol and fatty acids. Cholesterol liberated is transferred to the cytosol where it regulates the endogenous synthesis of cholesterol, the uptake of low density lipoprotein (LDL) and cholesterol esterification (Goldstein *et al.*, 1975).

Human LAL gene having 10 exons spread over 36 kb is mapped on chromosome 10q23.2-23.3 (Koch *et al.*, 1981; Anderson *et al.*, 1993; Ameis *et al.*, 1994; Aslanidis *et al.*, 1994). The deduced amino acid sequence of LAL has 378 amino acids including a putative 21 amino acid N-terminal signal peptide. Molecular cloning of the cDNA for LAL has revealed significant amino acid similarity with human gastric lipase and rat lingual lipase (58 and 57% amino acid sequence identical, respectively), indicating that these enzymes belong to a common family of acid lipases (Anderson and Sando, 1991). However, no similarity was found with neutral lipases, such as lipoprotein lipase, hepatic lipase and pancreatic lipase, suggesting different gene families of acid and neutral lipases (Ameis *et al.*, 1992; Hide *et al.*, 1992).

Deficiencies of human LAL have been found in two autosomal recessive storage disorders, cholesteryl ester storage disease (CESD) and Wolman disease (Assman and Seedorf, 1995). In both disorders, the enzyme deficiency leads to a progressive accumulation of cholesteryl esters and triacylglycerols in lysosomes of affected tissues. Even though both disorders are characterized by deficiencies in LAL activity, they display a striking difference of clinical and biochemical phenotypes. In Wolman disease, death usually occurs within 6 months after birth with massive and widespread intracellular storage of cholesteryl esters and triglycerides. On the other hand, CESD has a less severe phenotype. Moderate dyslipidemia and hepatomegaly are clinical signs of CESD. Low plasma level of

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high density lipoprotein (HDL)-cholesterol, which is an important risk factor for the premature coronary artery disease (CAD), is also commonly observed in CESD patients. Mutations of LAL gene are therefore to be useful markers for the development of CAD. Several mutations have been detected in patients with LAL deficiency, such as splicing defects resulting in the deletions (Klima *et al.*, 1993; Ameis *et al.*, 1995; Muntoni *et al.*, 1995), point mutations resulting in single amino acid substitutions (Anderson *et al.*, 1994; Seedorf *et al.*, 1995; Pagani *et al.*, 1994, 1996), insertion or deletion of nucleotides changing the reading frame (Anderson *et al.*, 1994; Ameis *et al.*, 1995).

Recently, a mutation within exon 2 of LAL gene has been found from two human LAL cDNA clones. The codon -6 at cDNA position 86 was either ACC in a liver cDNA (Ameis *et al.*, 1994) or CCC in a fibroblast cDNA (Anderson and Sando, 1991). The A to C missense mutation results in an amino acids substitution of threonine to proline of the putative signal peptide (Thr-6Pro). The Thr-6Pro mutation has been studied at the population level only by Muntoni *et al.* (1996). They revealed that it presents with a high frequency in three different normal populations. We could also detect the missense mutation independently of the other studies by the methods of single-strand conformation polymorphism (SSCP) and DNA sequencing analysis.

In this study, we analyzed the mutation in subjects with patients with familial hypercholesterolemia (FH) as well as normal population, to uncover whether the mutation is related to an increased risk for the development of hypercholesterolemia. Here, this study suggests that the mutation is present in Koreans with high frequency and may have no association with the FH development.

MATERIALS AND METHODS

Subjects and DNA isolation

The blood samples were collected into EDTA-containing tubes from 221 randomly selected unrelated individuals (control group: aged 19-25 years old) and 86 patients with FH (FH group: aged 21-67 years old; provided from Hospital in Seoul National University) in Korea. Genomic DNAs were purified from the whole bloods using a DNA purification system (Promega, USA).

Amplification and *Hae*III digestion of exon 2

The PCR was carried out in a 20 μl of premixed reaction solution containing 50-100 ng of genomic DNA template and 20 pmol of following primers designed by Anderson *et al.* (1994): ex2F (5'-GTGGGAGCATTAAGTTACC-3') and ex2R (5'-TGGATCGGGAAATAGATGC-3') corresponding to just upstream and downstream regions of exon 2, respectively. The reactions were performed in a thermal cycler through 30 cycles consisting of 30 sec at 94°C, 1 min at 55°C and 1 min at 72°C, followed by a final extension step of 7 min at 72°C (Perkin Elmer Cetus, USA). Amplified DNA fragments were purified from the PCR mixture using a PCR product purification system (Promega, USA), and then digested with *Hae*III restriction endonuclease (10 units/sample) at 37°C for 2 h. After the digested fragments were separated in 1.5% agarose gel, the bands were visualized by ethidium bromide staining.

Single-strand conformation polymorphism (SSCP)

The SSCP was carried out by the method of Orita *et al.* (1989) with some modification. After aliquots of PCR products (3-5 μl) were mixed with two volumes of stop solution (95% formamide, 10 mM NaOH, 0.25% bromophenol blue and 0.25% xylene cyanol), they were denatured at 94°C for 4 min, and then immediately chilled on ice. The samples were electrophoresed at 6 W constant power for 12 h on a 0.5X MDE gel (FMC, USA). The separated DNA bands were visualized by CYBR Green I staining (FMC, USA).

DNA sequencing

The DNA sequencing was performed according to the chain termination method of Sanger *et al.* (1977) using a PCR product DNA sequencing kit (USB-Amersham, USA). PCR amplified mixtures were treated with shrimp alkaline phosphatase and exonuclease I at 37°C for 20 min to remove excess dNTPs and primers added at the PCR step. After inactivation of these enzymes by heating the mixtures at 80°C for 15 min, the mixtures were subsequently used as templates for DNA sequencing reaction.

Determination of lipids concentration

Concentrations of plasma total cholesterol and triglycerides were measured by enzymatic colorimetry methods using a commercially available kits (Boehringer Mannheim, Germany). The concentrations of HDL-cholesterol were determined from the supernatant after precipitation of the plasma with $MgCl_2$ and dextran sulfate. LDL-cholesterol levels were then calculated by the formula of Friedwald *et al.* (1972).

Data analysis

Allele frequencies were determined by gene counting, and expected numbers were compared with observed numbers assuming Hardy-Weinberg equilibrium using χ^2-analysis with Yates' correction. Statistical significance was accepted at the level of P=0.05. Plasma lipid and lipoprotein levels were compared between genotypes using one-way analysis of variance.

RESULTS

Detection of Thr-6Pro missense mutation

The analysis of SSCP for the PCR-amplified exon 2 DNA fragment showed several clearly distinguishing bands of electromobility, which suggests existence of a mutation at this region. The DNA sequencing analysis revealed a missense mutation of A to C at the cDNA position 86 which has been already reported from the analyses of liver and fibroblast LAL cDNA clones (Anderson and Sando, 1991; Ameis *et al.*, 1994). The missense mutation of A to C leads to the production of a *Hae*III restriction site, whereas the loss of an *Ava*II restriction site.

In this study, the mutation was analyzed by digestion of the PCR products with *Hae*III restriction endonuclease, followed by agarose gel electrophoresis (Fig. 1A). Digestion of 242-bp PCR fragment with *Hae*III produced either one fragment of non-digested 242-bp (allele *T*, encoding threonine) or two fragments of 124- and 118-bp fragments (allele *P*, encoding proline). In the heterozygous state, all bands were detected simultaneously. The two fragments of 124- and 118-bp in *P* allele were nondistinguished in the 1.5% agarose gel.

As shown in Fig. 1B, the Thr-6Pro mutation was also demonstrable by the SSCP analysis. Each allele showed an allele-specific band as well as a common band. The *T* allele-specific band showed slightly slower electromobility than the *P* allele-specific band. Heterozygote showed all three bands. When the PCR products from 20 individuals were examined by the method of SSCP, it revealed complete concordance of genotypes with the results from *Hae*III RFLP analysis. The DNA sequencing analysis confirmed the existence of the missense mutation of A to C which results in the substitution of threonine to proline

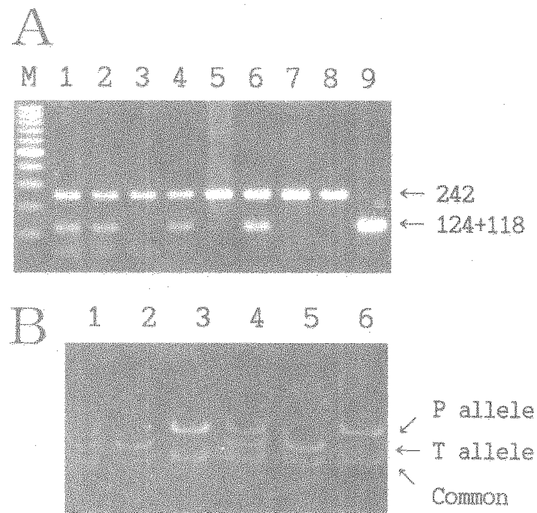

Fig. 1. Electrophoretic patterns of HaeIII RFLP and SSCP for the 242-bp PCR products encoding exon 2 of human LAL. A. Analysis of HaeIII RFLP. After the PCR products were digested with HaeIII endonuclease at 37°C for 2 hrs, they were separated on 1.5% agarose gel. M=100-bp DNA ladder; lanes 3, 5, 7 and 8=TT type; lanes 1, 2, 4 and 6=TP type; lane 9=PP type. B. Analysis of SSCP. The denatured PCR products were electrophoresed at 6 W constant power for 12 hrs on 0.5X MDE gel. Lanes 2 and 5=TT type; lanes 1 and 4=TP type; lanes 3 and 7=PP type.

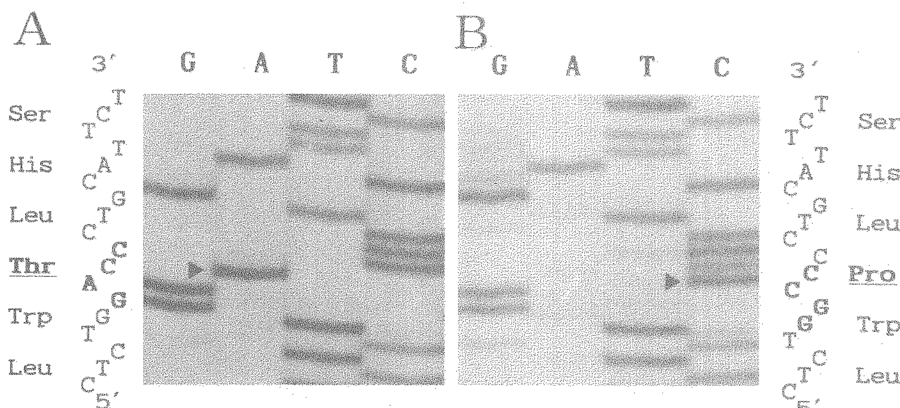

Fig. 2. Detection of A→C missense mutation by PCR product direct DNA sequencing. The non-purified PCR products were treated with shrimp alkaline phosphatase and exonuclease I, and then subjected to sequencing reaction of the chain termination method. The used primer was ex2F. The mutation of A to C (triangles) produced a HaeIII restriction site (bold letters) and an amino acid substitution of threonine to proline (bold and underlined letters). A. TT genotype. B. PP genotype.

and production of a *Hae*III restriction site (Fig. 2).

Population analysis of the mutation

When the mutation was analyzed in 221 control population, frequencies of allele *T* and *P* were 0.710 and 0.290, respectively (Table 1). The genotype distribution was 110 *TT*, 94 *TP* and 17 *PP*, which was well agreed with the Hardy-Weinberg equilibrium (χ^2=0.255, P>0.75). The observed and expected heterozygosities were 42.5 and 41.2%, respectively. The frequencies of allele *T* and *P* from the 86 FH group were 0.703 and 0.297, respectively. The genotype distribution was 47 *TT*, 27 *TP* and 12 *PP*. The observed number of *PP* genotype was somewhat high compared to the expected number, however, the genotype distribution was shown to fit the Hardy-Weinberg equilibrium (χ^2=5.229, P>0.050). The observed and expected heterozygosities of FH group were 31.4 and 41.8%, respectively. Within the total 307 examined samples, the allele frequencies of *T* and *P* were estimated to 0.708 and 0.292, respectively.

Concentrations of plasma triglyceride, total-, LDL- and HDL-cholesterol were determined to clarify association between lipid profiles and genotype. As shown in Table 2, an increase of about 60 mg/dl of triglyceride level was observed in *TP* heterozygotes of FH group, however, there was no statistically significant difference according to genotypes. The serum lipids levels between two groups were not compared in this study, since the average ages of control groups and patient groups are markedly different (average ages of control groups and FH groups are 22 and 37, respectively).

Table 1. Allele frequencies in several defined ethnic groups.

Populations	Studied No.	Frequencies	
		T	*P*
Koreans			
Control	221	0.710	0.290
FH	86	0.703	0.297
Total	307	0.708	0.292
Germans[a]	426	0.731	0.269
Sardinians (Italians)[a]	216	0.755	0.245
Japanese[a]	361	0.762	0.238

[a] Data from Muntoni *et al.* (1996).

DISCUSSION

The human LAL is required for the breakdown of cholesteryl esters and triglycerides that cells acquire from receptor-mediated uptake of LDL (Assman and Seedorf, 1995). The importance of LAL in the cholesterol metabolism is supported by the fact that the reduced enzyme activities are related to the development of hereditary atherosclerosis (Yatsu *et al.*, 1980; Coates *et al.*, 1986). Several mutations have been reported in the LAL gene as yet. One of them, a missense mutation A to C transversion at cDNA position 86 of exon 2 (Thr-6Pro) was found from the analysis of human liver and fibroblast cDNA (Anderson and Sando, 1991; Ameis *et al.*, 1994). Muntoni *et al.* (1996) performed the genetic analysis of the Thr-6Pro mutation at the population level for the first time using the mutagenically separated polymerase chain reaction (MS-PCR). They revealed that this mutation is present with high frequency in three randomly selected normal populations, Germans, Sardinians (Italians) and Japanese.

Here, we investigated the mutation in subjects with not only normal population, but also patients with FH using the method of SSCP and RFLP instead of MS-PCR. This is the first study analyzing the Thr-6Pro mutation in subjects with non-healthy group. This study revealed that Korean population has also a high mutation rate. When the mutation rate was compared with other previously studied groups, Korean population showed slightly higher rate than the

Table 2. Genotype distribution and serum lipid values between control and FH groups.

Lipids	Controls			FH		
	TT	*TP*	*PP*	*TT*	*TP*	*PP*
Observed number	110 (111.4a)	94 (91.0)	17 (18.6)	47 (42.5)	27 (35.9)	12 (7.6)
Total-cholesterol	178 ± 35	173 ± 17	182 ± 41	270 ± 47	256 ± 27	272 ± 39
LDL-cholesterol	109 ± 27	97 ± 32	103 ± 42	203 ± 49	176 ± 45	200 ± 27
HDL-cholesterol	37 ± 8	35 ± 7	39 ± 9	40 ± 12	45 ± 18	38 ± 6
Triglycerides	96 ± 45	93 ± 43	89 ± 31	132 ± 56	199 ± 106	140 ± 41

Lipid values are mean ±SD (mg/dl).
[a] The expected numbers are indicated in parentheses.

other populations (Table 1). However, allele distribution between populations was shown to agree with Hardy-Weinberg equilibrium.

The comparison of mutation distribution between control groups and FH groups showed that the mutation rate (*P* allele frequency) of FH group was slightly higher than that of the controls. However, it was no significant allele frequency difference between the two groups. It was interesting that the frequency of *PP* homozygous individuals of FH group was considerably higher than that of the controls. The appearance rate of *PP* homozygote in each group was 14.0 and 7.7%, respectively. However, we failed to find any significant deviation in the genotype distributions between two groups (χ^2=5.709, P>0.050). We also couldn't find any statistical difference between genotype and serum lipid levels in each group (Table 2). These results suggest that the Thr-6Pro mutation in the putative signal peptide may have no association with the increased risk of FH development. This is consistent with the suggestion of Muntoni *et al.* (1996) that the mutation may have minor or no functional significance. In biochemical point of view, it seems unlikely that the mutation produces a structural change of the LAL protein reducing the enzyme activity, since it locates on the putative signal peptide. However, we can not completely rule out the possibility that the mutation may have a mild defect on the localization of the protein and post-translational modification. Thus, to confirm the suggestion of no relevance between the mutation and risk for development of FH more evidently, further biochemical and genetic studies should be carried out in subjects with more ethnic populations.

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