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Genetic Analysis of Indian Subjects With Clinical Features of Possible Type IIa Hypercholesterolemia

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We performed genetic analysis in 55 patients with clinical features of possible type IIa hypercholesterolemia and 76 normolipemic healthy subjects for mutations and polymorphisms in the low-density lipoprotein (LDL) receptor (LDLR), apolipoprotein B-100 (APOB), apolipoprotein E (APOE), and hepatic lipase (LIPC) genes to elucidate the important genetic factors that can influence cholesterol levels in our population. None of the subjects showed mutations in part of exon 26 of the APOB gene, whereas two class 5 mutations were identified in exon 9 of the LDLR gene. First, an *E387K* mutation was observed in a Gujarati family in which both the parents were heterozygous for the mutation. Second, a *L393R* mutation was observed in a 38-year-old female. We found no correlation between LIPC −514C/T genotypes and cholesterol levels whereas the apoε4 allele frequency was significantly higher in cases and the apoE4 genotype was found to influence total cholesterol levels. J. Clin. Lab. Anal. 21:375–381, 2007. © 2007 Wiley-Liss, Inc.

Key words: apo B-100; apo E; FH; hepatic lipase; LDL receptor; mutations; polymorphisms

INTRODUCTION

Hypercholesterolemia is one of the most common disorders in India with a complex and mostly unknown genetic background (1). Several important genetic factors that can influence cholesterol levels include mutations in the low-density lipoprotein (LDL) receptor (LDLR) (2), apolipoprotein B-100 (APOB) genes (3), and polymorphisms in the apolipoprotein E (APOE) (4) and hepatic lipase (LIPC) genes (5). Mutations in the LDLR gene produce familial hypercholesterolemia (FH), a common autosomal dominant disorder characterized by tendinous xanthomas, a two- to three-fold elevation in serum LDL cholesterol, and increased risk of premature coronary artery disease (CAD) (2). In addition, mutations have been identified in the ligand for the LDLR, resulting in familial defective APOB (FDB) (3). FDB is most commonly caused by G-to-A substitution at position 10708 in exon 26 of the APOB gene (chromosome 2p23.24) creating an arginine to glutamine change (R3500Q). Additionally, the R3500W, R3531C, and R3486W changes in exon 26 are rare causes of FDB (6). Unlike in the APOB gene, to date over 800 mutations has been identified across the entire length of the LDLR gene (2,7). Neither the prevalence nor the type of mutations causing FH in India is known. However, a few point mutations have been reported in Indian immigrants residing in South Africa (8), of which the P664L mutation (FH Gujarat) was found to be most common (9).

Apo E and LIPC plays a pivotal role in the metabolism of both LDL and high-density lipoprotein (HDL) (4,5). A strong association of apoE4 with higher levels of total cholesterol and LDL has been reported in hypercholesterolemia, normolipidemia, and general population (10). Subjects with the apoε4 allele appear to be at increased risk of coronary heart disease (CHD) (10). Polymorphisms in the promoter region of the LIPC

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gene are associated with high LIPC activity, reduced levels of HDL small, dense LDL particles, and response to intensive lipid-lowering therapy (5,11).

In this study we performed genetic analyses in 55 patients with clinical features of possible type IIa hypercholesterolemia and 76 normolipemic healthy subjects for mutations and polymorphisms in the LDLR, APOB, APOE, and LIPC genes to elucidate the important genetic factors that can influence cholesterol levels in our population.

MATERIALS AND METHODS

Subjects

Blood samples were collected in plain and anticoagulated tubes after an overnight fast of 12 hr from 55 unrelated and related hypercholesterolemic individuals with clinical features of possible type IIa hypercholesterolemia (12). A total of 76 apparently healthy individuals who were normolipemic, nondiabetic, and normotensive with no past history of CHD were included as normal controls. Subjects were selected randomly from among those attending the regular health checkup program at the P.D. Hinduja National Hospital and Medical Research Centre, Mumbai, India.

Biochemical Analysis

Total serum cholesterol and triglyceride levels were measured on a Synchron Cx_7 autoanalyzer using standard enzymatic methods (Beckman Instruments, Fullerton, CA). HDL cholesterol levels were determined in the supernatant after the serum was subjected to precipitation with $MgCl_2$ and phosphotungstic acid (Beckman Instruments). LDL cholesterol was calculated by Friedewald's formula (13). Apolipoprotein A1 and B levels were measured by a rate nephelometry protein array system (Beckman Instruments). Lipoprotein(a) [Lp(a)] estimations were carried out by enzyme-linked immunosorbent assay (ELISA) (Innotest Lp(a); Innogenetics, Ghent, Belgium).

Genetic Analysis

DNA Extraction

The salting out procedure of Miller et al. (14) was used for extraction of genomic DNA from whole blood.

PCR Amplification, Mutation Detection, and Genotyping

Exons 9–10 (540 bp) of the LDLR gene was amplified using primers as described by Soutar et al. (9). The PCR-amplified DNA samples were screened for mutations by simultaneous heteroduplex (HDX) and single-strand conformation polymorphism (SSCP) analyses on low cross-linked polyacrylamide gels according to Kotze et al. (15) and with automated sequencing.

HDX analysis was performed to screen for mutations in part of exon 26 of the APOB gene according to the method of Kotze et al. (6).

APOE genotyping was performed by amplification refractory mutation system (ARMS)-PCR as described previously (16).

PCR-based restriction enzyme analysis was used to detect the −514C/T polymorphism of the LIPC gene (17). A C-to-T substitution 514 bp upstream of the transcription site creates an *Nla III* restriction site (5′-CATG-3′).

Statistical Analysis

Biochemical levels were expressed as mean ± standard deviation (SD). Statistical significances for these parameters between patients and controls were calculated by Student's *t*-test. Lp(a) levels were expressed as the median and the significance was calculated using the Mann-Whitney test. For all statistical measurements a value of $P < 0.05$ was considered to be statistically significant.

RESULTS

The lipids, lipoproteins, apolipoproteins, and Lp(a) levels of both patients and controls are shown in Table 1. The Lp(a) levels showed skewed distribution and were found to be significantly elevated in the patient group as compared to normolipemic controls.

From all the DNA samples analyzed, we identified a family (designated as "Family A") and a patient (designated as "Patient C") showing mutation in exon 9 of the LDLR gene (Fig. 1). Sequence analysis of Family A revealed a G-to-A transition at nucleotide position 1222 in exon 9. This substitution results in a Glu to Lys change at codon 387 (E387K). Mobility shift, observed in patient C, revealed a T-to-G transversion at nucleotide position 1241 in exon 9 on sequencing. The transversion results in a Leu to Arg substitution at codon 393 (L393R) of exon 9.

Both of these mutations can be rapidly detected using PCR-based restriction enzyme analysis. The E387K mutation abolishes an *Mnl I* restriction site and the L393R mutation creates a new site for *Aci I* restriction enzyme. The E387K mutation in Family A was readily confirmed by the presence of a 63-bp fragment in patients heterozygous for this change, whereas Patient C, exhibiting heterozygosity for L393R, showed the presence of additional 149-bp and 79-bp fragments.

TABLE 1. Lipid and lipoprotein results of patients with clinical features of possible FH and normal controls

Parameters	Controls (n = 76)	Patients (n = 55)
Male/female	37/39	30/25
Age (years)	40.17 ± 14.11	48.8 ± 12.97
Total cholesterol (mg/dL)	177.36 ± 26.79	295.44 ± 48.20*
Triglycerides (mg/dL)	95.49 ± 35.67	171.4 ± 78.32*
HDL-cholesterol (mg/dL)	47.32 ± 10.74	47.55 ± 11.42
Cholesterol/HDL ratio	3.79 ± 0.82	6.53 ± 1.94*
LDL-cholesterol (mg/dL)	110.58 ± 23.88	213.33 ± 45.14*
Apo A1 (g/L)	1.62 ± 0.52*	1.35 ± 0.36
Apo B (g/L)	1.03 ± 0.28	1.71 ± 0.56*
Lp(a) levels (mg/dL)		
Median	17.2	36
Range	1.0–106	4–131
Significance	$P<0.001$	
> 30 mg/dL	15 (19.74%)	30 (54.55%)
≤ 30 mg/dL	61 (80.26%)	25 (45.45%)
Significance	$\chi^2 = 17.14$, df = 1, $P<0.001$, OR, 4.9; 95% CI, 2.25–10.69	

*$P<0.001$.

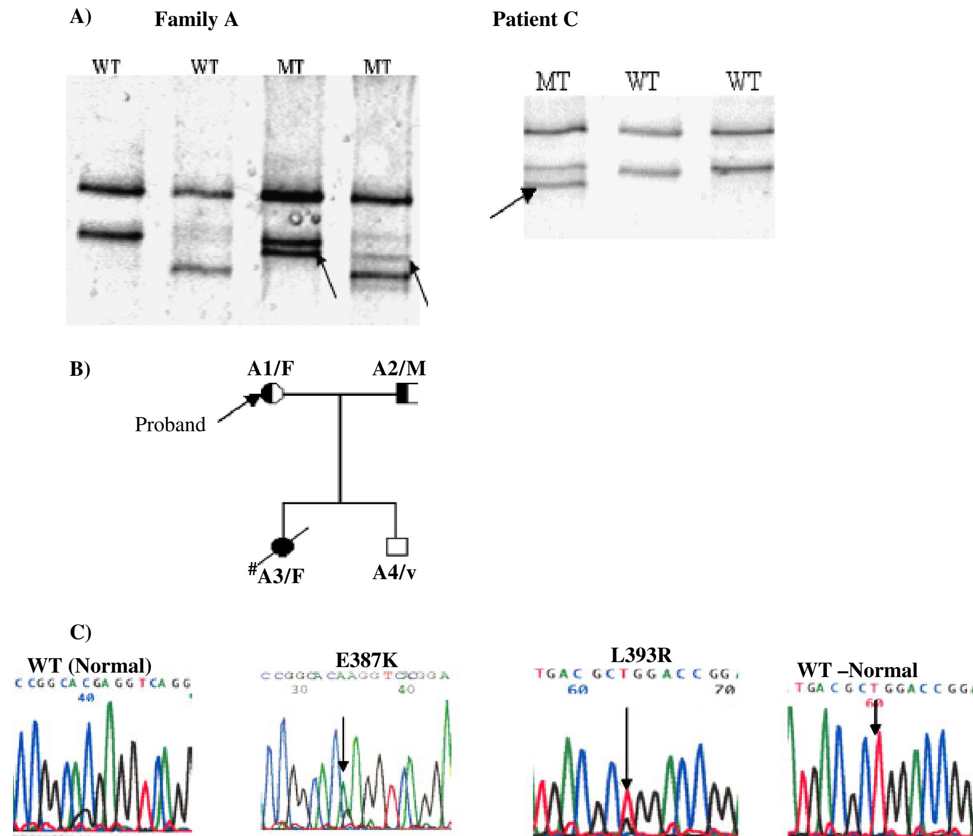

Fig. 1. **A:** Mobility shifts (indicated by arrows) seen as SSCP in Family A and Patient C in the region of exon 9-10 (~540 bp). WT, wild type; MT, mutant type. **B:** Pedigree of Family A. ♯, Deceased child (A3/F), no DNA sample available, deduced to be homozygous for E387K. **C:** Sequencing results of exon 9 showing transition resulting in Glu to Lys change at codon 387 (E387K) and transversion resulting in Leu to Arg change at codon 393 (L393R) as compared to normal WT.

Using PCR-based restriction enzyme analysis, we identified the E387K mutation in a 10-year-old male child in the first-degree relatives of the Patient A2/M (father) in Family A, confirming the diagnosis of FH. His total cholesterol was 202 mg/dL and the LDL cholesterol was 135 mg/dL (data not shown).

TABLE 2. Comparison of lipid and lipoprotein levels according to genotype

	Controls		Patients	
Parameters	E4-present (n = 8)	E4-absent (n = 68)	E4-present (n = 18)[a]	E4-absent (n = 33)
Age (years)	37.37±24.30	40.5±12.65	46.38±13.18	47.52±13.06
T.Chol (mg/dL)	192.13±43.98	175.61±23.91	327.88±44.58	290.30±27.89***
Triglyceride (mg/dL)	105.75±36.77	94.27±35.62	219.22±100.5	148.48±52.59**
HDL-C (mg/dL)	50.12±17.81	46.98±9.74	48.38±14.08	48.24±9.3
LDL-C (mg/dL)	119.88±36.72	109.48±22.04	232.74±50.22	211.79±27.28
Apo A1 (g/dL)	1.86±0.52	1.59±0.51	1.47±0.44	1.24±0.27*
Apo B (g/dL)	1.10±0.24	0.98±0.19	2.21±0.61	1.5±0.31***
Lp(a) median	13.0	17.5	29.5	40

*$P<0.05$.
**$P<0.01$.
***$P<0.001$.
[a]Four patients on treatment were excluded.
T.Chol, total cholesterol; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol.

TABLE 3. Comparison of lipid and lipoprotein levels according to genotype

	Controls			Patients		
Parameters	CC (n = 53)	CT (n = 22)	TT (n = 1)	CC (n = 34)	CT (n = 18)	TT (n = 3)
Age (years)[a]	39.66±13.73	41.68±15.76	44	47.53±12.63	52.56±12.92	40.67±15.53
Cholesterol (mg/dL)[a]	176.42±28.27	179.91±23.92	171	285.44±49.85	312.22±42.16	308±46.36
Triglyceride (mg/dL)[a]	96.62±35.13	93.68±38.24	75	160.06±64.87	189.17±87.53	193.33±159.16
HDL-C (mg/dL)[a]	47.92±10.64	45±10.44	66	47.12±11.49	49.44±12.03	41±3.61
LDL-C (mg/dL)[a]	108.04±25.34	117.64±18.93	90	206.4±47.94	225.22±34.27	220.33±70.36
ApoA1 (g/dL)[a]	1.68±0.52	1.51±0.5	1.29	1.3±0.28	1.36±0.43	1.85±0.34*
Apo B (g/dL)[a]	1.06±0.3	0.98±0.19	0.89	1.59±0.57	1.87±0.56	2.02±0.06
Lp(a)[b]	16.5	22.5	17	42	29	11

*$P<0.05$.
[a]Significance was tested using one-way ANOVA.
[b]Significance was tested using Kruskal-Wallis test.

No mobility shifts were observed in any other subjects included in the study in any exons and the promoter region of the LDLR gene, apart from those due to known polymorphic sites.

None of the DNA samples screened for FDB showed the presence of HDXs, indicating the absence of mutation in the part of exon 26 of the APOB gene.

The overall distribution of APOE genotypes was significantly different ($P<0.01$) in the patients as compared to the controls. The APOE genotype distribution was in Hardy-Weinberg equilibrium. No E2/E2 and E2/E4 genotypes were observed. The ε3 were the most common allele in both the groups, being highest in the controls (92.7%). The prevalence of the ε2 allele was similar in the normolipemic controls (1.9%) as compared to the patient group (1.8%). The frequency of the ε4 allele was higher in the patient group (21%) as compared to 5.2% in the controls ($\chi^2 = 19.87$, degrees of freedom [df] = 1, $P<0.001$, odds ratio [OR] 6.03; 95% confidence interval [CI] 2.56–14.01). None of the lipid and lipoprotein levels were influenced by apoE4 genotypes in the control group. In the patient group, the triglycerides, apo A1, and apo B levels were significantly higher in the apoE4-present group than in the apoE4-absent group. The total cholesterol and LDL cholesterol tended to be higher, but the difference was not statistically significant. Table 2 shows the lipids and lipoprotein profiles in these two groups.

The genotype distribution for the −514 C-to-T polymorphic locus did not deviate significantly from Hardy-Weinberg equilibrium. Also, the allele frequencies between the control and patient groups were not significantly different. The frequency of the rare "T" (+) allele was found to be 0.16 and 0.22 in the control and the patient group, respectively. The association of the three genotypes of the LIPC gene with age, lipid,

and lipoprotein levels in our population was examined by one-way analysis of variance (ANOVA) (Table 3). The T/(+) allele of the −514 promoter polymorphism was associated with higher apo A1 levels ($P = 0.035$) in the patient group. None of the other parameters differed within the genotypes. Since the number of homozygotes observed was very few (one in controls and three in patients) we also evaluated the effect of T allele by dividing the two study groups into: T-present and T-absent. None of the parameters varied significantly in the control and patient groups.

DISCUSSION

In this first study on complete mutation analysis of the entire coding and promoter region of the LDLR gene in Indian individuals with clinical features of possible type IIa hypercholesterolemia, we identified two missense mutations in the LDLR gene.

First, an E387K mutation in exon 9 was observed in a Gujarati family (Family A) in which both the parents harbored the same mutation (Fig. 1B). Their family history showed that they had a child (A3/F), who expired at 8 years of age and whose total cholesterol was >600 mg/dL (>15 mmol/L). Unfortunately, the DNA sample was not available for analysis. Since both the parents are heterozygous for the E387K mutation it can be predicted that the child must have been homozygous for the change, resulting in classical features of homozygous FH. DNA analysis of their 4-year-old child (A4/M) did not show the presence of this or any other mutation and thus it is unlikely that he may have FH. The E387K mutation has been previously reported designated as FH Algeria-1 (2) and has also been identified in an Asian Indian (of Gujarati origin) residing in the United Kingdom (18). This mutation occurs at a CpG dinucleotide, a mutational hotspot underlying most human genetic diseases. Such mutations had been detected in different ethnic groups and might have occurred independently.

The second missense mutation, L393R, was also identified in exon 9, in another subject designated Patient C. This L393R mutation has been identified for the first time in an FH patient from India. However, the same has been previously reported in homozygous FH patient from South Africa (19) and also accounts for 7% of FH cases in China (7,20). It is highly unlikely, that they would share a common ancestral gene or a haplotype and thus this could be a recurrent mutation.

None of the above patients identified with LDLR mutations had any history of coronary events during the course of the study.

Based on the studies of similar mutations in the epidermal growth factor (EGF)-precursor homology domain of LDLR (2), both of the mutant alleles are predicted to code for an LDLR protein that fails to recycle back to the cell surface, resulting in a recycling defective allele (class 5 phenotype) and therefore pathogenic.

To date, over 10 different point mutations have been identified in different exons of the LDLR gene among Indian immigrants in South Africa and the United Kingdom (8). We have previously identified two novel insertion mutations (21) and in this study we identified two missense mutations. However, no common mutations of diagnostic value for FH in India were observed. In a highly heterogeneous population like India, with various ethnic groups, it is unlikely that a common founder mutation could exist.

No carriers of APOB gene mutation(s) in part of exon 26 were observed, indicating that this gene does not influence cholesterol levels in our population. However, the possibility of a low rate or other mutations and polymorphisms at the APOB locus cannot be ruled out.

Though studies have shown that ε4 allele is associated with elevated levels of total and LDL cholesterol (22,23), there are many studies that have failed to report any association of APOE genotypes in definitive type IIa (FH) subjects (24,25). In our study a high frequency of apoE4-present genotype and a greater tendency toward the ε4 allele frequency in the patient group than in the controls was observed. Furthermore, when the patient population was divided into two groups—those with apoE4-present (n = 22) and those with apoE4-absent (n = 33)—no effect of apoE4 genotype was observed on total and LDL cholesterol levels. The absence of any significance for total cholesterol and LDL cholesterol may be attributed to the fact that there were four patients with the E3/E4 genotype who were on treatment for hypercholesterolemia and their total cholesterol levels were within the normal range of 130–220 mg/dL. When these patients were excluded from the analysis, the total cholesterol levels were found to be significantly elevated in the apoE4-present subjects along with triglyceride, apo A1, and apo B levels, but yet the difference for LDL cholesterol remained nonsignificant. These findings are in accordance with a study by Eto et al. (26) in heterozygous FH subjects. ApoE4 is known to promote the development of CHD through its hypercholesterolemic effect (10). In addition, hypertriglyceridemia observed in apoE4-present cases might cause a higher incidence of CHD in such subjects.

Association studies indicate that the rare T (−514 C-to-T) allele is associated with increased concentrations of HDL and apo A1 levels (17,27). The frequency of the T allele has been reported to be 0.52 to 0.582 in African Americans (28), 0.14 to 0.24 in the white population (29), and 0.21 in Dutch families with familial combined

hyperlipidemia (FCH) (30). In our study the frequency of the T allele was found to be 0.16 in the normolipemic healthy group and 0.22 in individuals with clinical features of type IIa hypercholesterolemia. The polymorphism was not significantly associated with any of the lipid and lipoprotein levels except apo A1 in the patient group, indicating that this variant may affect HDL metabolism but does not influence total and LDL cholesterol levels. There have been studies in which no association of the polymorphism with lipids and lipoprotein levels has been reported (29,31).

In summary, we investigated 55 patients with clinical features of possible type IIa hypercholesterolemia. We identified two missense mutations in the LDLR gene. No carriers of APOB mutations were detected. The apoE4 genotype was found to influence total cholesterol levels and no correlation was found between the −514C/T genotypes of LIPC gene and the cholesterol levels.

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