Vol. 179, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1991 Pages 78-84

COMPOUND HETEROZYGOSITY FOR MUTANT HEPATIC LIPASE IN FAMILIAL HEPATIC LIPASE DEFICIENCY

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Received July 8, 1991

SUMMARY: In a kindred with three hyperlipidemic subjects who had premature atherosclerosis and complete deficiency of hepatic lipase activity, we had previously identified a novel structural hepatic lipase gene variant. We now report the identification of three more hepatic lipase gene mutations in this family and demonstrate that compound heterozygosity for two hepatic lipase mutations (designated S267F and T383M) underlies hepatic lipase deficiency.

Hepatic lipase (HL) is important in the metabolism of very low density lipoprotein (VLDL) remnants and high density lipoproteins (HDL) (1-3). Subjects with a complete deficiency of HL have premature atherosclerosis and elevated total cholesterol, triglycerides (TG), very low density lipoprotein (VLDL) and TG-rich low density lipoprotein (LDL) and HDL subfractions (4,5). The human HL gene on chromosome 15q21 is 35 kilobases (kb) in size with 9 exons that encode a 1.6 kb mRNA that is translated into a 477 amino acid protein (6-10). There are four reported families with heritable HL deficiency (4,11-13).

We had analyzed genomic DNA from two unrelated HL deficient families, one each from Ontario (OHLD) and Quebec (QHLD) (13). Screening of the HL exons of affected subjects with a method based on the polymerase chain reaction (PCR) (14) revealed a new C->T substitution that caused a threonine to methionine change at position 383 (T383M). All 6 individuals with complete HL deficiency from both families had T383M, while none of 50 random unaffected subjects had T383M. Most carriers of T383M had decreased HL activity compared with non-carriers (13). Given that subjects with complete HL deficiency were possibly compound heterozygotes for two HL mutations, we sequenced the HL gene in OHLD.

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0006-291X/91 $1.50

MATERIALS AND METHODS

Subjects and biochemical analyses

Members of kindred OHLD and unrelated normal individuals were studied in accordance with criteria of the University of Toronto Ethics Committee (4,5). The pedigree structure has been reported (5,13). All biochemical analyses in kindred OHLD were performed as reported (5).

HL genotype analysis

DNA was prepared as described (13). PCR primers for amplification of all 9 HL exons were synthesized based upon HL genomic sequence information (9,10). DNA from subject B2 with complete HL deficiency was amplified in a Tempcycler (Coy) with each pair of PCR primers using the motif described (13), but substituting various annealing temperatures optimized for each exonal reaction: 60 C for exons 1,2,3 and 5; 55 C for exon 7,8 and 9; and 63 C for exons 4 and 6. DNA from each exonal reaction was purified and sequenced directly from the 5′ and 3′ ends as described (13) using the PCR primers as sequencing primers. When a missense mutation (i.e. one each in exon 5 and 6) was identified in the affected subjects, all family members were genotyped with direct sequencing of exon 5 (N193S) and digestion of the PCR-amplified exon 6 (S267F) with HinfI (Boehringer Mannheim). Segregation patterns of the missense mutations in exons 5 and 6 were compared to the segregation of T383M. In addition, 10 normal control subjects were genotyped for N193S and 72 normal control subjects were genotyped for S267F.

Statistical and linkage analysis

Statistical analyses were performed with SAS (15). Linkage analysis was performed with LIPED (16).

RESULTS

New HL gene mutations

Heterozygosity for a new HL mutation in exon 4 (G->T at base 472, Val->Val at codon 133) that does not alter the amino acid sequence (V133V) was identified

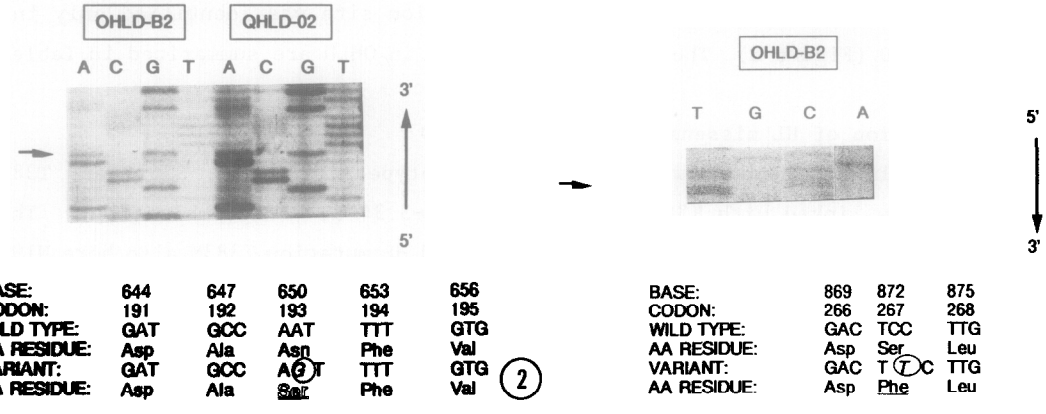

Figure 1. **DNA sequencing of HL exon 5.**
Two species of HL exon 5 were identified: the "wild type", which contained the normal reported HL DNA sequence (an A) at position 651 in codon 193, and a "variant" (a G) detected in HL deficient subjects from Ontario (OHLD-B2) and Quebec (QHLD-02).

Figure 2. **DNA sequencing of HL exon 6.**
Two species of HL exon 6 were identified: the "wild type", which contained the normal reported HL DNA sequence (a C) at position 873 in codon 267, and a "variant" (a T) detected only in the HL deficient subject from Ontario (OHLD-B2).

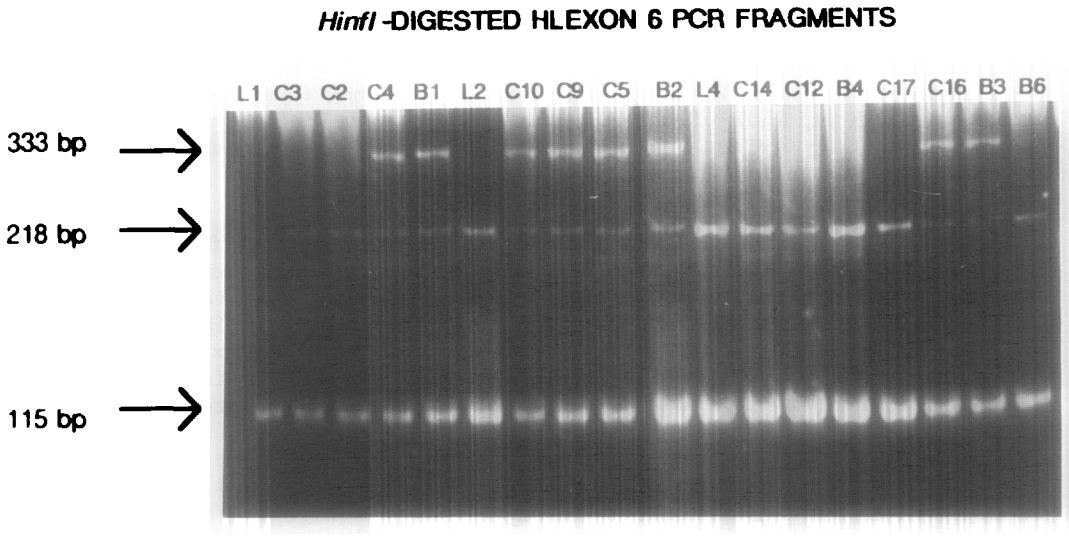

Figure 3. **HL exon 6 HinfI RFLP typing in OHLD.**
The S267F allele obliterates a HinfI site, creating a larger RFLP band in the heterozygous family members. Identification numbers were assigned according to reference 5. The three compound heterozygotes are B1, B2 and B3. Unrelated spouses are designated with the letter "L". The children of the compound heterozygotes and their siblings are designated with the letter "C". HinfI fragment sizes in base pairs (bp) are shown for alleles containing the normal (218 and 115 bp) and the mutant (333 bp) HL exon 6 sequence (S267F).

in B2 (data not shown). Heterozygosity for a second new HL mutation in HL exon 5 (A->G at base 651, Asn->Ser at codon 193) that alters the amino acid sequence (N193S) was identified in B2, and in an affected subject from the QHLD kindred (Figure 1). Heterozygosity for a third new mutation in exon 6 (C->T at base 1221, Ser->Phe at codon 267) that both alters the amino acid sequence (S267F) (Figure 2) and obliterates a HinfI restriction site was identified only in B2 from OHLD (Figure 3). The HL mutations found in OHLD are summarized in Table 1.

Segregation of HL missense mutations in OHLD

All OHLD members were completely genotyped for N193S, S267F and T383M. N193S was linked with T383M (peak LOD score=3.34 at 0% recombination). Thus, the allele that carried our initially defined HL mutation T383M also bore N193S. However, normal spouses (e.g L1) who lacked T383M were also found to have N193S, with the result that one offspring (C17) was homozygous for N193S, but had detectable HL activity. Two of 10 unrelated normal control subjects were heterozygous for N193S (data not shown).

TABLE 1. SUMMARY OF KNOWN HL MUTATIONS

DESIGNATION	EXON	BASE	CODON	DNA MUTATION	AMINO ACID CHANGE
V133V	4	472	133	G->T	Val->Val
N193S	5	651	193	A->G	Asn->Ser
S267F	6	873	267	C->T	Ser->Phe
T383M	8	1221	383	C->T	Thr->Met

TABLE 2. HL ACTIVITIES IN OHLD FAMILY MEMBERS CLASSIFIED ACCORDING TO HL GENOTYPE

GENOTYPE	NUMBER	HL ACTIVITY
S267F-/T383M-	2	9.71±1.46
S267F+/T383M-	5	3.88±1.93
S267F-/T383M+	4	7.18±3.77
S267F+/T383M+	3	none detectable

Post heparin HL activity (mean±SEM) expressed in μmol of free fatty acid/mL/h in OHLD family members (excluding unrelated spouses). The range in normal subjects of HL activity is 8 to 11 μmol/mL/h.

S267F was unique to OHLD, occurring only in the three subjects with complete HL deficiency and their first degree relatives. Furthermore, we observed that S267F was inherited on the opposite HL allele (i.e. *trans* to T383M) and that there were three compound heterozygotes for T383M and S267F. These three subjects were the three brothers who had complete HL deficiency. None of 72 unrelated normal control subjects had S267F, and this mutation was not found in HL deficient subjects from QHLD (data not shown).

HL activities in OHLD family members classified by HL genotype

When third generation OHLD family members were classified according to the presence or absence of S267F and T383M we observed three categories of subjects: normal (S267F-/T383M-), simple S267F heterozygotes (S267F+/T383M-) and simple T383M heterozygotes (S267F-/T383M+). Results of the assay of HL activities among the three groups are shown in Table 2. Mean (±SD) HL activity in S267F-/T383M- S267F+/T383M- and S267F-/T383M+ subjects were, respectively, 9.71±1.46, 3.88±1.93, and 7.18±3.77 μmol/mL/h. The three S267F+/T383M+ compound heterozygotes each had no detectable HL activity. General linear models analysis using age and sex as co-variates revealed a significant effect of HL genotype on levels of HL activity in the HL deficient subjects and their first degree relatives (P=0.004). Among simple T383M heterozygotes there was a wide range of HL activities (from 2.6 to 10.6 μmol/mL/h). Mean HL activities in simple S267F and T383M heterozygotes were significantly less than mean HL activity in normal relatives (P=0.001 and 0.03, respectively, t-test). Mean HL activity in simple heterozygotes for S267F and T383M were significantly different from each other (P=0.02).

DISCUSSION

The principal finding of this study is the identification of three novel HL mutations, designated as V133V, N193S and S267F, the latter two of which are missense mutations. Significantly, S267F was co-inherited in each of three brothers with complete HL deficiency along with a previously reported HL missense mutation (T383M). The new mutations were found by sequencing all the coding regions of the HL gene, and not with PCR screening of exons (14).

S267F and T383M appear to be etiologic in this family. V133V is a silent mutation with no physiologic relevance. Review of published HL sequence data revealed that V133V has been documented but not commented upon (6-11). Among 9 alleles sequenced to date, the G at base 472 is present in 6, whereas the T at base 472 is present in three, implying that it is a common DNA variant.

S267F appears to be clinically relevant based upon four facts. First, S267F is present in subjects with complete HL deficiency and their first degree relatives. Second, HL activity in simple S267F heterozygotes is significantly depressed compared to genotypically normal family members. Third, none of 72 normal controls had S267F. Finally, serines at various positions appear to be important in lipases (17-19). The normal serine at HL position 267 is conserved amongst all members of the lipase family in all species (5), and mutagenesis of this serine to alanine in lipoprotein lipase resulted in an expressed protein with immunoreactive mass but greatly reduced lipolytic activity (17). Phenylalanine would be expected to be even more disruptive. Thus, the genetic, biochemical and molecular data indicate that S267F is functionally relevant in clinical HL deficiency in OHLD. S267F was not seen in QHLD suggesting that for HL deficiency, Quebecois are distinct.

We previously demonstrated that T383M is associated with HL deficiency (13). It is also etiologic in HL deficiency based upon four facts. First, T383M was found in subjects with complete HL deficiency and their first degree relatives. Second, mean HL activity in simple T383M heterozygotes was significantly depressed compared to genotypically normal family members. Third, none of 50 normal control subjects had T383M. Finally, T383M occurs within a region that is adjacent to an N-linked glycosylation site. The normal threonine at position 383 is conserved in the HL gene among species, and is unique to the HL gene compared with other members of the lipase family within humans. Thus, T383M is likely also to be clinically relevant. However, we noticed a wide variation in HL activities amongst T383M heterozygotes in OHLD (13). This has now been shown to be due in part to the fact that three T383M heterozygotes with undetectable HL activity are actually compound heterozygotes for T383M and S267F. However, among simple T383M heterozygotes, there still appears to be variability in the level of HL activity. This would imply that the deficiency imparted by T383M might be modulated by other factors such as age or sex, or by the effects of variation at other genes.

We also documented that N193S occurs on alleles that encode a protein that is normal at position 383 in subjects with normal HL activity. This suggests that the HL encoded by an allele that harbours N193S in isolation is a normal variant. The normal asparagine at position 193 is not predicted to be involved in glycosylation. It is however possible that the protein translated from an allele that encodes both N193S and T383M may be defective as a result of either

T383M alone or perhaps through some interaction with N193S. Since we do not have an allele with T383M that does not also have N193S, a possible interaction between the *cis* mutations may be difficult to prove without site-directed mutagenesis that creates 3 species of HL: one that contains N193S alone, one that contains T383M alone and one that contains N193S and T383M together. *In vitro* expression and functional assays of each species of mutant will be necessary. However, because N193S is prevalent (allele frequency approximately 10%) in normal control subjects, we postulate that in isolation it has no clinically relevant effect.

In summary, subjects with complete HL deficiency are compound heterozygotes for mutant HL. T383M likely has a deleterious effect on HL activity, but also is associated with a wide range of HL activity. S267F likely has a more severe deleterious effect on HL activity. HL deficiency has been considered a rare disease because the phenotype had been difficult to diagnose without specialized biochemical tests. It has been suggested that HL deficiency among dyslipidemic subjects may be more prevalent than traditionally thought (20). DNA-based screening for HL mutations will allow for this to be tested.

ACKNOWLEDGMENTS

We acknowledge the valued assistance of Camilla Vezina, Liling Tu and Graham Maguire. This work was supported by operating grants from the Heart and Stroke Foundation of Ontario and the MRC (Canada). Dr. Hegele is a McDonald Scholar of the Heart and Stroke Foundation of Canada.

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