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HMG CoA Lyase Deficiency: Identification of Five Causal Point Mutations in Codons 41 and 42, Including a Frequent Saudi Arabian Mutation, R41Q

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Summary

The hereditary deficiency of 3-hydroxy–3-methylglutaryl (HMG) CoA lyase (HL; OMIM 246450 [http://www3.ncbi.nlm.nih.gov:80/htbin-post/Omim/dispmim?246450]) results in episodes of hypoketotic hypoglycemia and coma and is reported to be frequent and clinically severe in Saudi Arabia. We found genetic diversity among nine Saudi HL-deficient probands: six were homozygous for the missense mutation R41Q, and two were homozygous for the frameshift mutation F305fs(−2). In 32 non-Saudi HL-deficient probands, we found three R41Q alleles and also discovered four other deleterious point mutations in codons 41 and 42: R41X, D42E, D42G, and D42H. In purified mutant recombinant HL, all four missense mutations in codons 41 and 42 cause a marked decrease in HL activity. We developed a screening procedure for HL missense mutations that yields residual activity at levels comparable to those obtained using purified HL peptides. Codons 41 and 42 are important for normal HL catalysis and account for a disproportionate 21 (26%) of 82 of mutant alleles in our group of HL-deficient probands.

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Introduction

Hereditary deficiency of 3-hydroxy–3-methylglutaryl (HMG) CoA lyase (HL; E.C. 4.1.3.4) (OMIM 246450 [http://www3.ncbi.nlm.nih.gov:80/htbin-post/Omim/dispmim?246450]) causes up to 16% of inherited metabolic disease in Saudi Arabia (Ozand et al. 1992). Furthermore, the clinical signs of HL deficiency, which include hypoketotic hypoglycemia, fatty liver, coma, mental retardation, and a characteristic pattern of urinary organic acids (Gibson et al. 1988), may be particularly severe in Saudi patients (Ozand et al. 1991). Defining the molecular basis of HL deficiency in Saudi Arabia has potential significance for patient care, metabolism, and population genetics.

HL is a mitochondrial and peroxisomal enzyme (Ashmarina et al. 1994). In mitochondria, HL catalyzes the last step of both leucine degradation and ketogenesis. Its role in peroxisomes is unknown. We have cloned the human HL cDNA (Mitchell et al. 1993) and gene (Wang et al. 1996) and have reported two frameshift mutations (Mitchell et al. 1993, 1995) and two large HL gene deletions (Wang et al. 1996) in HL-deficient patients. A splice-site mutation has also been reported (Buesa et al. 1996). The only missense mutation reported, to date, is H233R. We expressed and purified mutant HL containing an arginine at residue 233 and used this to demonstrate that H233 is essential for HL catalysis (Roberts et al. 1996).

In this article, we report six new HL point mutations, including two in Saudi patients, R41Q and F305fs(−2). We also adapt the bacterial expression system for rapid screening of the activity of HL mutant proteins. In 32 non-Saudi patients, we found four other deleterious point mutations clustered within a radius of 4 nt of R41Q. This demonstrates genetic diversity among Saudi HL-deficient patients and implicates HL residues 41 and 42 as important for normal catalytic function of HL.

Material and Methods

Patients

Patients described in this article are summarized in table 1. In our group of 41 HL-deficient probands, 9 originate from Saudi Arabia. Affected siblings are excluded as probands. Extensive genealogical information is not available, and we cannot formally exclude kinship among some of the probands.

SSCP Detection of Mutations

We used flanking intronic primers (Wang et al. 1996) to amplify exons 2–9 of the HL gene. Specifically, 50 μmol of each primer and 250 ng of genomic DNA were added to a mixture containing a final concentration of dNTPs, each 12.5 μM; ^{35}S-dATP and ^{35}S-dCTP (New England Nuclear), each 12.5 μCi, and *Taq* polymerase, 2.5 U; Tris HCl (pH 8.4), 20 mM; KCl, 50 mM; $MgCl_2$, 1.5 mM in a volume of 50 μl. Following a hot start, 25 amplification cycles were performed as follows: 94°C, 15 s; 58°C, 15 s; and 72°C, 15 s, with a final 5-min extension at 72°C. Electrophoresis was performed under each of three different conditions as described elsewhere (Michaud et al. 1992).

Allele-Specific Oligonucleotide (ASO) Detection of Mutations

HL exon 2 was amplified (Wang et al. 1996), and samples were slot blotted and hybridized to radiolabeled ASOs. Oligonucleotides were end labeled using α^{32}P-ATP as described by Sambrook et al. (1989), except that spermidine HCl, 1 mM, and EDTA, 1 mM, were not used in the kinase buffer. The hybridization buffer was 5 SSPE (1 × SSPE is NaCl, 15 mM; NaH2PO4, 10 mM; and EDTA, 1mM), 1% SDS, and 5 × Denhardt's solution (1 × Denhardt's is Ficoll, BSA, and polyvinyl pyrrolidone, each 0.2 mg/ml). Following a 30-min prehybridization, hybridization was performed for 3 h. Washing was performed in 2 × SSC (1 × SSC is sodium citrate, 1.5 mM, and NaCl, 15 mM) and 0.5% SDS, once for 10 min. The oligonucleotides, their orientations, and their positions in the HL cDNA were as follows. The normal sequence oligonucleotide is 5′-GGTCCCCGAGATGG-3′ (the R41 codon is underlined), sense strand, hybridization temperature 35°C; washing temperature 51°C. The mutant oligonucleotide is 5′-CCCCAAGATGGACTACA-3′ (the Q41 codon is underlined), sense strand, hybridization temperature 45°C; washing temperature 48°C. For the D42E mutation, the oligonucleotide was 5′-CGAGAGGGACTACA-3′ (E42 underlined) sense strand, hybridation temperature 40°C; washing temperature 48°C.

Table 1

Ethnic Origin, Previous Descriptions, and Mutations of the HL Patients Studied

Patient	Ethnic Origin	Clinical Report	Genotype[a]
HL3	Saudi	Ozand et al. (1991), patient 10	R41Q/R41Q
HL4	Saudi	Ozand et al. (1991), patient 7	R41Q/R41Q
HL5	Saudi	Ozand et al. (1991), patient 11	R41Q/R41Q
HL6	Saudi	Ozand et al. (1991), patient 8	R41Q/R41Q
HL33	Saudi	…	R41Q/R41Q
HL37	Saudi	…	R41Q/R41Q
HL41	Turkish	…	R41Q/R41Q
HL36	Italian	…	R41Q/?
HL8	Saudi	Greene et al. (1984)	F305fs(−2)/F305fs(−2)
HL9	Saudi	Dasouki et al. (1987)	F305fs(−2)/F305fs(−2)
HL25	English/German	…	R41X/?
HL14	Cajun Black	Gibson et al. (1988), patient 1	D42H/?
HL26	Dutch	…	D42G/D42G
HL13	Austrian	Plöchl et al. (1989, 1990, 1992)	D42E/D42E
HL7	Saudi	…	?/?

[a] A question mark (?) signifies an as-yet-uncharacterized mutant allele(s).

Cloning of Mutant HL Alleles

Our initial experiments were performed using the pGEX-2T plasmid (Pharmacia) that expresses fusion proteins with glutathione S-transferase. Into this vector we cloned a *Bam*HI-*Eco*RI fragment of the human HL cDNA that contains the sequence of the mature HL peptide. We introduced the 5′ *Bam*HI site immediately upstream of the Thr 28 residue that is the first residue of mature HL and used a 3′ cloning *Eco*RI site that was present in the original vector. Mutant cDNAs from patients were prepared for cloning into this vector and sequencing. Reverse transcription was performed from patient fibroblast RNA by use of the primer 5′-TTGATGTTCTTCTTGGT-3′, complementary to HL cDNA residues 419–403. The normal and mutant fusion proteins produced by this vector were unstable.

We, therefore, adopted an expression system in the pTrc HL-C323S vector (Roberts et al. 1994). This vector contains the coding sequence of the normal, mature HL peptide plus 5′ Met and Gly codons that contain a *Nco*I cloning site. Also the codon of the reactive Cys 323 residue has been mutated to a Ser codon. Purified re-

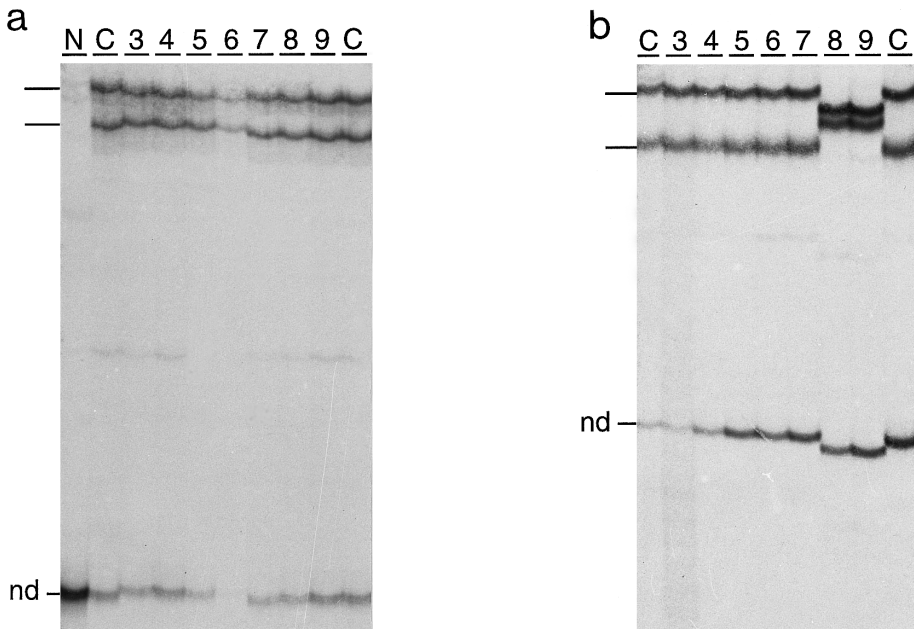

Figure 1 SSCP detection of R41Q and F305fs(−2). *a,* Exon 2. *b,* Exon 9. Patient numbers are shown above the lanes. Migration was performed at 4°C in the absence of glycerol. Horizontal lines indicate the normally migrating fragments, which contrast with the abnormal migration in panel *a* of HL3–6 and in panel *b* of HL8 and HL9. C = normal control; nd = nondenatured fragment.

combinant C323S HL closely resembles wild-type HL enzymatically but is more stable (Roberts et al. 1994). We transfered the mutant sequences from the PGEX-2T vectors by use of the above 3′ primer and the 5′ primer 5′-ATCCATGGGCACTTTACCAAAGCGG-3′ (residues 76–96, plus a 5′ *Nco*I site, underlined). Thirty cycles were performed as follows: 94°, 15 s; 57°, 15 s; and 72°, 15 s. The reaction products were digested with *Nco*I and *Sst*I and were used to replace the corresponding cassette in the pTrcHL-C323S plasmid. The mutant cassette was sequenced on each strand.

Bacterial Expression of Mutant HL, Enzyme Assays, Western Blot Analysis, and Protein Purification

The following were performed as described elsewhere: protein determination (Bradford 1976); HL assays in crude lysates (Ashmarina et al. 1994) and in purified HL (Kramer and Miziorko 1980); and HL expression (Roberts et al. 1994). Lysate supernatants obtained following centrifugation at 100,000 *g* for 60 min were resolved by PAGE, and Western blotting was performed as described by Ashmarina et al. (1994), by use of a rabbit polyclonal anti-HL antibody. Recombinant HL was purified as described by Roberts et al. (1994).

Table 2

Comparison of HL-Specific Activity in Crude Lysate Preparations and in Purified Normal and Mutant HL Peptides

	HL-Specific Activity in (μmol acetoacetate produced/min/mg protein)	
Mutation	Crude Supernatant	Purified Protein
Normal	9.6 (100)	159.0 (100)
R41Q	.06 (.6)	.002 (.00125)
D42H	.00 (0)	<.001 (<.0006)
D42G	.03 (.3)	.012 (.0075)
D42E	1.14 (12.0)	7.0 (4.4)

Note.—Values in parentheses represent percentage of wild-type values.

Results and Discussion

In SSCP studies of patients HL3–9, we found two different abnormalities in Saudi patients, one in exon 2 (fig. 1*a;* HL3–6), the other in exon 9 (fig. 1*b;* HL8 and 9).

The subtle migration abnormality of exon 2 was due to a G→A transition at residue 122 that changes the normal Arg 41 codon (CGA) to a Gln codon (CAA) and is designated R41Q (fig. 1). In exon 9, we found a deletion of a TT dinucleotide in the F305 codon, F305fs(−2). We searched for R41Q in our set of 41 HL-deficient probands by ASO analysis (fig. 2). As judged by SSCP, direct sequencing, and ASO analysis, six of nine Saudi patients were homozygous for R41Q, and two were F305fs(−2) homozygotes. Samples from the parents of the affected patients were unavailable, so we cannot formally confirm homozygosity in these patients, as opposed to compound heterozygosity with a second mutant allele not detectable by the procedures described above. We tentatively classify these patients as homo-

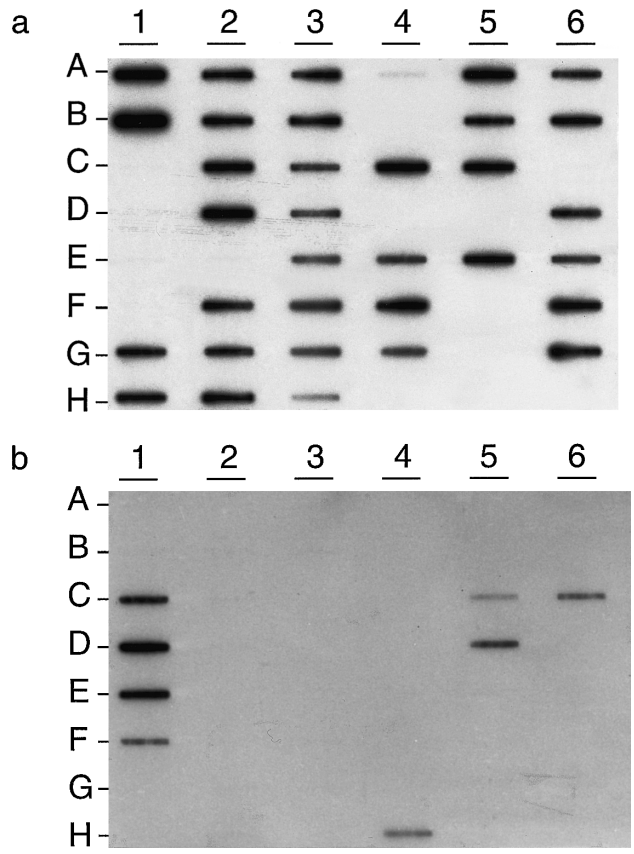

Figure 2 ASO screening. *a*, R41R, and *b*, R41Q, in 41 HL-deficient probands. The positions of the array correspond to the following patients: 1C, HL3; 1D, HL4; 1E, HL5; 1F, HL6; 1G, HL7; 1H, HL8; 2A, HL9; 2E, HL13; 2F, HL14; 3H, HL25; 4A, HL26; 4H, HL33; 5C, HL36; 5D, HL37; and 6C, HL41. 5E and 6G are normal controls. 5F–H and 6H are blanks. 4B and 4D are, respectively, from the previously reported patients HL26 and HL29, who have deletions encompassing HL exon 2 (Wang et al. 1996).

zygotes (table 1). One patient, HL7, was normal on SSCP analysis, and his causal mutation has not yet been defined. Therefore, at least three mutant alleles cause HL deficiency in Saudis.

One patient (HL41, Turkish) was a R41Q homozygote; another (HL36, Italian) was a compound of R41Q and another mutant allele (3 [4.7%] of 64 of non-Saudi mutant alleles) (fig. 2). By SSCP analysis, F305fs(−2) was not detected in the non-Saudi probands (not shown). We also examined amplified HL exon 2 fragments from 86 normal controls of diverse ethnic origins. None hybridized to the R41Q oligonucleotide, and all hybridized to the normal (R41R) allele (not shown).

Interestingly, on ASO analysis, samples from HL13 and HL26 hybridized with neither the R41Q nor the normal oligonucleotide (fig. 2). Furthermore, SSCP analysis of HL exon 2 from four non-Saudi probands (HL13, -14, -25, and -26) showed abnormal migration, distinct from that of R41Q (not shown). By direct sequencing, all patients had mutations in codons 41 or 42: R41X (HL25, heterozygous), D42E (HL13, homozygous), D42H (HL14, heterozygous), and D42G (HL26, homozygous) (fig. 3; table 1). R41 and D42 are conserved in HL from mouse (Wang et al. 1993), chicken (Mitchell et al. 1993), *Pseudomonas mevalonii* (Anderson and Rodwell 1989), and *Rhodospirillum rubrum* (Baltscheffsky et al. 1997), suggesting possible functional significance.

We then performed bacterial expression of HL peptides containing missense mutations at codons 41 and 42. Despite the presence of approximately normal amounts of HL antigen (fig. 4), HL activity was markedly reduced, both in the supernatants of cells expressing each mutant tested and in the purified mutant HL peptides (table 2). As a control for possible artifactual mutations outside of the restriction cassette containing codons 41 and 42, we replaced the mutant cassette with one of normal sequence. In each case, normal levels of HL activity were restored (data not shown).

We conclude that R41Q, D42E, D42G, and D42H

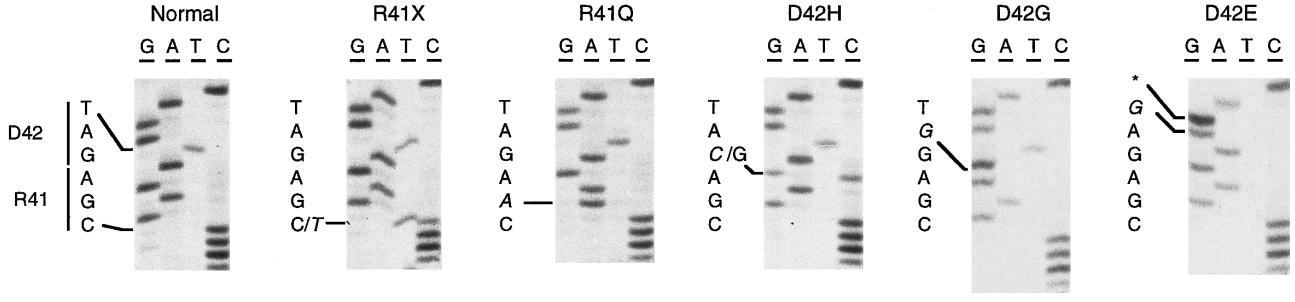

Figure 3 R41 and D42 codons. Sequence of normal and mutant genomic DNA are shown. The mutated residues are shown in italics. A compression artifact is present in the D42E sequence and is indicated with an asterisk (*), but the sequence was confirmed by sequencing the opposite strand and by ASO hybridization (not shown).

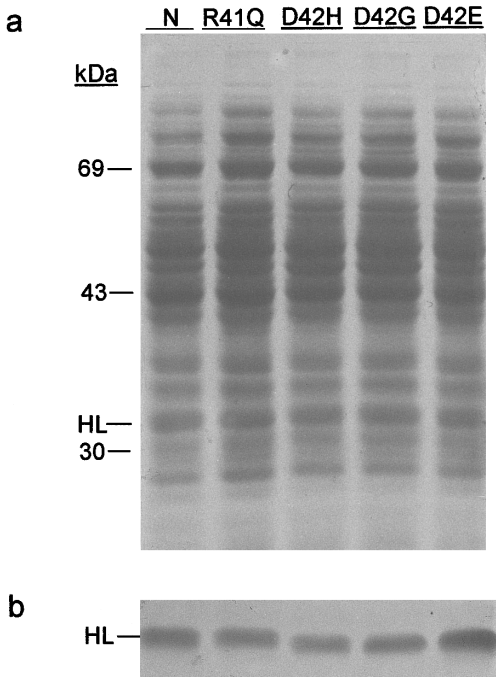

Figure 4 Expression of normal and mutant HL cDNAs in bacteria. *a*, Coomassie blue–stained gel of bacterial lysates. *b*, Western blot. Ten micrograms of protein were loaded in each lane. The position of HL is indicated in each panel.

are causal in HL deficiency. Even D42E, which in view of the similarity of the two residues involved is intuitively expected to be mild, substantially reduces HL activity. The two premature termination mutations described here, R41X and F305fs(−2), are predicted to generate HL peptides that lack essential amino acid residues and are also considered to be causal for HL deficiency.

Of note for diagnosis, our technique of bacterial expression of mutant HL cDNAs and assay of HL activity in crude cell lysates proved in all cases to be a conservative estimate of the extent to which HL activity was reduced. The method cannot be used to test splicing mutations or mutations in the mitochondrial leader. Also, a small number of causal mutations may, in theory, be more stable in bacteria than in mammalian cells and give a falsely high estimate of the patients' residual HL activity. With these provisos, our method should be useful for screening the enzymatic activity of HL missense mutations in patients with HL deficiency, most of whom have very low residual HL activity in fibroblasts. Our method also yields large amounts of mutant HL peptide, which can be purified for detailed enzymatic studies. We have reported such studies for the H233R mutation (Roberts et al. 1996), and similar work is underway for mutations in codons 41 and 42.

Both known Saudi mutations are predicted to have negligible enzymatic activity, which correlates with the reported clinical severity of Saudi HL deficiency. The D42E homozygote HL13, who has 4.4% residual activity in the purified recombinant HL peptide, presented only at 1 year of age but previously had feeding difficulties and at least one episode of hypoglycemic convulsions (Plöchl et al. 1989, 1990, 1992), suggesting that substantial HL activity is required for normal clinical function during catabolic stress. It will be of particular interest to determine the K_m value for HMG-CoA of mutant HL that contains D42E. HL13 also had the unusual findings of raised serum amylase values and a family history of pancreatic carcinoma. It is too early to draw conclusions from these observations, but we will continue to correlate clinical phenotypes to genotypes as mutation analysis proceeds.

Mutations in codons 41 and 42 account for $\geqslant$19 (29.2%) of 65 of mutant alleles in our group of HL-deficient probands and 9 (14%) of 64 of the non-Saudi mutant alleles. Although our sample size is small, it represents a substantial portion of known HL-deficient patients, and the finding of five different point mutations within a 6-nt interval is notable. As shown in figure 3, R41Q and R41ter are reciprocal mutations resulting from transitions at a CpG dinucleotide. CpG dinucleotides are known to mutate frequently (Cooper et al. 1995). We currently have no explanation for the apparent clustering of mutations in codon 42.

R41Q occurs in HL-deficient patients of diverse ethnic backgrounds, suggesting that this mutation may have arisen independently more than once. We are searching for polymorphic markers in and around the HL gene with which to test this hypothesis. The genetic heterogeneity among Saudi HL-deficient patients is not unexpected, in view of the size of the Saudi population (9 million) and the technically advanced detection of organic acidemias available in Saudi Arabia (Rashed et al. 1995, 1997). As more information becomes available about the family structure of the R41Q probands, our findings may prove useful for evaluating population subgroups in Saudi Arabia.

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