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Ten Novel *HMGCL* Mutations in 24 Patients of Different Origin with 3-Hydroxy-3-Methyl-Glutaric Aciduria

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ABSTRACT: 3-Hydroxy-3-methylglutaric aciduria is a rare autosomal recessive genetic disorder that affects ketogenesis and L-leucine catabolism. The clinical acute symptoms include vomiting, convulsions, metabolic acidosis, hypoketotic hypoglycaemia and lethargy. To date, 33 mutations in 100 patients have been reported in the *HMGCL* gene. In this study 10 new mutations in 24 patients are described. They include: 5 missense mutations: c.109G>A, c.425C>T, c.521G>A, c.575T>C and c.598A>T, 2 nonsense mutations: c.242G>A and c.559G>T, one small deletion: c.853delC, and 2 mutations in intron regions: c.497+4A>G and c.750+1G>A. Two prevalent mutations were detected, 109G>T (E37X) in 38% of disease alleles analyzed and c.504_505delCT in 10% of them. Although patients are mainly of European origin (71%) and mostly Spanish (54%), the group is ethnically diverse and includes, for the first time, patients from Pakistan, Palestine and Ecuador. We also present a simple, efficient method to express the enzyme and we analyze the possible functional effects of missense mutations. The finding that all identified missense mutations cause a >95% decrease in the enzyme activity, indicates that the disease appears only in very severe genotypes." © 2009 Wiley-Liss, Inc.

KEY WORDS: 3-hydroxy-3-methylglutaric aciduria, *HMGCL* , HMG-CoA lyase, mutations, ketone bodies

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INTRODUCTION

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase (HL) (EC 4.1.3.4) is a TIM Barrel structure (Casals, et al., 2003; Fu, et al., 2006) mitochondrial enzyme that catalyzes the cleavage of HMG-CoA into acetyl-CoA and acetoacetic acid. Its absence causes HL deficiency or 3-hydroxy-3-methylglutaric aciduria (MIM# 246450), a rare autosomal recessive genetic disorder that affects ketogenesis and L-leucine catabolism. The disease usually appears in the first year of life after a fasting period and its clinical acute symptoms include vomiting, seizures, metabolic acidosis, hypoketotic hypoglycaemia and lethargy (Faull, et al., 1976). These symptoms sometimes progress to coma, with fatal outcome in about 20% of cases (Sweetman and Williams, 2001). Its prevalence is less than 1/100,000 liveborns. This disease belongs to a group of 29 genetic conditions for which effective treatment is available. The American College of Medical Genetics (ACMG) (Watson, et al., 2006) and the Health Council of the Netherlands (Bolhuis and Page-Christiaens, 2005) have recommended that hospitals with neonate units should be able to diagnose this condition.

The *HMGCL* gene (GenBank NM_000191.2), located in the short arm of chromosome 1 (1p36.1-p35), between *FUCA1* and *TCEB3*, has 9 exons with a total of 24,336 base pairs and codifies human HL. Its 5'-untranslated region bears the characteristic elements of a housekeeping gene, as well as a CpG island that contains binding sites for SP1. There is no evidence for either a TATA box or a CAAT box (Wang, et al., 1996).

To date, 34 variant alleles (33 mutations and 1 SNP) in 100 patients (Figure 1) have been reported in the *HMGCL* gene (two cases were fetuses) (Koling, et al., 2000; Pié, et al., 2007; Vargas, et al., 2007; Zafeiriou, et al., 2007). In the coding region, missense mutations are the most frequent (16), followed by frameshift deletions (5) or insertions (1), nonsense mutations (4) and large deletions (3). Three mutations that cause aberrant splicing have been found in intron sequences. These mutations are uniformly distributed along the gene sequence, although some clustering is observed in exon 2, which has been considered a hot spot (Pié, et al., 2003). The most frequent mutation reported worldwide is c.122G>A, found almost exclusively in patients from Saudi Arabia (Al-Sayed, et al., 2006; Mitchell, et al., 1998; Pie, et al., 2007).

This paper reviews the retrospective genetic studies performed in the last 9 years in 24 patients from different ethnic backgrounds affected by 3-Hydroxy-3-methylglutaric aciduria. The new allelic variants described here comprise about one fourth of all the variants known for this gene. We also propose a simple, efficient method to express the enzyme and we analyze the possible functional effects of missense mutations.

MATERIAL AND METHODS

Patients

Twenty-four cases (one was a fetus) with HL deficiency were studied. Nine patients (cases 3, 5, 6, 7, 8, 10, 14, 20 and 21) become symptomatic during the neonatal period, five (cases 2, 4, 13, 22 and 23) between 3 and 12 months of age and three (cases 11, 12 and 18) during the second year of life. Special cases were: case 1, whose symptoms started at 12 years of age; case 9, who did not develop any symptoms; and case 15, who was a fetus (prenatal diagnosis). In most patients, the triggering event was hypoglycemia, although three of them became sick after an intercurrent illness (cases 1, 12 and 18). All patients developed an acute disease with hypoketotic hypoglycemia and metabolic acidosis. Case 4 had 3 acute episodes. The most frequent complication was hepatic involvement with hepatomegaly (cases 8, 9, 10, 21 and 22) and elevation of transaminases (cases 10, 18 and 20). Functional neurologic symptoms (cases 7, 13 and 18) included axial hypotonia and limb hypertonia (case 7), and clonic movements (case 18). Elevation of organic acids levels in urine was reported in 14 out of 24 patients, who showed increased 3-hydroxy-isovaleric, 3-methylglutaric, 3-methylglutaconic, methylcrotonyl glycine and 3-hydroxy-3-methylglutaric acids. In cases 12, 22 and 23, HL deficiency was confirmed by low fibroblast enzyme activity (<5%). Although all patients received appropriate treatment, three died (cases 6, 10 and 14). DNAs were sent to our center for genetic analysis. Relatives were examined when available. Informed consent for genetic analysis was obtained in all the families studied.

Mutational analysis

Genomic DNA was extracted from blood samples and amniotic fluid (case 15) with "DNAzol®Reagent Kit" from Invitrogen. In case 11, DNA was extracted from blood impregnated cardboard samples with Promega "Ready Amp Genomic DNA Purification Resin" Kit. Oligonucleotides used to amplify nine exons of the *HMGCL* gene and their splice junctions are reported elsewhere (Wang, et al., 1996). When RNA or fibroblasts were available (cases 5 and 16), five overlapping RT-PCR fragments were generated to cover the cDNA sequence of the coding region completely. PCR products were purified with "QIAquick PCR Purification Kit" or "QIAquick Gel Extraction Kit" from Qiagen and sequenced in two directions with the Big Dye Terminator Cycle Sequencing Kit and the ABI Prism 377 Genetic analyzer from Applied Biosystems. We checked the sequence variants using the Mutalizer program (Wildeman, et al., 2008).

Construction of the expression plasmid pMAL-c2x-HL

A DNA fragment encoding HL without the signal peptide was amplified by PCR using the previously constructed plasmid pTr-HLwt (Casals, et al., 2003) as a template, and primers HL*Bam*F (5'-GCATGGATCCACTTTACCAAAGCGGGTG-3') and HL*Bam*R (5'-CGGGTGGACCTAGGGACCCC-3'). These primers contain the *Bam*HI restriction sequence. This fragment was subsequently cloned into the expression plasmid pMAL-c2x, which incorporates the codifying sequence of the fusion protein Maltose Binding Protein (MBP). The construct was transformed into the strain BL21-Codon Plus (DE3)-RIPL (Novagen). Finally, positive clones were confirmed by nucleotide sequencing.

Expression and purification of the HL

E. coli strain BL21-Codon Plus (DE3)-RIPL expressing MBP-HL was grown in LB medium containing 0.1 mg/mL ampicillin to an A_{600} of 1.0 at 37 °C. Optimal protein expression was induced with 0.2 mM IPTG at 18 ºC for 16 h. Cells were recovered by centrifugation at 4000xg at 4 ºC for 15 min. Then, the cells were resuspended in lysis buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mg/mL Lysozyme, 10 µg/mL DNase, 10 µg/mL RNase, 100 µg/mL PMSF, 1 mM DTT, 0.1% Triton X-100 and 10% glycerol) and disrupted by sonication at 4 ºC. The soluble fraction containing the MBP-HL fusion protein was loaded into an amylase affinity column, which had been washed with 3 column volumes of buffer 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA and 1 mM DTT. The HL was eluted from the affinity resin using a buffer containing the protease factor Xa.

Protein and enzyme assays

Protein was quantified by Bradford's method and analyzed by SDS-PAGE using a 12% acrylamide running gel and a 5% acrylamide stacking gel. HMG-CoA lyase activity was measured by a simple spectrophotometric method that determines the amount of acetoacetate produced (Wanders, et al., 1988).

Mutants

Using Quick Change PCR-based mutagenesis (Stratagene), new missense mutations were induced in the wild-type HL cDNA. Five primer pairs were used and these are available on request. DNA sequencing of the new constructs was performed to confirm target mutations. Conditions of expression and enzymatic assay were the same as those used for the wild-type protein

Bioinformatic analysis

Sequences of homologous proteins to human HL were obtained using BLAST (Altschul and Lipman, 1990). Multiple alignments to analyze conservation were performed using ClustalW (Thompson, et al., 1994). Location of residues and generation of protein plot were performed using PyMOL (DeLano Scientific, San Carlos, CA). Atomic coordinates of the 3-D structure of human HMG-CoA lyase in complex with 3-hydroxypentanedioic acid (3-hydroxyglutaric acid) (Fu, et al., 2006) were obtained from the Protein Data Bank (entry 2CW6).

RESULTS

Twenty-four patients were diagnosed at molecular level. More than 50% were Spanish (13 cases), and the remaining were: 2 English, 2 Pakistan, 2 Palestinian, 1 Argentinian, 1 Ecuatorian, 1 French, 1 Moroccoan, and 1 Portuguese (Table 1). In 23 patients mutations were identifyed in both alleles, and in patient 17 only in one allele. Previously, in patient 22, c.144G>T (K48N) mutation of one allele was reported (Carrasco, et al., 2007). In all the patients in which parental DNA was available (cases 6, 7, 8, 9, 10, 11, 14, 15, 17 and 18), inheritance was confirmed.

We identified a total of 16 different allelic variants, 11 of which are reported for the first time (10 mutations and 1 polymorphism) (Table 1) (Figure 1). Of these new mutations, 5 were missense mutations: c.109G>A (p.E37K), c.425C>T (p.S142F), c.521G>A (p.C174Y), c.575T>C (p.F192S) and c.598A>T (I200F), 2 were nonsense mutations: c.242G>A (p.W81X) and c.559G>T (p.E187X), one was a small deletion: c.853delC (p.L285X), and 2 were mutations in intronic regions: c.497+4A>G (p.A118RfsX10/p.V117_E187del) and c.750+1G>A (r.spl?). The SNP c.252+34C>T identified in intron 3 of patient 11 was already registered in the dbSNP database (rs2076344). We also found 5 mutations previously reported: 2 missense mutation c.125A>G (p.D42G) (Mitchell, et al., 1998) and c.698A>G (p.H233R) (Roberts, et al., 1996), one nonsense mutation c.109G>T (p.E37X) (Pié, et al., 1997), and 2 deletions c.202_207delCT (p.S69CfsX11) (Mitchell, et al., 1993) and c.504_505delCT (p.S169LfsX8) (Casals, et al., 1997) (Table 1) (Figure 1).

To examine the effect of the new missense mutations on enzyme activity, we validated a method of expression of HL in *E.coli* by using the pMAL-c2x plasmid, which encodes the MBP fusion protein. The HL assay (Wanders et al., 1988) was performed in reductive conditions because the enzyme was more active than in oxidative conditions (Roberts et al., 1994; Hruz and Miziorko, 1992). Although the enzyme in nature is a dimer linked by disulfide bonds, in this redox state HL is a monomer, indicating that there is no protein-protein interaction that might modify the activity values. All recombinant proteins (p.E37K, p.S142F, p.C174Y, p.F192S, p.I200F) generated from these mutations showed an specific activity and Km no detectable (Table 2).

Table 1. Allelic variants of the *HMGCL* gene found in patients of this study.

Allelic variants	Exon/ Intron	Aminoacid or mRNA changes	Patient code	Ethnic Origin	Mutant alleles	References
Non reported before						
Missense mutations						
c.109G>A	E2	p.E37K*	8, 9	2 Pakistani	4	This study
c.425C>T	E5	p.S142F*	18	1 Spanish	1 (1 ht)	This study
c.521G>A	E6	p.C174Y*	11	1 Palestinian	2	This study
c.575T>C	E7	p.F192S*	12	1 Spanish	2	This study
c.598A>T	E7	p.I200F*	13	1 French	2	This study
Nonsense mutations						
c.242G>A	E3	p.W81X	10	1 Ecuadorian	2	This study
c.559G>T	E6	p.E187X	18	1 Spanish	1 (1 ht)	This study
Deletions						
c.853delC	E8	p.L285X	16	1 English	1 (1 ht)	This study
Intronic mutations						
c.497+4A>G	I5	p.A118RfsX10 (exon 5 skipping), p.V117_E187del (exons 5-6 skipping)	16	1 English	1 (1 ht)	This study
c.750+1G>A	I7	r.spl?	14, 15	2 Spanish	2 (2 ht)	This study
Polymorphism						
c.252+34C>T	I3		11	1 Palestinian	1 (1 ht)	This study
Reported before						
c.109G>T	E2	p.E37X	1, 3, 5, 6, 7, 19, 23, 2, 4, 21	7 Spanish, 1 Argentinian, 1 Moroccoan, 1 Portuguese	18 (2 ht)	Pié et al. 1997
c.125A>G	E2	p.D42G	24	1 Palestinian	2	Mitchell et al. 1998
c.202_207delCT	E3	p.S69CfsX11	20	1 Spanish	2	Mitchell el al 1993
c.504_505delCT	E6	p.S169LfsX8	14, 15, 19, 22, 21	4 Spanish and 1 Portuguese	5 (5 ht)	Casals et al. 1997
c.698A>G	E7	p.H233R	17	1 English	1 (1 ht)	Roberts et al. 1996

cDNA numbering is based on reference sequence GenBank NM_000191.2; +1 corresponds to the A of the ATG initiation translation codon; ht, heterozygous;*all recombinant protein (p.E37K, p.S142F, p.C174Y, p.F192S, p.I200F) generated from these mutations, showed an activity lower than 5% respect to the wild-type (see material and methods, and results).

Table 2. Kinetic Parameters of the wild-type and mutants of human HMG-CoA lyase

Protein	Specific activity (nmol acetoacetate/ min/ mg protein)	Km (HMG-CoA, µM)
Wild-type	50	25
p.E37K	<5%	n.d.
p.S142F	<5%	n.d.
p.C174Y	<5%	n.d.
p.F192S	<5%	n.d.
p.I200F	<5%	n.d.

Values of mutant specific activity represent percentage of wild-type values. nd: not detected

The mutation c.497+4A>G found in intron 5 of patient 16 generated an alternative splicing with 2 transcripts, one bearing a five-exon deletion and the other with deletion of exons 5 and 6. In the other intronic mutation (c.750+1G>A), the splicing study could not be performed because the patient (case 14) died. When his mother became pregnant again, we performed a prenatal study in the fetus (case15), which showed the same mutations as his brother (c.[750+1G>A] +[504_505delCT]) (case 14). In previously reported mutations, alternative splicings were confirmed, as in c.109G>T, with the variant without exon 2 and in c.504_505delCT, with the variants without exons 5 and 6 and without exon 6 (Figure 1).

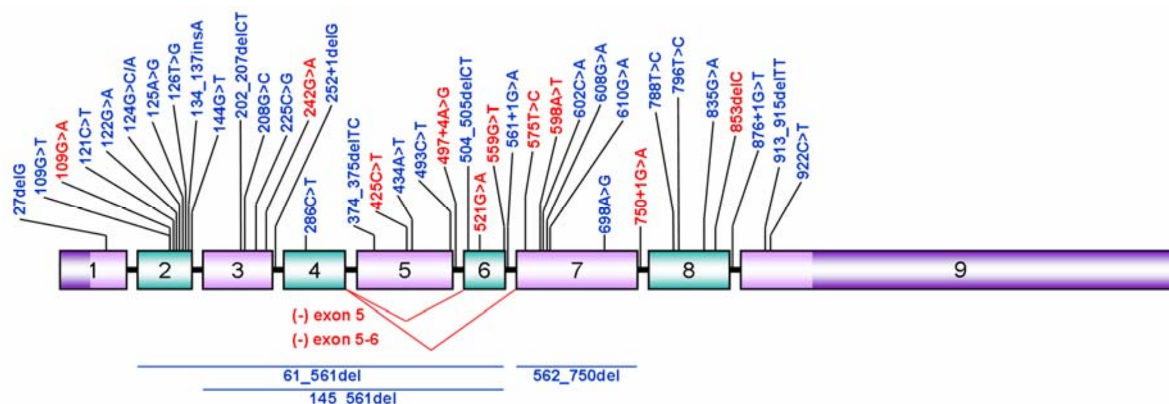

Figure 1. Scheme of the mutations located in the human *HMGCL* gene. The newly reported mutations are indicated in red.

In patients 12 and 16 the diagnosis was confirmed by genetic analysis. In the first case because he died prematurely, the mutations were identified in the parents, and the second was a prenatal diagnosis.

To establish the correlation between structure-function of the newly identified missense mutations, an evolutive and structural analysis was performed using the HMGCL protein sequence from several organisms (from human to bacteria) and the crystallized structure (Fu, et al., 2006) of the human enzyme (Figure 2) as references. All five mutated residues were completely conserved in the sequences analyzed (Figure 2A), with the exception of F192 (see below), which is consistent with the lack of activity of the corresponding mutant enzymes. The charged side chain of E37, located in beta strand 1, is linked through hydrogen bonds to polar residues S258 and S259, in beta strand 8, and T278, in alpha helix 8, contributing to the structural stability of the enzyme. Mutation of E37 to Lys may modify the geometry of this interaction. Due to the proximity of E37 to catalytic residues R41 and D42 (Figure 2B), such a change may disturb the arrangement of the active center. Mutation C174Y may have a similarleading to the generation of an abnormal shape of the active center. Both F192 and I200 residues are located in a hydrophobic cluster formed by residues I126, F150, V168 and V188 (Figure 2C). Although F192 is not conserved through evolution, the hydrophobic nature of the residue in the homologous position is maintained (Figure 2A), this being Leu or Met in the rest of the organisms studied. Replacing Phe by Ser in this position may reorientate the residues involved in substrate positioning. A similar mechanism, although not as dramatic as the F192S change, may explain I200F mutant malfunction, probably due to the larger shape of the newly introduced

Phe residue in the packed cluster instead of Ile. Mutation of S142 to Phe may generate a similar effect: as the side chain of S142 contacts charged residues E132, K136 and E145, the introduction of a large hydrophobic Phe aminoacid in this polar environment (Figure 2D) may disrupt the enzyme structure in the vicinity of the catalytic centre.

A

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HMGCL_HUMAN 30 PKRVKIVEVGPRD 42 135 TKKNINCSIEESFQRF 150 166 GYVSCALGCPY 176 185 VAEVTKKFYSMGCYEISLGDTIG 207
HMGCL_RAT   30 PKRVKIVEVGPRD 42 135 TRKNVNCSIEESFQRF 150 166 GYVSCALGCPY 176 185 VAEVAKKLYSMGCYEISLGDTIG 207
HMGCL_MOUSE 30 PKQVKIVEVGPRD 42 135 TRKNANCSIEESFQRF 150 166 GYVSCALGCPY 176 185 VAEVAKKLYSMGCYEISLGDTIG 207
HMGCL_XENLA 33 PKEVKIVEVGPRD 45 138 SKKNINCSIDESLQRF 153 169 GYVSCVLGCPY 179 188 VAEVAYKMFSMGCYEISLGDTIG 210
HMGCL_PSEMV  1 MQAVKVFEVGPRD 13 106 SRNNINCSIDESFERF 121 137 GYVSCVLGCPF 147 156 VAKVARRLYELGCYEISLGDTIG 178
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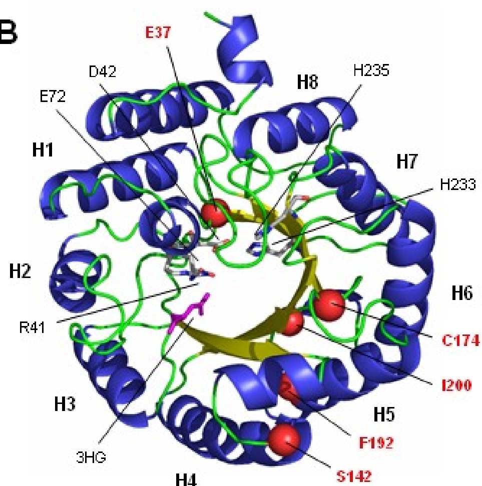

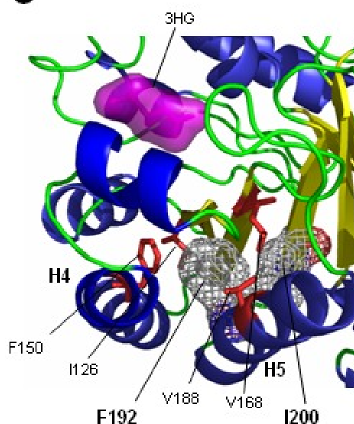

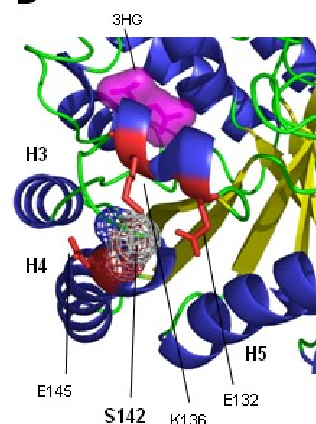

Figure 2. Structural location of missense mutations. **A**. Multiple alignment of several HMGCL protein sequences (from human; rat, mouse, *Xenopus laevis* -XENLA- and *Pseudomonas mevalonii* -PSEMV-, respectively), in the neighborhood of mutated residues E37, S142, C174, F192 and I200. **B**. 3-D structure of human 3-hydroxy-3-methylglutaryl-CoA lyase (PDB entry 2CW6). Position of newly reported mutations, catalytic residues R41, D42, E72, H233 and H235, as well as a molecule of co-crystallized 3-hydroxyglutaric acid (3HG), is indicated. Alpha helices in the $(ab)_8$ barrel structure are labeled H1 to H8. **C**. Area surrounding missense mutations F192 and I200. Location of 3HG molecule and residues in the same hydrophobic cluster (I126, F150, V168 and V188) is indicated. **D**. Location of residues in the vicinity of S142, indicating the close situation of 3HG in the catalytic center of the enzyme.

DISCUSSION

Mutational update

This article reports the genetic study of the largest and most heterogeneous cohort of patients with 3-hydroxy-3-methylglutaric aciduria. Previously, 34 allelic variants were characterized in 100 patients with this disease (Figure 1). Here, we analyzed 11 new variants in 24 patients, which accounts for 24% of the known allelic variants and 24% of the cases reported. Although patients are mainly of European origin (71%) and mostly Spanish (54%), we have also studied patients from different ethnic backgrounds, including for the first time Pakistani, Palestinian and Ecuatorian patients, some of whom were found to bear mutations hitherto unknown.

The mutational profile is heterogeneous with no prevalent sequence variations detected, except for 109G>T (E37X), known as Mediterranean mutation (Casale, et al., 1998), which was found in 18 of the 48 disease alleles analyzed (38%) and c.504_505delCT in 5 out of 48 (10%). These results confirm the Mediterranean mutation as the second most frequent mutation in the world (31 cases), with a specific location in the Iberian Peninsula, where

it was carried by 100% of the Portuguese patients (13 cases) (Carrasco, et al., 2007; Pié, et al., 1997) and by 61% of Spanish (11 cases) (Casale, et al., 1998; Puisac, et al., 2005). This mutation was also found in an Argentinian patient of Spanish ancestry and in one Moroccoan; and since the other studied Moroccoan patient carries it (Pié, et al., 1997), the Mediterranean mutation may have a high incidence in that country. The present study also demonstrates that the mutation c.504_505delCT is the third most frequent worldwide although its incidence is much lower than the first two, and it seems to be exclusively located in the Iberian Peninsula, where 15% of Portuguese (2 cases) (Cardoso, et al., 2004) and 27% of Spanish patients have it (Casals, et al., 1997). In spite of genetic similarities between the Iberian Peninsula populations, the Portuguese seem to be more homogeneous, bearing only one of these two mutations. Our results show that the Spanish have the highest known allelic variety, with 7 different mutations, 4 new and 1 previously described in Acadian French Canadian patients (c.202_207delCT) (Mitchell, et al., 1993).

Characterization of 2 English patients with 3 mutations, 2 of them new, is an advance in the study of that population, where just 2 mutations on 2 patients were known so far (Casals, et al., 2003; Wang, et al., 1996). A new mutation is described in a French patient, resulting in 6 mutations in 6 patients known in the French group (including Acadians and Cajuns) (Mitchell, et al., 1992; Mitchell, et al., 1993; Zapater, et al., 1998). Surprisingly, one of the mutations (c.125A>G) located on Palestinian patients was previously reported on a Dutch patient (Mitchell, et al., 1998). These results reinforce the hypothesis that there are 3 countries, Saudi Arabia (> 45 patients), Spain (18 patients) and Portugal (13 patients) in which a large number of cases bear the same mutations, and that the remaining countries have few patients affected by a large variety of mutations.

The prenatal study performed in a living foetus (case 15) is the third reported prenatal molecular diagnosis performed in an affected family (case 14). The first two were carried out on an Italian and a Turkish family, respectively (Koling, et al., 2000; Mitchell, et al., 1995). These studies allowed the accurate genetic counselling of those families.

Functional and structural analysis

Missense mutations were studied with *E. coli* expression of HL with plasmid pMAL-c2x-HL. However, other methods have been described in bacteria (Casals, et al., 2003; Roberts, et al., 1996) and eukaryotes (Muroi, et al., 2000). The most efficient is that based on pTrc99A vector, in which the enzyme was crystallized with 3 chromatography steps (Fu, et al., 2006; Roberts, et al., 1994). The new system proposed here has been shown to be efficient in enzymatic and directed mutagenesis studies. It is simple and it can be performed in a single chromatography step.

New mutation c.109G>A (cases 8 and 9), is located on exon 2, at the beginning of a previously suggested *hot spot* for mutations (Pié, et al., 2003) (Figure 1). In the 3-D structure of the protein, the changed aminoacid (p.E37K) is located very close to residue R41, involved in the stabilization of the intermediate enolate of the reaction, and to D42, involved in the coordination of magnesium (Fu, et al., 2006). These two residues, in addition to residues E72, H233 and H235, are located in the active center of the enzyme. The replacement of an acidic residue (Glu37) by a basic one (Lys) in the vicinity explains the total loss of activity of mutant protein in affected patients. Another two missense mutations (p.F192S and p.I200F) are located on exon 7. Four different mutations have been found in this exon, although only one, c.698A>G (p.H233R), appears to affect a residue in the active center of the enzyme. The other appear to be more related to changes in protein structure shape and stability. This high incidence of mutations in exon 7 thus suggests a new hot spot. Variations in protein structure, in positions located close to the active center, are also the most probable cause of the effects observed in enzyme activity of mutants p.S142F and p.C174Y, the latter probably also implicated in the maintenance of a disulphide bond.

Genotype-phenotype correlations

The fact that all studied missense mutations cause a loss of enzyme activity greater than 95% is consistent with other published data (Mitchell, et al., 1998; Carrasco, et al., 2007) and suggests that the illness appears only in very severe genotypes, and that partial disruption of the enzyme is probably compatible with normal function. Therefore, it is very difficult to establish genotype-phenotype correlations because we only see the effects of very severe genotypes. The expansion of neonate screening would help us to detect asymptomatic cases or mild cases with no clinical expression. We hypothesize that phenotype variations should be sought in external factors, like

those that may cause hypoglycaemia (fasting or acute illness). Clinical observations seem to support this hypothesis. On one hand, two brothers (patients 8 and 9) bearing the same mutation in homozygosis (c.109G>A) had very different evolution. One of them had an acute crisis with hypoglycaemia and lethargy and the other has not showed any symptom of the illness. On the other hand, patients with the same mutation in homozygosis (c.109G>T) sometimes presented low to moderate symptoms, such as fever or mild hypoglycaemia (cases 1 and 2), or more severe symptoms that were life-threatening (case 6). Therefore, it is very important to avoid situations that might induce hypoglycaemia in these patients.

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