Analysis of aberrant splicing and nonsense-mediated decay of the stop codon mutations c.109G>T and c.504_505delCT in 7 patients with HMG-CoA lyase deficiency

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Abstract

Eukaryotic cells can be protected against mutations that generate stop codons by nonsense-mediated mRNA decay (NMD) and/or nonsense-associated altered splicing (NAS). However, the processes are only partially understood and do not always occur. In this work, we study these phenomena in the stop codon mutations c.109G>T (p.Glu37*) and c.504_505delCT; the second and third most frequent mutations in HMG-CoA lyase deficiency (MIM #246450). The deficiency affects the synthesis of ketone bodies and produces severe disorders during early childhood. We used a minigene approach, real-time quantitative PCR and the inhibition of NMD by puromycin treatment, to study the effect of stop codons on splicing (NAS) and NMD in seven patients. Surprisingly, none of the stop codons studied appears to be the direct cause of aberrant splicing. In the mutation c.109G>T, the splicing is due to the base change G>T at position 109, which is critical and cannot be explained by disruption of exonic splicing enhancer (ESE) elements, by the appearance of exonic splicing silencer (ESS) elements which were predicted by bioinformatic tools or by the stop codons. Moreover, the mutation c.504_505delCT produces two mRNA transcripts both with stop codons that generate simultaneous NMD phenomena. The effects of the mutations studied on splicing seemed to be similar in all the patients. Furthermore, we report a Spanish patient with 3-hydroxy-3-methylglutaric aciduria and a novel missense mutation: c.825C>G (p.Asn275Lys).

1. Introduction

3-Hydroxy-3-methylglutaric aciduria is a rare autosomal recessive inborn error of metabolism (MIM #246450) that usually manifests during fasting and intercurrent illness with hypoglycemia, hyperketonemia and metabolic acidosis that can lead to coma and death [1–3]. It was caused by deficiency of the enzyme 3-hydroxy-3-methylglutaryl-CoA lyase (HL, EC 4.1.3.4), which is involved in the final step of the synthesis of ketone bodies and leucine catabolism. Two isoforms of the HL protein have been described, one mitochondrial and the other peroxisomal, encoded by the same gene, HMGCL. Recently, another HMG-CoA lyase enzyme codified by a different gene HMGCLL1 has been characterized with a dual location in endoplasmic reticulum and cytosol [4]. All these enzymes have a homodimer TIM-barrel structure with a catalytic center containing a divalent-cation binding site [5,6]. The HMGCL gene (Gene ID 3155, ENSG00000117305) codifies human HL and has 9 exons with a total of 23,582 base pairs (bp)[7]. Exons size varies between 64 and 527 bp and the introns range between 600 and 3400 bp. This gene presents a physiological splicing with three variants, one with all exons encoding the active protein (NM_000191) and two with deletion of exons 5 and 6 (NM_001166059.1) and deletion of exons 5, 6 and 7 (GU433941) both maintain the coding frame but do not produce active protein [8].

Thirteen of the 48 mutations described in this gene generate a stop codon [9,10]. Among those 13 are the second and third most common mutations: the nonsense mutation c.109G>T (p.Glu37*) and the frameshift mutation c.504_505delCT. The first is located in the middle of HMGCL exon 2 and is associated with the skipping of this exon [11,12]. The second is located at the beginning of exon 6 and determines the occurrence of three mature transcripts: the mRNA transcript corresponding to the CT deletion itself, other containing a frameshift deletion of exon 6 and the physiological transcript with exon 5 plus 6 deletion [13].
Two mechanisms associated with stop codon mutations that offer protection through preventing the formation of truncated proteins have been reported. One is nonsense-mediated mRNA decay (NMD) which degrades the affected mRNA transcripts and leads to decreased mRNA levels [14]; the other is nonsense-associated altered splicing (NAS) which results in the exon where the stop codon is situated being skipped [15,16]. However, not all mutations behave in a similar way and questions remain about the underlying mechanisms [17,18].

Using a minigene approach, real-time quantitative PCR, and the inhibition of NMD by puromycin treatment, we studied whether the stop codons that the mutations c.109G>T and c.504_505delCT generate, cause aberrant splicing by NAS or NMD processes. We further analyzed splicing variability between patients and the effect of the mutations on physiological splicing. Finally, we identified a new missense mutation in a patient with HL deficiency.

2. Materials and methods

2.1. Patients and controls

The study is based on seven Spanish patients with 3-hydroxy-3-methylglutaric aciduria [10,12,13]. The clinical data relating to the patients and their genetic diagnoses are shown in Table 1. Patient 4 is reported here for the first time. She is a girl born to healthy non-consanguineous Spanish parents after a normal pregnancy and delivery. At 5 days of age she was hospitalized with dehydration and hypotonia. Biochemical analysis showed hypoketotic hypoglycemia, metabolic acidosis, and elevated levels of transaminases. A characteristic excretory pattern of organic acids in urine together with an assay of the HL enzyme in cultured fibroblasts, con

2.2. In silico prediction analysis

Putative exonic splicing enhancer (ESE) and exonic splicing silencer (ESS) sequences in exons 2 and 6 of the HMGCL gene were assessed using two web-based programs: Human Splice Finder (http://www.umd.be/HSF/) and RESCUE-ESE (http://genes.mit.edu/burgelab/rescue-ese/).

2.3. Cell culture

Fibroblasts from skin biopsies from the patients and control individuals were cultured in a monolayer system at 37°C under conditions of 5% CO₂. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine 2 mM.

2.4. DNA extraction and sequence analysis

Genomic DNA was extracted from the cultured fibroblasts by following a standard procedure [19]. The oligonucleotides used to amplify nine exons in the HMGCL gene and their splice junctions are reported elsewhere [7]. The procedure for the PCR were performed as described in Casals et al. [5] using a proof reading polymerase (Bio Rad, Hércules, USA) The PCR products were purified using a QiAquick PCR Purification Kit (QiAGEN, Hilden, Germany) and were sequenced on an ADN 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.5. RNA extraction and identification of splice transcripts by RT-PCR

Total RNA was extracted from the cultured fibroblasts using a RNeasy Protect Mini Kit (QiAGEN) according to the manufacturer's instructions. Single-stranded cDNAs were synthesized from 2 μg of RNA from each patient using a SuperScript First-Strand RT-PCR kit (Invitrogen Corporation, Carlsbad, CA, USA) with random hexamers. To show the different mRNA transcripts generated, the subsequent PCR reactions used 2 μl of the cDNA product and specific HMGCL primers for exploring exon 2 (11: Exon 1, -7-12 position,

### Table 1

<table>
<thead>
<tr>
<th>Case</th>
<th>HMGCL mutation</th>
<th>Splicing alteration</th>
<th>Protein alteration</th>
<th>HMG-CoA lyase activity</th>
<th>Clinical data</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P5</td>
<td>c.109G&gt;T/c.504_505delCT</td>
<td>Exon 2 skipping</td>
<td>p.Glu37*/p.Val168Valfs*8</td>
<td>n.d. b</td>
<td>Debut at 5 days with hypoglycemia and hypotonia At 3 years favorable evolution</td>
<td>[10]</td>
</tr>
<tr>
<td>P6</td>
<td>c.144G&gt;T/c.504_505delCT</td>
<td>Exon 2 skipping</td>
<td>p.Lys48Asn/p.Val168Valfs*8</td>
<td>n.d. b</td>
<td>Debut at 6 months with hypoglycemia At 6 years favorable evolution</td>
<td>[10]</td>
</tr>
</tbody>
</table>

a Control activity: 5.5 nmol/min/mg prot.

b n.d.: not detectable.
transcripts generated by the mutations, we used the boundary
2.7. Real-time quantitative PCR
μ out with 1
harvested and the RNA extracted. RT-PCR transcription was carried
manufacturer's instructions. At 48 h posttransfection the cells were
using JetPEI reagent (Qbiogene Inc., Irvine, CA, USA) following the
wild-type or mutant minigenes were transfected into HepG2 cells
(Invitrogen Corporation). PCR was carried out on 5
of genomic DNA
in a 20 μl PCR reaction with 20 pmol of each primer, 200 μM of each
dNTP, 1.5 mM MgCl2, and 2.5 units of Taq polymerase and 1× PCR
buffer. The PCR amplification program was as follows: 1 one step of
3 min at 98 °C, 35 subsequent cycles of 30 s at 96 °C, 30 s at 57 °C,
and 30 s at 72 °C and a final 5-min extension at 72 °C.

The fragments were cloned into the TOPO vector (Invitrogen
Corporation) following the manufacturer’s protocol. The inserts
were excited with EcoRI and were cloned into pSP3. Ligation was
performed at 25 °C for 5 min, using T4 DNA ligase (Invitrogen
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were selected for correct fragment orientation and sequenced. An identical process was carried out with
each of the fragments
or
and p.Gly39* (c.115_117delinsTAA)). After that, and based on predic-
tion c.504_505delCT.

2.8. Inhibition of NMD by puromycin treatment

The fibroblasts obtained from patient 7, who was homozygous for
the c.504_505delCT mutation, were subdivided into two cultures. One
of them was treated with the protein synthesis inhibitor puromycin,
which was added to the medium at a concentration of 100 μg/ml.
and the other was untreated. Puromycin is known to abrogate NMD.
After 6 h of incubation, the fibroblasts from both subcultures were
harvested and total RNA extraction and RT-PCR reactions were
performed as described above. Quantitative real-time PCR experi-
ments were performed as described in Section 2.7. to analyze the
level of three alternative mRNA transcripts before and after
puromycin treatment: with the c.504_505delCT mutation; deletion
of exon 6 only; and deletion of exons 5 and 6.

3. Results

3.1. Genetic diagnosis of a new patient with HL deficiency

Patient 4 is a heterozygote for the nonsense mutation c.109G>T
(GLU37*) and a new missense mutation: c.825C>G. The missense
mutation results in a substitution of Asn 275 by Lys (p.Asn275Lys).

2.6. Minigene construction and site-directed mutagenesis

To evaluate the splicing mechanism, minigene constructs were
generated using the vector pSPL3 (Exon Trapping System, Gibco,
BRL, Carlsbad, CA; kindly provided by Dr. B. Pérez). The HMGCL
gene fragments corresponding to exon 2 and the intronic flanking
regions from the DNA of a patient who was homozygous for c.109G>T mutation
and from that of a control individual were amplified by PCR using the
primers: HLM2F (5′-GCCAGTGGAAAGGATCTG-3′) and HLM2R
(5′-TATTGTGCCAGACTGCTCTCA-3′). We used 50 ng of genomic DNA
in a 20 μl PCR reaction with 20 pmol of each primer, 200 μM of each
dNTP, 1.5 mM MgCl2, and 2.5 units of Taq polymerase and 1× PCR
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Multiple sequence analysis of the mutated residue, Asn275, shows high
conservation across species. Residue Asn275 is located in the
same manner as the catalytic residues Asp42,
His233 and His235 ( Figs. 1D and E). Modeled mutant residue
Lys275 has a higher length (Fig. 1E) and this suggest that the interaction
of the mutated amino acid to Mg++ atom of the active center
may be altered.

3.2. Splice transcripts of the 109G>T and c.504_505delCT mutations

Amplification using the pair II/R1 (Fig. 1B) of cDNA from patients
1, 2, 3, 4, 5 and 6 showed two bands: one of the expected size
(247 bp) and the other containing only 163 bp. The band of 247 bp
had a stop codon that replaced amino acid 37 and disrupt the coding

GGCCCAATGGCGAAGCATG and R1: Exon 4, 240–221 position, CTAGGAGACAACAGCTGG and also exons 5, 6 (F1, Exon 3, 177–197 position, ATAGACATGTTCTGAGG and R4, Exon 8, 832–813 position, TGGCCAATTCTTCTGAGCC). The resulting PCR products were analyzed by electrophoresis in 2% agarose gels and the excised band was purified using a QIAEX Gel Extraction Kit (QIAGEN). The identity of the bands was confirmed by sequencing.
Fig. 1. RT-PCR screening for HMGCL mRNA transcripts in patient and control fibroblasts. A. Scheme of the HMGCL gene with mutations and splice variants. B. HMGCL cDNA from exons 1 to 3 was amplified by PCR using the primer pair I1/R1. The normal-sized band contains 247 bp. The band containing 163 bp corresponds to the product in which exon 2 is skipped. Electropherogram showing the deletion of exon 2 is provided. C. HMGCL cDNA from exons 4 to 8 was amplified by PCR using the primer pair F1/R4. The normal-sized band contains 655 bp. The band containing 591 bp corresponds to the skipping of exon 6. The band containing 442 bp corresponds to the mRNA transcript with exons 5 and 6 deleted. Electropherograms showing the deletion of exon 5 and 6 and deletion of exon 6 is provided. Patients with HMGCL mutations: P1: c.109G>T/c.109G>T; P2: c.109G>T/c.109G>T; P3: c.109G>T/c.109G>T; P4: c.109G>T/c.825C>G; P5: c.109G>T/c.504_505delCT; P6: c.144G>T/c.504_505delCT; CT: control. D. Structural location of mutated Asn275Lys residue. 3D structure of human 3-hydroxy-3-methylglutaryl-CoA lyase (PDB entry 2CW6). E. Position of catalytic residues Asp42, His233 and His235, as well as a molecule of co-crystallized 3-hydroxyglutaric acid and the Mg++ atom, are indicated. Location of wt Asn275 residue (light grey) and predicted mutant Lys275 (black sticks) are also indicated.
frame. The smaller band contained an in-frame deletion of exon 2. The patient who is heterozygous for mutation c.144G>T (patient 6) also produced the band reflecting deletion of exon 2 (Fig. 1B).

Amplification using the pair F1/R4 (Fig. 1C) resulted in different patterns depending on the patients’ mutations. Patient 7, who is homozygous for the mutation c.504_505delCT, produced an almost negligible 655 bp mRNA transcript with CT deletion, and two new mRNA transcripts: one with deletion of exon 6 (591 bp) and the other with deletion of exons 5 and 6 (442 bp). Both mRNA transcripts, that containing the CT deletion and that containing only deletion of exon 6, result in reading frame disruptions. The mRNA transcript with the deletion of exons 5 and 6 also appeared in patients who are heterozygous for this mutation (patients 5 and 6) but only appeared very slightly in the rest of the patients (patients 1, 2, 3 and 4).

3.3. Minigene analysis of the c.109G>T and c.504_505delCT mutations

Transfection using the construct with exon 2 of the HMGCL gene in HepG2 cells and subsequent amplification by RT-PCR, yielded a 357 bp fragment containing the exon (Fig. 2A: Exon2 WT), while the introduction of the mutation c.109G>T in the construct produced only a smaller fragment of 268 bp that lacked exon 2 (Fig. 2A; c.109G>T).

The introduction into the construct with the mutation c.109G>T (p.Glu37*) of the change c.110A>T (Glu37Leu) or c.111A>T (Glu37Tyr), caused the stop codon to disappear and maintain the deletion of exon 2 (Fig. 2A; c.110A>T (Glu37Leu) and c.111A>T (Glu37Tyr)). However, the introduction of stop codons at the nearby amino acids, p.Ile35* and p.Gly39*, led to a normal PCR product (Fig. 2A; p.Ile35* (c.103_105delinsTAA) and p.Gly39* (c.115_117delinsTAA)).

The removal of three potential ESE elements that overlap at position 109 (TTGTGA, TGGAG and GAAGT) by deletion of the entire sequence or by replacing the purines by pyrimidines, did not result in the exon being skipped (Fig. 2B; deESE and PYR). The elimination of the potential ESE element GTAAG created by the mutation c.109G>T, by substituting its original bases for cytosines, did not affect the production of a fragment reflecting the deletion of exon 2 (Fig. 2C; ESS-C).

In the minigene model, the substitutions c.109G>A (Glu37Asn) and c.109G>C (Glu37Tyr) produced the full mRNA transcript (Fig. 2D; c.109G>A and c.109G>C). Transfection using the construct with the normal exon 6 of the HMGCL gene resulted in a mRNA transcript of the expected size (337 bp) (Fig. 2E; exon 6 WT). The introduction into this construct of the c.504_505delCT mutation produced the full length mRNA transcript and a smaller fragment containing only 273 bp which lacked exon 6 (Fig. 2E; c.504_505delCT). The construct with the mutation that eliminates the stop codons, produced the full length mRNA transcript and the deletion of exon 6 (Fig. 2E; Only delCT). Meanwhile, the construct with stop codons but without the CT deletion produced only the full length mRNA transcript (Fig. 2E; Only STOP).

Bioinformatic analysis of the effect of the mutation c.504_505delCT using the Human Splice Finder program, revealed that there is no appearance or disappearance of either ESE or ESS elements.

3.4. Quantification of splice transcripts in patients

The quantification of total mRNA of the HMGCL gene in patients 1, 2 and 3, (who are homozygous for the mutation c.109G>T) and in patient 4 (who is heterozygous for this mutation) showed no significant differences from the levels in control individuals (reference value 1) (Fig. 3A). However, in patient 7, who is homozygous for the mutation c.504_505delCT, the mRNA values were about 5.5 times lower than those of the control individuals (0.18 ± 0.06). The patients who are heterozygous for this mutation (patients 5 and 6) also had decreased values: 0.55 ± 0.03 and 0.66 ± 0.15 respectively (Fig. 3A).

Quantification of the mRNA transcript with deletion of exon 2 yielded similar results in all the patients who are homozygous for the mutation c.109G>T (patients 1, 2 and 3) with values ranging between 14 and 20% (Fig. 3B); values were relative to total HMGCL transcripts. In patient 4, who is heterozygous for this mutation, the levels were approximately half of these values (8% of total transcripts) (Fig. 3B). In patient 5 who is heterozygous for this mutation too, the percentage was greater (15% of total transcripts), but this was distorted because the other allele has a mutation c.504_505delCT which causes NMD and thus decreased total transcripts. In patient 6, NMD phenomenon also occurs, but it is evident that the mutation c.144G>T in heterozygosity produced deletion of exon 2 at greater levels (35.8%) than the mutation c.109G>T in homozygosis (Fig. 3B).

Quantification of the mRNA transcript with deletion of exon 6 in patient 7, who is homozygous for the mutation c.504_505delCT, yielded a value (4% of total transcripts) that was approximately twice that of the patients P5 and P6 who are heterozygous for this mutation (Fig. 3C).

Quantification of the physiological splicing variant of the HMGCL gene with deletion of exons 5 and 6 in patients 1, 2, 3 and 4 who all have the mutation c.109G>T, produced similar levels to those of the control (3–5%) (Fig. 3D). However, in patient 7 who is homozygous for the mutation c.504_505delCT, the level of this transcript represents almost 80% of total transcripts. However, we must consider that in this patient the phenomenon of NMD caused a drastic decrease in the total transcripts (5.5 times less than the control). In patients heterozygous for this mutation P5 and P6, this transcript values represented 15–19% of total transcripts.

3.5. NMD analysis in the c.504_505delCT mutation

To study the mechanism of NMD in patient 7 (who is homozygous for the mutation c.504_505delCT), fibroblasts were treated with puromycin and changes in the three splicing mRNA transcripts were quantified by real-time PCR. Fig. 4A shows that before treatment (untreated), the levels of normal mRNA transcript with CT deletion were very low; about 7% of those of the control individuals. After treatment with puromycin, the levels increased 10-fold to stand at 70% of those of the control individuals.

Fig. 4B shows the quantification of the mRNA transcript with deletion of exon 6, which did not occur in control subjects. Puromycin treatment resulted in a considerable increase in its levels (10 times). Fig. 4C shows that the levels of the mRNA transcript with deletion of exons 5 and 6 were 3 times higher in patient than in control subjects, but treatment with puromycin did not modify them.

4. Discussion

Stop codon mutations generate truncated proteins which usually have serious consequences for subjects with the mutations. In such a situation, eukaryotic cells are protected by two mechanisms: mRNA transcript degradation by NMD; or the skipping of the exon with the mutation, NAS. In this work, we study the two most common stop codon mutations associated with HMG-CoA lyase deficiency: c.109G>T (p.Glu37*), also called the Mediterranean mutation; and c.504_505delCT [10-12,21-23]. The mutations appear to be associated with the NMD and NAS mechanisms.

It has been reported that the mutation c.109G>T (p.Glu37*) is associated with the skipping of exon 2, but the NAS mechanism which results in such skipping is not known [11,12,21,22]. Two types of NAS have been described [24] in the first (NAS1), the mutation acts on splicing regulatory elements, such as ESEs or ESSs [25,26]. In the second (NAS2), the mutation generates a stop codon which acts as an elimination signal for the exon that contains it.
To study the NAS mechanism that affects the mutation c.109G>T, we devised a minigene model for exon 2 and it confirms the splicing of the mutation, although only the mRNA transcript with exon 2 deleted is present (Fig. 2A; c.109G>T).

According to our model, the skipping does not depend directly on the triplet that codes the stop codon (NAS2 mechanism). If we eliminate the stop codon while maintaining the G109T, also resulted in deletion of the exon. Nonsense mutations at nearby positions (p.Ile35*; p.Gly39*) did not result in exon skipping. The Mediterranean mutation did not disrupt an ESE. Elimination of the hypothetically predicted ESEs by in-frame deletion (DEL2) or by purine to pyrimidine transversion (PYR), did not result in exon skipping in our minigene system. Disruption of the hypothetically created ESSs by replacement with cytosines did not avoid the skipping of exon 2. D. Only the C>T substitution at position c.109 caused exon skipping. Minigene analysis of other possible substitutions at position c.109 (c.109G>A and c.109G>C) resulted in only the normal mRNA transcript being obtained. E. The exon skipping associated with mutation c.504_505delCT only occurred when there was a lack of CT nucleotides (Only delCT): it did not occur when only the stop codons were present (Only STOP).

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Moreover, the hypothesis that the G/T transversion alters a purine-rich sequence which acts as an ESE [11] (NAS2) does not seem possible, since the deletion of the probable enhancer sequence or its substitution for a pyrimidine base sequence [25], does not cause the skipping of exon 2 (Fig. 2B; delESE and PYR). ESE elements
with non-purine sequences as A/C rich splicing enhancers (ACEs) [28] have also been reported but the sequence surrounding the mutation does not include these types of bases and the deletion of the sequence also eliminates these elements. It was further considered that the mutation could generate an ESS element, which, unlike the ESE, would act by promoting the skipping of the exon that contains it [29]. According to bioinformatic analysis, the mutation creates two possible ESS elements: one of them, GTAAGT, has been reported previously [30]. However, the elimination of the supposed ESS by replacing its bases for cytosines (GTAAGT to CTCCCC) which are the least frequent bases in the ESS [30], does not avoid the skipping of exon 2 (Fig. 2C; ESS-C).

In summary, all these results suggest that the skipping of exon 2 is not produced by the triplet with the stop codon by the disruption of an ESE or by the appearance of an ESS predicted according to the bioinformatics programs. Anyway, it is not excluded that the mutation is acting on complex regulatory sequences CERES (Composite Exonic Regulatory Elements of Splicing) [31]. However the only thing that seems critical is the change of the G at position 109 of the cDNA of the gene for a T (Fig. 2D). Thus, the changes c.109G>C and c.109G>A do not produce the skipping of the exon (Fig. 2D; c.109G>A and c.109G>C).

The frameshift mutation c.504_505delCT causes multiple splicing with three mRNA transcripts one with the CT deletion, another with the deletion of exon 6, and one with the deletion of the exons 5 and 6 [13]. The last of them has also been described as a physiological mRNA transcript [8]. Bioinformatics predictions rule out this mutation altering ESE or ESS elements (NAS1). Neither does it seem to act via an NAS2 mechanism. In the minigenes model, the presence of the stop codon without the frameshift deletion does not cause the skipping of exon 6 (Fig. 2E, Only STOP). However, maintaining the deletion coupled with the elimination by directed mutagenesis of the stop codon, preserves the skipping of exon 6 (Fig. 2E; Only delCT). Taken together, these results do not rule out the possibility that the frameshift deletion affects the splice acceptor site [13].

NMD is unlikely in the mutation c.109G>T because a decrease in the expression of the gene HMGCL was not observed in the affected patients (Fig. 3A). However, in the mutation c.504_505delCT, the homozygote patient had only 18% of the corresponding mRNA of control individuals (Fig. 3A). Two of the three mRNA transcripts associated with this mutation, with CT deletion and with deletion of exon 6, give rise to a stop codon [13]. Using puromycin, a known NMD inhibitor, we demonstrate that both mRNA transcripts are affected by this process, increasing their levels by 10-fold after treatment (Figs. 4A and B). This is surprising because the mRNA transcript with the CT deletion generates a stop codon 31 nucleotides upstream from the 3′ exon–exon junction and this does not satisfy the rule in which the stop codon must be located further than 50 nucleotides upstream.
to be affected by NMD [32]. This is in agreement with a new model of regulation of NMD which takes into consideration other sequence signals [33] or distance parameters [34].

The effect of the mutation c.504_505delCT does not circumscribe the production of two pathological mRNA transcripts and it also enhances the physiological mRNA transcript with the deletion of exons 5 and 6 (Fig. 3D). This phenomenon does not occur in the patients with the mutation c.109G>T (Fig. 3D). The location of the mutation c.504_505delCT in one of the deleted exons seems to affect the regulation of the splicing, as described in other cases [35].

Although it has been described that the splicing mechanism may vary between individuals thereby modifying the phenotype and severity of the disease [36], the effects of the mutations studied on splicing seem to be similar in all the patients. Thus, the quantification of the deletion of exon 2 in patients who are homozygous for the mutation c.109G>T led to similar results (Fig. 3B). Moreover, the study of the heterozygote patients revealed that the quantity of mRNA transcript with deletion of exon 2 (P4 and P5) (Fig. 3B) or with deletion of exon 6 (P5 and P6) (Fig. 3C) is proportional to the presence in an allele of the causal mutation. In patients with transcripts suffering NMD the percentage is distorted by the decrease of total transcripts.

Comparison of the effects on pre-mRNA splicing of the mutations c.109G>T and c.144G>T, shows that the latter mutation in heterozygosis causes more deletion of exon 2 than the first mutation in homozygosis (Fig. 3B). The alteration of a consensus intron region appears to have a greater impact on the splicing than the introduction of the change in the middle of the exon: c.109G>T.

In this work, a novel Spanish patient with 3-hydroxy-3-methylglutaric aciduria is also reported. The patient is a double heterozygote for the mutation c.109G>T (p.Glu37*) and a novel missense mutation (c.825C>G; p.Asn275Lys), located in exon 8 of the HMGCL gene. The amino acid Asn275 is highly conserved at the evolutionary scale and bioinformatic predictions suggest that this change is the cause of the disease. In the crystallized structure of the HL wt protein [6], the Mg++ atom present in the active center is coordinated by essential residues Asp42, His233 and His235, as well as by Asn275. Lack of enzymatic activity of Asn275Lys mutant can be explained as a consequence of the predicted differences in the geometry of the active center of the protein due to the presence of a residue, Lys275, not able to interact correctly to the Mg++ atom and thus modifying the position of the rest of catalytic residues.

Although the patients with the mutation c.109G>T share a lack of enzyme activity and have similar splicing patterns, their clinical course is variable (Table 1). As has been suggested, it seems probable that the phenotypic variations depend more on external factors which cause hypoglycemia (fasting or intercurrent illness) than on the characteristics of the mutation [10].

In conclusion, in the two mutations studied here, the stop codon does not appear to be the cause of the aberrant splicing; but in one of them, it seems to cause NMD. In the Mediterranean mutation, the splicing is due to the base change G>T at position 109, which is critical and is not explained by disruption of ESE elements, or by the generation of ESS elements or of stop codons. The mutation c.504_505delCT causes two mRNA transcripts with a stop codon that generate two simultaneous NMD phenomena.

Conflict of interest statement

There is no conflict of interest.

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