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Characterization of splice variants of the genes encoding human mitochondrial HMG-CoA lyase and HMG-CoA synthase, the main enzymes of the ketogenesis pathway

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Abstract The genes *HMGCS2* and *HMGCL* encode the two main enzymes for ketone-body synthesis, mitochondrial HMG-CoA synthase and HMG-CoA lyase. Here, we identify and describe possible splice variants of these genes in human tissues. We detected an alternative transcript of *HMGCS2* carrying a deletion of exon 4, and two alternative transcripts of *HMGCL* with deletions of exons 5 and 6, and exons 5, 6 and 7, respectively. All splice variants maintained the reading frame. However, Western blot studies and overexpression measurements in eukaryotic or prokaryotic cell models did not reveal HL or mHS protein variants. Both genes showed a similar distribution of the inactive variants in different tissues. Surprisingly, the highest percentages were found in tissues where almost no ketone bodies are synthesized: heart, skeletal muscle and brain. Our results suggest that alternative splicing might coordinately block the two main enzymes of ketogenesis in specific human tissues.

Keywords *HMGCL* · *HMGCS2* · Ketone bodies · Alternative splicing

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Introduction

The genes *HMGCS2* and *HMGCL* encode the two main enzymes for ketone-body synthesis, mitochondrial HMG-CoA synthase (mHS) and HMG-CoA lyase (HL). *HMGCS2* lies on chromosome 1 [1] and has 10 exons and 9 introns. Expression studies show a differential distribution of the gene transcripts, with the highest concentrations in liver, and lower levels in kidney, intestine and testis [2, 3]. *HMGCL* lies on the distal short arm of chromosome 1 [4] and has 9 exons and 8 introns. Unlike *HMGCS2*, the *HMGCL* gene is broadly distributed [5], since HL is also involved in leucine catabolism. Both mHS and HL catalyze two consecutive reactions in which the acetyl-CoA, mainly produced from the fatty-acid β-oxidation pathway, is converted to ketone bodies: β-hydroxybutyrate, acetoacetate and acetone in the mitochondria. Ketone bodies are essential as an alternative source of energy to glucose, as lipid precursors and as regulators of metabolism [6]. The key enzyme in the regulation of ketogenesis is HMG-CoA synthase. Its regulatory mechanisms have been described at transcription and at protein levels [7]. Despite the increasingly evident role of alternative splicing mechanism

in the regulation and diversification of the function of genes [8, 9] to date, no splicing variants from the *HMGCS2* gene and only mutation-bearing variants in the *HMGCL* gene have been reported [10–13]. Moreover, splicing is believed to coordinate functionally related genes [9, 14]. The aim of this study is the detailed characterization of the possible physiological splicing variants of two main genes of ketogenesis. We have examined whether the alternative transcripts result in functional proteins and, additionally, we have quantified the transcript variants in numerous human tissues. The results can help us to understand the functional role of the physiological splicing variants of the *HMGCS2* and *HMGCL* genes and also to improve our interpretation of the pathological splicing variants related with mutations in these genes.

Materials and methods

Identification of splice variants

RNA was isolated from fibroblasts, pooled from four normal controls, using ULTRASPEC RNA (Biotecx, Houston, USA). Total RNA (1 μg) was reverse transcribed using the Ready-to-go T-primed First-Strand kit (Pharmacia Biotech, Uppsala, Sweden). In addition, cDNA from different human tissues: liver, skeletal muscle, brain, kidney, heart, pancreas, lung, small intestine, colon, ovary, testis, prostate, leukocyte, thymus, and spleen were purchased from Clontech (Mountain View, USA).

An exhaustive analysis of different *HMGCS2* mRNA transcripts was performed using ten primers that produced nine overlapping fragments. *HMGCL* cDNA was amplified in eight overlapping fragments with nine primers. The primers are available on request. Initially, we explored *HMGCS2* and *HMGCL* variants in cDNA from fibroblasts, liver and skeletal muscle tissues. We then used the amplifications that showed additional bands in the other tissues. The identity of all PCR products was confirmed by DNA sequencing using the "ABI Prism BigDye Terminator cycle Sequencing v2.0" (Applied Biosystems, Foster City, USA) and analyzed on an Applied Biosystems 5700 DNA sequencer.

Real-time PCR

Real-time quantitative PCR assay was used to measure the full-length and deleted *HMGCS2* and *HMGCL* transcripts in accordance with Vandenbroucke et al. [15]. Boundary–spanning primers that specifically amplify each splice variant were designed and their specificity was tested using plasmids containing mHS or HL cDNA with or without the particular exons deleted. The sequences for primers are shown in Table 1. Real-time PCR was carried out in a 20 μl volume containing 1× SYBR Green PCR Master mix (Applied Biosystems), 1 ng of Clontech cDNA and 100 nM of each primer. To quantify each transcript we used different absolute standard curves based on a dilution series of fluorometrically quantified PCR products [15]. To generate these curves, PCR products of each spliced and full-length transcripts were run on an agarose gel. The fragments were excised and eluted using QIAquick Gel Purification (QUIAGEN, Venlo, The Netherlands). The concentrations of PCR products were measured using the Fluorescent DNA quantitation (Biorad, Hercules, USA) on a TKO-100 HSI fluorometer. The level of each transcript was quantified by measuring the Ct value and using the absolute standard curve to determine the copy number. Data are expressed as the ratio of the copy number for the alternative transcript to the copy number of the full-length transcript.

Recombinant expression of HL and mHS variants and enzymatic activity

Plasmid construction

To study the expression of these variants in a prokaryotic model, mutants with the deletion of exon 4 of the *HMGCS2* gene and deletions of exons 5 and 6, and 5, 6 and 7 of the *HMGCL* gene were constructed using the QuickChange PCR-based mutagenesis procedure (Stratagene, La Jolla, USA) with the pMAL-c2x-HL and pMAL-c2x-mHS as template [16]. Primers 5′-TGGCCCTGGAGCGAGCTGGCAGCGCGAGCTGGCAGCGATCGAC-3′ and 5′-GTCGATCGCTGCCAGCTCGCTCCAGGGCCAG-3′ were used to construct pMAL-c2x-mHSΔ4. Primers 5′-GGCTTCAG

Table 1 Sequence of primers used for real-time PCR

mRNA transcript	Forward primer (5′→3′)	Reverse primer (5′→3′)	Amplicon size (pb)
mHS full-length transcript	GAATCAGTGGAAGCAAGCTGG	GAATCAGTGGAAGCAAGCTGG	116
*HMGCS2*Δ4	GGCCCTGGAGCGAGCT	GAATCAGTGGAAGCAAGCTGG	218
HL full-length transcript	TGAAGGGCATTCAGAAGTTTCC	TCTGAGGCAGCTCCAAAGATG	121
*HMGCL*Δ5,6	GAGGCAGCGGTCACCA	AGCCAGGGCAGCCAGAG	142
*HMGCL*Δ5,6,7	TGGGTGACCACACTGAAGTCTT	CGACACTCACTCCCATCGCT	111

AGCAGCGGTCACCAAGAAGTTC-3′ and 5′-GAACTT CTTGGTGACCGCTGCCTCGAAGCC-3′ were used to construct plasmid pMAL-c2x-HLΔ5,6 and primers 5′-GGC TTCAGAGCAGCGATGGGAGTGAGTGTC-3′ and 5′-G ACACTCACTCCCATCGCTGCTCTCTGAAGCC-3′ were used to construct pMAL-c2x-HLΔ5,6, 7. All constructs were validated by sequence analysis.

To study the expression of these variants in a eukaryotic model, mutants with the deletion of exon 4 of the *HMGCS2* gene and deletion of exons 5 and 6 of the *HMGCL* gene were constructed using the QuickChange PCR-based mutagenesis procedure (Stratagene) with the pIRES2-EGFP-HL or the pIRES2-EGFP-mHS as a template (pIRES2-EGFP from Clontech). Primers 5′-CTGGCCCTG GAGCGAGCTG GCAGCGCGAGCTGGCAGCGATCG AC-3′ and 5′-GTCGATCGCTGCCAGCTCGCTCCAGG GCCAG-3′ were used to construct pIRES2-EGFP-mHSΔ4, and primers 5′-GAAAGGCTTCGAGGCAGCGGTCACC AAGAAGTTCTACTC-3′ and 5′-GAGTAGAACTTCTTG GTGACCGCTGCCTCGAAGCCTTTC-3′ were used to construct plasmid pIRES2-EGFP-HLΔ5,6. All constructs were validated by sequence analysis.

Expression in a prokaryotic model

E. coli strain BL21-Codon Plus (DE3)-RIPL expressing MBP-mHSΔ4, MBP-HLΔ5,6 and MBP-HLΔ5,6,7 were grown in LB medium and recombinant proteins were purified according to the method described in Menao et al. [16].

Cell culture and expression in a eukaryotic model

Human embryonic kidney (HEK-293) cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum and 1% penicillin and streptomycin, in 100-mm plates. 4×10^6 Cells were transiently transfected with 15 μg of each plasmid, using JetPei transfection reagent (Polyplus Transfection, NY, USA) and harvested after 48 h.

Enzymatic activity

The amount of protein in each fraction was quantified by Bradford's method [17] and was analyzed by SDS-PAGE. HMG-CoA lyase activity was assayed by the spectrophotometric assay described by Wanders, which measures the amounts of acetoacetate produced [18]. HMG-CoA synthase activity was assayed by a spectrophotometric method based on that described by Clinkenbeard et al. [5].

Western blotting protein analysis

Human tissues: liver, brain, skeletal muscle and pancreas were obtained from autopsies performed at the Department of Pathology of the University of Zaragoza. To isolate the mitochondrial fraction of human tissues, we performed the subcellular separation method reported by Clinkenbeard et al. [5]. Mitochondrial proteins separated on a 15% SDS-PAGE were transferred to a nitrocellulose membrane. Blotted membranes were probed with a polyclonal antibody against aminoacids 425-509 of mHS (Abnova 1:1000 dilution) and a monoclonal antibody against HL (Abnova 1:1000 dilution) or a polyclonal antibody against aminoacids 300–325 of HL (Sigma 1:1000 dilution). All antibodies were expected to bind to the complete and putative deleted forms of the protein. The results were revealed with the kit SuperSignal West Dura Extended Duration substrate (Thermo Scientific, Waltham, USA) using a peroxidase-conjugated secondary anti-mouse antibody (1:1000, Sigma).

Role of splice site strength in splice site selection

The strength of splice sites for a given exon of both *HMGCS2* and *HMGCL* genes was calculated from the consensus values determined by the Splice Site Prediction program by Neural Networks (SSPNN, http://www.fruitfly.org/seq_tools/splice.html) and the gene prediction program *Geneid* [19]. The output of the network is a score between 0 and 1 for a potential splice site, whereas *Geneid* reports a real number as a score.

Protein structure analysis

Bioinformatic analysis was performed to predict the effects of deleted exons on mHS and HL structure. We used the crystallized structure of these proteins as reported by Fu et al. [20] and Shafqat et al. [21]. The ribbon diagrams were drawn with the RASMOL program [22].

Results

Identification of splice variants

To identify alternatively spliced variants of these genes, overlapping RT-PCR amplifications were performed with mHS and HL cDNAs from fibroblasts, liver and skeletal muscle. In the case of *HMGCS2*, the amplification that explored exons 3, 4 and 5 generated a smaller band including deletion of exon 4 (Fig. 1). This deletion is an in-frame splicing. We refer to this novel *HMGCS2* mRNA splicing variant as *HMGCS2*Δ4 (GeneBank accession no. GU433940). PCR amplification that explored exons 4–7 of HL cDNA showed two additional shorter bands. These bands corresponded to a deletion of exons 5 and 6, and to deletion of exons 5–7 of *HMGCL* gene. Both deletions are in-frame splicing. We refer to these novel *HMGCL*

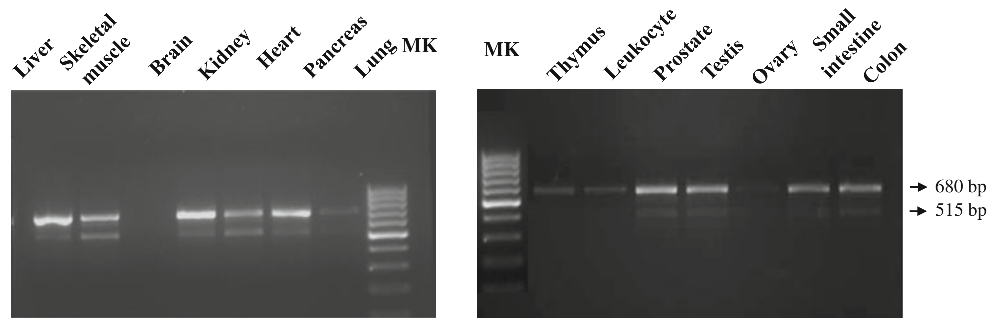

Fig. 1 RT-PCR screening for *HMGCS2* splice variants expression in human tissues. PCR products were separated by agarose gel electrophoresis (2%). The band of normal size has 680 bp. The band of 515 bp corresponds to the amplification of the variant with the deletion of exon 4. MK: Molecular weight marker

mRNA variants as *HMGCL*Δ5,6 (NM_001166059.1) and *HMGCL*Δ5,6,7 (GeneBank accession no. GU433941). The expression pattern of these transcripts in various human tissues is shown in Fig. 2. The *HMGCS2*Δ4 and *HMGCL*Δ5,6 variants were also confirmed in the Alternative Splicing Database (ASD) http://www.ebi.ac.uk/asd/.

Quantification of splice variants

We quantified full-length *HMGCS2* and *HMGCL* and their alternative transcripts in eight human tissues by quantitative PCR. The highest *HMGCS2* expression was found in liver (320,528 copies), while kidney, pancreas and testis had lower values, around 2,000 copies. Much lower values were found in heart (773 copies) and skeletal muscle (750 copies) while no mRNA *HMGCS2* expression was found in brain. The tissues with the highest levels of full-length *HMGCL* transcript were liver (7,734 copies), kidney (2,060 copies) and intestine (1,113 copies). However, we also found high expression in tissues that are not classically considered ketogenic: pancreas (2,946 copies), and testis (1,979 copies). In contrast, other tissues showed low mRNA levels: brain (121 copies) and skeletal muscle (117 copies).

The percentages of alternative splicing transcripts relative to the full-length *HMGCS2* transcript in several tissues are shown in Fig. 3. The highest values were found in heart (70.3%) and skeletal muscle (60.2%). Tissues such as kidney and pancreas had values between 11 and 13% and testis and liver had values under 5%.

The percentages of deleted splice transcripts relative to the full-length transcript of *HMGCL* in different tissues are shown in Fig. 3. The *HMGCL*Δ5,6 variant is the most abundant, but the percentages vary over a large range, depending on the tissue. The highest values were found in heart with 29.5%, followed by skeletal muscle with 22.4% and brain with 14.4%. In contrast, tissues such as small intestine, liver, kidney, testis and pancreas contained less than 2% of the variant genes. The values of the *HMGCL*Δ5,6,7 variant are lower than those of the *HMGCL*Δ5,6 variant. Only skeletal muscle had values over 5%, and the remaining tissues had values below 1%.

In vitro expression of splice variants

Splice variants detected as mRNA were cloned in pMAL and pIRES2-EGFP vectors to analyze their expression as proteins in prokaryotic or eukaryotic systems respectively. The resulting wild-type proteins were expressed in active and soluble form with mHS activity, 22.1 U/mg protein and HL activity, 29.5 U/mg protein. However, when attempts were made to overexpress the deleted proteins in *E. coli* or HEK-293 cells we did not find them in the soluble fraction.

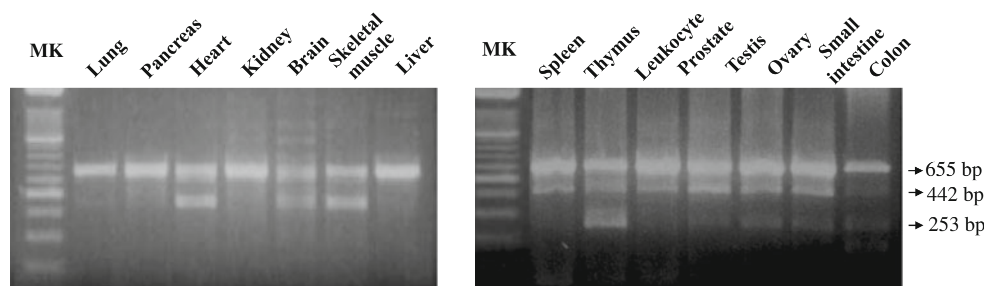

Fig. 2 *HMGCL* splice variants RT-PCR screening for PCR products were separated by agarose gel electrophoresis (2%). The band of normal size appears to 655 bp. The band to 442 bp corresponds to the amplification of the variant with deletion of exons 5 and 6. The band of 253 bp corresponds to the variant with deletion of exons 5, 6 and 7. MK: Molecular weight marker

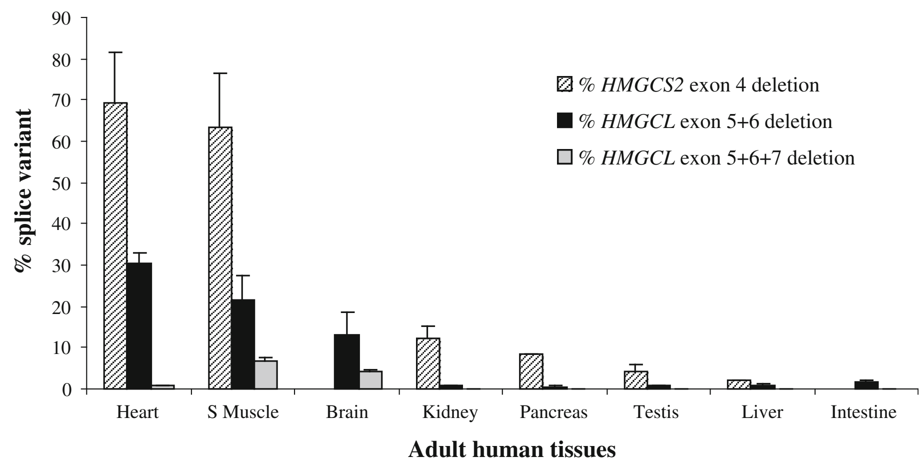

Fig. 3 Quantitative real-time PCR analysis of *HMGCS2* and *HMGCL* splice variants. Specific amplifications of full-length transcripts and deleted transcripts were performed using primers described in Table 1 and commercial cDNA from human tissues. The bars in the graph represent the percentage of the number of molecules of splice variant relative to number of molecules of the full transcript. The values are means of triplicate determination ±SD

Western blotting protein HL and mHS analysis

In the Western blot of the mHS protein (Fig. 4a), we observe a more intense band corresponding to the complete protein at approximately 53 kDa. Western blot, using different polyclonal or monoclonal antibodies against HL in the mitochondrial fraction obtained from various human tissues showed a band corresponding to 31.5 kDa, the size of the complete HL protein (Fig. 4b). However, we did not detect proteins from transcripts *HMGCL*Δ5,6, or *HMGCL*Δ5,6,7. These proteins should have a molecular mass of approximately 24 and 17 kDa, smaller than the complete protein.

Bioinformatic analysis

The SSPNN program, used to calculate the strength of the 5′ and 3′ splice sites of nine exons of *HMGCL* gene, revealed that the weakest exons are 2 and 5. Both exons have a weak acceptor site with values of 0.35 and 0.53, respectively. *Geneid*, however, only reported a negative score for the acceptor site of exon 5 (−0.90) and reported positive scores for all other splice sites. Of the ten exons of the *HMGCS2* gene only exon 3 showed a weak donor site (0.53).

To illustrate the effect of deletions, the deleted exons are highlighted in the structure described for mHS and HL proteins (Fig. 5). The splice variant *HMGCS2*Δ4 with exon 4 deleted (55 aa) caused the loss of beta sheet 8 and alpha helix 7 in the core of the mHS protein (Fig. 5a). Exon 5 plus 6 skipping (71 aa) in HL protein produced the loss of part of the alpha helices 3, 4 and 5, and beta-sheets 4 and 5 (Fig. 5b). The transcript with the skipping of exons 5, 6 and 7 (134 aa) caused, in addition, the disappearance of alpha helices 6 and 7 and beta-sheets 6 and 7, which contain Histidine-233 and Histidine-235 (Fig. 5c). These aminoacids have been identified as metal-binding ligands that are important for enzyme activity [20].

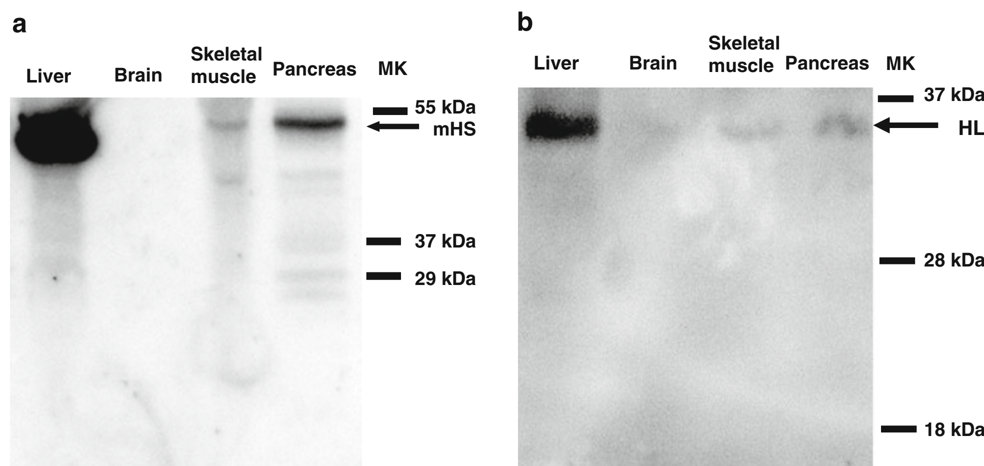

Fig. 4 mHS and HL protein expression in human tissues. Twenty micrograms of protein from mitochondrial extracts from human liver, brain, skeletal muscle and pancreas was separated by SDS/PAGE and subjected to immunoblotting by using mHS and HL antibodies. **a** In mHS Western blot a band corresponding to the complete protein at approximately 53 kDa is observed. **b** Only the bands corresponding to the complete HL protein (31.5 kDa) were observed in all tissues. MK: Molecular weight marker

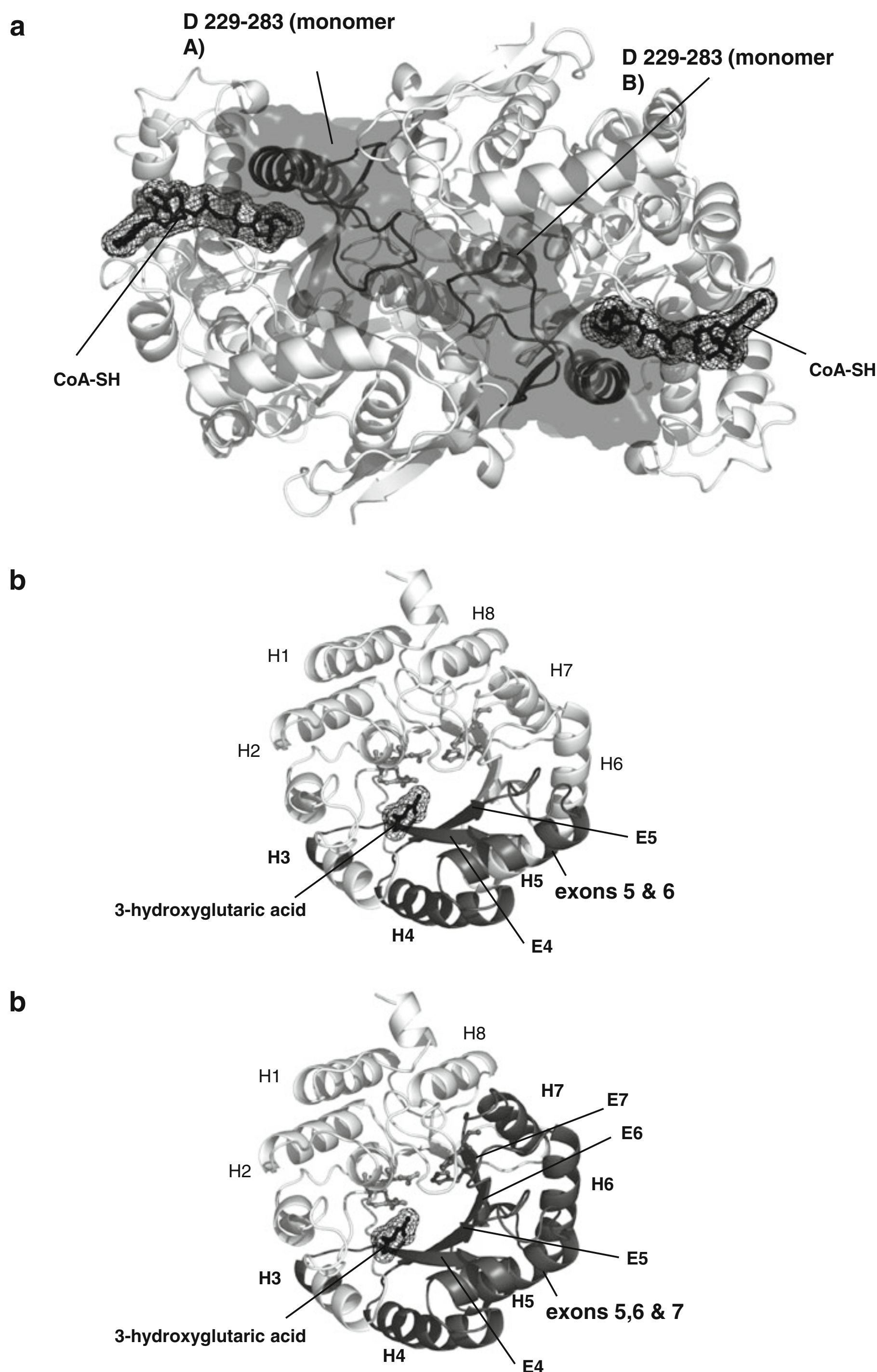

Fig. 5 Ribbon diagrams of structure of wild type mHS and HL. **a** Ribbon diagrams of structure of wild type mHS showing deletion of exon 4. The position of the aminoacids deleted (229–283) is highlighted in gray in the main zone of interaction between monomers. **b** Structure of wild type HL showing deletion of exons 5 and 6 and **c** Structure of wild type HL showing deletion of exons 5, 6 and 7. Secondary structure elements which were deleted are shown in *black*, and the position of the substrate in the protein structure is highlighted

Discussion

This is the first study of the physiological splice variants of two key enzymes of ketogenesis, mHS and HL. Transcripts were identified by amplifying overlapping fragments of cDNA of these genes. For human *HMGCS2* gene only one isoform with exon 3 deletion is present in the NCBI database (NM_000191.2), but it was identified in a liver tumor. We could not find this splice variant in eight normal human tissues (data not shown), which suggests that it is only present in pathological tissues. However, in this study we detected a physiological alternative transcript with exon 4 skipping (*HMGCS2*Δ4). In the *HMGCL* gene two physiological alternative transcripts were identified: one bearing a deletion of exons 5 and 6 (*HMGCL*Δ5,6) and the other with a deletion of exons 5, 6 and 7 (*HMGCL*Δ5,6,7). The

*HMGCL*Δ5,6 transcript was previously reported in association with the c.504_5delCT mutation [23] and in normal tissues [24] but it was not fully characterized. The existence of the splice variants *HMGCL*Δ5,6 and *HMGCS2*Δ4 was confirmed by reference to the ASD (Alternative Splicing Database) http://www.ebi.ac.uk/asd/, but the *HMGCL*Δ5,6,7 transcript is reported here for first time. The knowledge of these physiological splicing variants may help in the correct interpretation of the pathological variants of both genes: on the one hand, it is essential to discriminate pathological transcripts, and on the other hand, the mutations may affect the ratio of the physiological splice variants.

It is difficult to determine the precise mechanisms by which the splicing variants of these genes are generated, since multiple parameters may be involved: splice site strength, exon/intron architecture, RNA structure or splicing sequences regulators [25]. According to the splice site strength, exons 2 and 5 are weak in the *HMGCL* gene. However, no physiological splice variants have been found with the exon 2 deletion. The role of exon 2 as a strong exon could be related to the presence of exonic splicing enhancers (ESEs) that would strengthen it [11, 26, 27]. The weakness of the definition of exon 5 could explain the existence of *HMGCL*Δ5,6 and *HMGCL*Δ5,6,7 variants. It may be a drag mechanism of exon 5, on the strong posterior exons 6 and 7 [12]. Although similar cases have been described with physiological [28, 29] or pathological splice variants [12, 30] the proposed drag mechanism is not completely understood. In the *HMGCS2* gene, the high splice site strength of exon 4 suggests that other parameters modulate its skipping [25].

Today, the role that the splicing mechanism can play in proteome expansion and gene regulation is evident [8, 9]. In the two genes studied, none of the alternative transcripts found disrupts the reading frame and they may all encode new proteins. However, the bioinformatic analyses of these variants show that these structural deletions may have compromised mHS and HL activity. In the *HMGCS2* gene, the deletion of exon 4 affects a protein core area next the substrate interaction and the area of interaction of the two monomers (Fig. 5a). Recently three missense mutations have been localized in exon 4 in patients with mHS deficiency [21], which highlights the importance of the segment encoded by this exon. As shown in the figure of the HL structure (Fig. 5b, c) deletions affect a significant fraction of the hydrophobic core and aminoacids that are essential for enzymatic activity: His-233, which is required for the correct cleavage of the substrate [31] and His-235, which is involved in the octahedral coordination of the divalent cation [32]. In order to discern whether the variant transcripts produced protein, we examined the expression in vitro of their recombinant proteins in prokaryotic and eukaryotic overexpression systems. However, we did not detect soluble proteins and we could not measure enzymatic activity, although we succeeded in expressing and measuring the activity of the wild-type protein. Moreover, we analyzed HL and mHS protein expression in various human tissues by Western blot. The blot clearly showed the wild-type proteins and we did not detect the predicted proteins with deletions, in the mitochondrial fraction. This suggests that the variants are present at the mRNA level but cannot be detected as proteins. The production of non-encoding transcripts is common in physiological alternative splicing processes and it has been interpreted as a mechanism of regulation of gene expression [33].

To study this further, we measured the full-length and deleted variants of *HMGCL* and *HMGCS2* genes in various human tissues, by qPCR. The expression results of the full-length transcript of *HMGCS2* gene agree with those obtained in other studies using semiquantitative Northern blot [3, 34]. The levels in skeletal muscle and heart were higher in Mascaró's study [34], although if we assume that the amount of the full-length and deleted transcript was quantified together, their results agree with ours. Interestingly, when we compare the percentage of alternative transcripts, ketone body-producing tissues with higher levels of the full-length transcript have low (<10%, kidney and pancreas) or very low (<5%, liver and testis) percentages of the inactive gene variant. In contrast, the low or non-ketogenic tissues (heart, skeletal muscle and brain), although ketone body-consumers, have less of the full-length transcript and more of the deleted variant (60–70%). These findings suggest that the mHS alternative splicing could reduce the level of active enzyme by generating shorter, inactive transcript variants in these tissues.

Surprisingly, the tissue distribution of inactive variants of *HMGCL* gene seems to follow a similar pattern to the *HMGCS2* gene, with higher values for non-ketone body producing tissues: heart, skeletal muscle and brain. However, the percentages are lower than the *HMGCS2* gene. This might be related with the role of HL in leucine catabolism, which requires its presence in all tissues [35]. We detected a high level of the *HMGCL*Δ5,6 variant in brain. However, this was not found in the *HMGCS2* gene variant because this gene is not expressed in brain [34].

Recently, a global study with microarrays reinforced the idea that alternative splicing can coordinate the activity of functionally related genes [14]. Our findings support this hypothesis and suggest that alternative splicing could coordinately block the two main enzymes of ketogenesis, mitochondrial HMG-CoA synthase and HMG-CoA lyase. Alternative splicing could act as a tissue-specific blocking mechanism rather than a regulatory mechanism in different metabolic situations, and it could be important in tissues that do not produce ketone bodies such as heart and skeletal

muscle. On the one hand, these tissues would have a low rate of *HMGCL* and *HMGCS2* gene transcription and on the other hand, they would have splicing machinery to produce inactive forms. The result would be that these tissues would have a low level of HL and mHS as a result of this double "lock". Similar mechanisms have been described in the coupling of splicing and nonsense-mediated decay that help the cell to achieve the proper level of expression for a given protein [36].

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