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Identification And Functional Analyses of *CBS* Alleles In Spanish And Argentinian Homocystinuric Patients

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ABSTRACT: Homocystinuria due to CBS deficiency (MIM#236200) is a rare autosomal recessive disorder characterized by elevated plasma levels of homocysteine (Hcy) and methionine (Met). Here we present the analysis of 22 unrelated patients of different geographical origins, mainly Spanish and Argentinian. Twenty-two different mutations were found, ten of which were novel. Five new mutations were missense and five were deletions of different sizes, including a 794-bp deletion (c.532-37_736+438del794) detected by Southern blot analysis. To assess the pathogenicity of these mutations, seven were expressed heterologously in *E. coli* and their enzyme activities were assayed *in vitro*, in the absence and presence of the CBS activators PLP and SAM. The presence of the mutant proteins was confirmed by Western-blotting. Mutations p.M173del, p.I278S, p.D281N and p.D321V showed null activity in all conditions tested, while mutations p.49L, p.P200L and p.A446S retained different degrees of activity and response to stimulation. Finally, a minigene strategy allowed us to demonstrate the pathogenicity of an 8-bp intronic deletion, which led to the skipping of exon 6. In general, frameshifting deletions correlated with a more severe phenotype, consistent with the concept that missense mutations may recover enzymatic activity under certain conditions.

KEY WORDS: CBS mutations; homocystinuria; Southern blot; mini-gene; heterologous expression; enzyme activity

Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid that occupies a major regulatory branch point in methionine metabolism. Homocysteine may be either remethylated to methionine or catabolized to form cysteine through the transsulfuration pathway. The first step of transsulfuration involves the condensation of homocysteine with serine to form cystathionine, a reaction catalyzed by the enzyme cystathionine β-synthase (CBS; EC 4.2.1.22).

Mutations in the *CBS* gene (MIM# 613381) cause classical homocystinuria (Hcu, MIM# 236200), an autosomal recessive disease characterized by severe hyperhomocysteinemia and homocystinuria, decreased plasma levels of cysteine and, often, hypermethioninemia. At the clinical level, classical homocystinuria mainly affects the eye, the skeleton, the vascular system and the central nervous system (CNS). Symptoms usually include ectopia lentis, osteoporosis, scoliosis, Marfanoid features, premature arteriosclerosis, thromboembolism and mental retardation (Mudd, et al., 1985). Age of onset and disease severity are highly variable, ranging from dramatically affected children to asymptomatic adults (Magner, et al., 2011; Skovby, et al., 2010; Walter, et al., 1998; Yap and Naughten, 1998). Treatments that lower tHcy, such as B-vitamins, dietary methionine restriction, and betaine supplementation, can significantly reduce the incidence of vascular events (the main cause of death in these patients) and improve the neurological problems (Wilcken and Wilcken, 1997; Yap, et al., 2001; Yap and Naughten, 1998).

Homocystinuria is a rare disease with variable incidence. While the worldwide incidence is estimated as 1/344000 born alive (Mudd, et al., 1995), in Qatar it is 1/3124 (El-Said, et al., 2006) and in Japan 1/800 000 (Mudd, et al., 1995). In Northern Europe the incidence may be of 1/20500 to 1/6400, as estimated from the high

number of p.I278T mutation carriers found in some populations (Gaustadnes, et al., 1999; Refsum, et al., 2004).

To date, more than 150 different *CBS* mutations have been found worldwide, some of them common. Mutation p.I278T is considered panethnic and is particularly frequent in Northern Europe (Janosik, et al., 2001b; Kraus, et al., 1999; Moat, et al., 2004). In Ireland, the p.G307S mutation accounts for 71% of the mutant alleles (Gallagher, et al., 1995), and in the Iberian Peninsula and Colombia, mutation p.T191M represents between 40% and 75% (Urreizti, et al., 2006a) of the mutant alleles.

A truncated form of the human CBS enzyme lacking the C-terminal regulatory domain has been crystallized (Janosik, et al., 2001a; Meier, et al., 2001) and structurally characterized (Banerjee and Zou, 2005; Kabil, et al., 2001; Kery, et al., 1999; Meier, et al., 2001; Taoka, et al., 2002). The active form of this cytoplasmic enzyme is a homotetramer of four 63-KDa subunits. Each subunit combines one heme group and one pyridoxal phosphate (PLP), the latter acting as a cofactor in the reaction. In addition, each subunit binds the allosteric activator S-Adenosil-Methionine (S-AdoMet or SAM), an intermediate in the methionine cycle. Heterologous expression in *E. coli* has been widely used to test the functionality of the *CBS* mutant alleles independently from the patient's genetic background (de Franchis, et al., 1994; Katsushima, et al., 2006; Maclean, et al., 2002). It has been proven to be a useful tool since the *E. coli*-expressed human CBS is indistinguishable from that obtained from cultured fibroblasts (Bukovska, et al., 1994; Kozich and Kraus, 1992). In addition, *in vitro,* its relative activity in response to PLP and SAM is comparable to that of the enzyme obtained from human tissues (Bukovska, et al., 1994; Kozich and Kraus, 1992).

This study updates the *CBS* mutation spectrum of the homocystinuric patients from the Iberian Peninsula by presenting the analysis of 16 new cases. It also includes one Norwegian, one Indian and four Argentinian patients. In this cohort of 22 cases, 22 mutations were found, ten of which were novel. The new mutations include a deletion of 794 bp (c.532-37_736+438del794) detected by Southern blot analysis and an intronic deletion that leads to the skipping of exon 6, which was characterized using a minigene strategy. The pathogenic role of seven of the changes was assessed by heterologous expression of these mutant proteins, and their stability and activity were analyzed.

Materials And Methods

Patients

Twenty-six patients with classical homocystinuria from 22 unrelated pedigrees were involved in this study. Patients were initially diagnosed by their physicians on the basis of clinical manifestations suggestive of homozygous CBS deficiency. Biochemically, these patients presented with a combination of severe hyperhomocysteinemia (typically above 150 μmol/l), and hypermethioninemia (typically above 40 μmol/l). Thirteen Spanish, three Portuguese, one Norwegian, one Indian and four Argentinian patients were included in the study. Our research was conducted in accordance with the tenets of the Declaration of Helsinki. The nature and possible consequences of the study were first explained to all patients and/or their parents, before their informed consent for inclusion in the research project was obtained.

PCR amplification and DNA sequencing

Genomic DNA was prepared from peripheral blood leukocytes, using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA).

All 16 *CBS* coding exons (including exon 15) and their intronic flanking regions, were amplified by PCR and sequenced as described previously (Urreizti, et al., 2006a) with some modifications. Briefly, PCR reactions were performed on a final volume of 50 µl with 50 ng gDNA, 0.2 mM of each dNTP, 0.4 mM of each primer, 1.5–2.5 mM $MgCl_2$ and 1.25 U of GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA). All mutations detected were confirmed by restriction analysis of the PCR products with the appropriate restriction enzyme, and the presence of all new mutations was assessed in 100 control chromosomes from Spanish anonymous donors. Primer sequences and PCR conditions have been described previously (Urreizti, et al., 2006a). *MTHFR* c.677C>T (rs1801133) was analyzed in all patients as described in Frosst et al. (Frosst, et al., 1995).

To characterize the deletion found in patient 87, genomic DNAs of the patient and her parents were PCR-amplified using primers 4F and 7R as described above with the addition of a final concentration of 5% DMSO. The PCR fragments were purified and sequenced.

For naming the mutations the following reference sequences were used: Genomic, GenBank NG_008938.1; cDNA, ENST00000352178. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Southern blot

A Southern blot to analyze a 36-kb fragment of the *CBS* genomic region (Fig. 1), between two *Drd*I sites, encompassing all 23 exons, plus 6 kb of the 5′ flanking region and 11.5 kb of the 3′ region, was performed as follows: 10 μg of patient and control gDNA were double digested by *Drd*I and *Afl*II (NEBiolabs, Ipswich, MA, USA), electrophoresed on a 0.9% agarose gel, blotted onto Amersham Hybond™-N+ (GE Healthcare, Waukesha, WI, USA) membrane using standard protocols, and fixed by UV crosslinking. The *Drd*I/*Afl*II double digestion of the 36 kb yields, in a wild-type setting, four fragments of around 15 kb, 10 kb, 8 kb and 3 kb, respectively (Fig. 1A).

Probe design, preparation and hybridization

Four probes were designed by selecting one unique sequence of approximately 600 bp within each restriction fragment. The sequences were aligned to the whole genome by BLAST, to confirm their specificity. These four sequences were PCR-amplified from total genomic DNA (as described above), cloned into pUC19 vector (Fermentas, Burlington, ON, Canada) and sequenced. Probes were obtained from the clones by using digoxigenin-dUTP and the "PCR DIG Probe Synthesis Kit" (Roche, Mannheim, Germany), according to the manufacturer's instructions. Primer sequences for the probes are available on request. Labelled probes were subsequently purified using Illustra™ GFX™ PCR DNA and a Gel Band Purification Kit (GE Healthcare, Waukesha, WI, USA).

All subsequent steps of the Southern protocol (prehybridization, hybridization and developing of the filters) were performed using reagents (Dig Easy Hyb) and protocols (Dig Application Manual) from Roche (Mannheim, Germany).

Site-directed mutagenesis, heterologous expression and *in vitro* enzyme activity assays of the *CBS* mutations

All *CBS* mutant constructs were derived from the wild-type *CBS* expression plasmid pHCS3 (Kozich and Kraus, 1992), a gift from the authors of that study. Each mutation was introduced into the wild-type expression plasmid using a Quik Change II XL ™ Site-Directed Mutagenesis Kit (Stratagene Cloning Systems, La Jolla, CA, USA) and expressed in XL-Gold *E. coli* cells as described in Urreizti et al. (Urreizti, et al., 2006b). Expression of pHCS3 was used as a positive control, and that of the empty vector pKK388.1 was included as a negative control. Protein extracts were obtained and CBS activity was measured as described in Kraus (Kraus, 1987) with some modifications (Urreizti, et al., 2006b).

CBS protein analysis

To assess the presence and relative amount of wild-type and mutant CBS proteins, Western blot analysis of the soluble fraction of the crude cell lysates was performed under denaturing conditions as described in Janosik et al. (Janosik, et al., 2001b), with some modifications (Urreizti, et al., 2006b).

Minigene construction and splicing assay

Genomic DNA from patient 68a (heterozygote for mutation c.667-14_-7del8) was amplified using *CBS* primers 4F and 6R to obtain a fragment of 626 or 618 bp (from the last 29 bp of intron 3 to the first 103 bp of intron 6). This PCR product, containing both wild-type and mutant alleles, was purified using Illustra™ GFX™

PCR DNA and a Gel Band Purification Kit (GE Healthcare, Waukesha, WI, USA) and cloned into the pGLB1 vector (Diaz-Font, et al., 2005). Plasmid pGLB1 is based on a pcDNA3.1 vector and contains exons 7, 8, and 9 and introns 7 and 8 of the *GLB1* gene, where intron 7 contains a *Pme*I restriction site, which was used to clone the *CBS* fragment. The resulting plasmids, named pGLB1-CBS_wt and pGLB1-CBS_mut, were confirmed by sequencing.

The splicing assay was performed by transfecting 1 μg of each minigene plasmid with 5 μl of Lipofectamine 2000 Reagent (Life Technologies, Basel, Switzerland) into 90% confluent HeLa cells. Total RNAs were isolated 24 h after transfection using the QIAshredder and the RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA concentration was determined spectrophotometrically, integrity was verified by gel electrophoresis and RNA quality was assessed by OD 260/230 and 260/280 ratios.

Two μg of each RNA was reverse-transcribed in a final volume of 20 μl using a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

PCR amplification of each cDNA product was performed using primers T7 and SP6 as previously described (Diaz-Font, et al., 2005; Santamaria, et al., 2008). The PCR products were electrophoresed and each band of interest was purified and analyzed by sequencing.

Results

Screening for *CBS* mutations

Twenty-two different mutations (Table 1), ten of them novel, were found in 22 unrelated patients of different geographical origin (Table 2). Forty-two mutant alleles were identified through sequencing of coding exons and flanking intronic regions, while an additional mutant allele was discovered by Southern blot analysis (see below). Only one allele remained unidentified. Five of the new mutations were missense while five were deletions.

After sequencing the complete *CBS* coding sequence, only mutation c.1566delG was found in heterozygosis in patient 87, which she inherited from her father. A Southern blot analysis of this patient and her mother, hybridized with a set of four probes covering the entire *CBS* genomic region (Fig. 1A), revealed the same pattern of bands observed in control samples but the relative intensities of fragments 2, 3 and 4 were altered (Fig. 1B). Hybridization of the same membrane separately with each individual probe revealed an additional band of nearly 10 kb detected by probes B and C (Fig. 1C). This result was consistent with a deletion removing the *Drd*I site present in intron 5. To test this hypothesis, a PCR amplification from intron 3 to intron 7 was performed. As shown in Fig. 1D, a new band of 1176 bp was observed in the samples of the patient and her mother. Sequencing revealed a 796-bp deletion (c.532-37_736+438del794), which spans from the last 37 bp of intron 4 to the first 438 bp of intron 6 (Fig. 1E). This deletion causes a frameshift starting from a valine to glycine substitution at position 178 and leading to a stop codon at residue 201 (p.V178GfsX23).

Analyses of pathogenicity of mutant CBS enzymes

None of the new mutations found was present in 100 control chromosomes, ruling out the possibility of common polymorphisms.

We assessed the pathogenicity of all five new missense mutations and of the novel in-frame deletion p.M173del using an *E. coli* heterologous expression system followed by Western blot and *in vitro* enzyme activity analysis (Fig. 2). Mutation p.P49L, previously described by de Franchis et al. (de Franchis, et al., 1998) was also analyzed.

All the proteins from the mutant alleles were found in amounts similar to those of the wild type (Fig. 2, upper panel). The activity of these mutant enzymes was assayed *in vitro,* either in the presence or absence of the cofactor PLP, or in the joint presence of PLP and SAM (Fig. 2, lower panel). As previously described (Kluijtmans, et al., 1996; Maclean, et al., 2002), we found that the wild-type CBS activity was strongly stimulated by both PLP and the combination of PLP and SAM. Mutations p.M173del, p.I278S, p.D281N and p.D321V showed null activity, either in the absence or presence of the activators. In contrast, mutations p.49L, p.P200L and p.A446S retained different amounts of activity and also the ability to be stimulated.

In particular, mutation p.P49L displayed null activity in the absence of any of the activators, retained 71% of the wild-type activity in the presence of PLP and showed reduced activation by SAM. In contrast, in the absence of any of the activators, mutation p.P200L displayed around a third of the activity of the wild type (15% versus 43%) and retained the ability to be induced by PLP and SAM. SAM stimulation was 2-fold, comparable to that of the wild type. Finally, p.A446S displayed highly variable levels of activity in different tests, in the absence or presence of activators, but in general the values where in the range of the wild type or higher.

Effect of a small intronic deletion on RNA splicing

The small deletion c.667-14_-7del8 involved intron 5 sequences within the acceptor site, without affecting the conserved AG dinucleotide. Since no RNA was available from patients with this mutation (68a and 68b), a minigene assay was performed to test the effect of this mutation on RNA splicing (Fig. 3A). The genomic region spanning from the 3' part of intron 3 to the 5' part of intron 6 of the *CBS* gene of the mutant and wild-type alleles was PCR-amplified and cloned within intron 7 of a construct containing several *GLB1* exons (see Material and Methods for details). Wild-type and mutant constructs were transfected into HeLa cells and splicing was assayed by RT-PCR using the T7 and SP6 primers (Fig. 3B). The major band observed in the wild-type sample corresponds to the inclusion of all three *CBS* exons, whereas in the mutant, exon 6 was skipped, as assessed by sequencing of all the bands.

Discussion

Mutation p.T191M was identified in 21.8% of the alleles of the Spanish and Portuguese patients included in this study (Table 2). Taking into consideration all 50 homocystinuric patients from the Iberian Peninsula included in this and in our previous studies (Urreizti, et al., 2003; 2006a), mutation p.T191M accounts for 44% of the mutant chromosomes. Mutation c.1566delG is the second most prevalent change (7% overall), and is particularly common in Portugal (21%). In the present study, we found the panethnic mutation p.I278T for the first time in two unrelated Spanish patients, both in the heterozygote state, and in a Norwegian patient, in homozygosis (Table 2). Missense mutations are the most common alterations in the *CBS* gene in the patients of the present study (73%). We also found six deletions of different sizes (1 bp to 794 bp), five of them novel.

Southern blot analysis allowed us to identify a large deletion of nearly 800 bp in the heterozygous state (c.532-37_736+438del794, p.V178GfsX23) in patient 87 and in her mother. Additionally, a qmfPCR assay was performed on the patient's sample yielding a consistent result (C. Ged, personal communication). This patient, diagnosed through neonatal screening, presented with dramatically increased plasma methionine (700 μM) and total homocysteine (145 μM). She has been on a normal diet and treated with pyridoxal, folate and betaine since her birth. Currently aged 4 years, her plasma homocysteine and methionine levels are 76 μM and 302 μM, respectively, and she is clinically asymptomatic, highlighting the importance of an early diagnosis.

An intronic 8-bp deletion (c.667-14_-7del8) was found in two siblings (#68a-#68b, Table 2). Prediction algorithms indicated a subtle reduction in the score of the splicing acceptor site of intron 5. However, a mini-gene analysis clearly showed that it led to the skipping of exon 6, and produced a frameshift. This is consistent with the severity of the patients' phenotypes. We also found a 5 bp deletion in exon 8 in patient #109, and a 1 bp deletion in exon 6 in patient #89, both in homozygosis (Table 2). The severity of these genotypes is in accordance with the severe clinical presentation of both patients. In general, frameshift-generating deletions correlate with a more consistently severe phenotype, as expected.

Seven mutations (six missense and a 3-bp deletion) were expressed in *E. coli* and analyzed by Western blot under denaturing conditions (Fig. 2, upper panel). Their presence in amounts similar to those of the wild-type CBS suggests that the mutations do not affect protein integrity. Alternatively, they may affect the catalytic core, proper protein folding or the ability to form tetramers. In this regard, several authors have studied the involvement of certain chaperones in the rescue of missense

CBS mutants, and demonstrated that the regulation of these chaperones could lead to almost complete recovery of the mutant protein activity (Kopecka, et al., 2010; Majtan, et al., 2010; Singh, et al., 2007; Singh, et al., 2010).

Four mutations showed null activity, which supports their pathogenic character. These mutations, p.D281N, p.I278S, p.D321V and p.M173del, were found in four unrelated patients, #82a (and his siblings), #94, #90, and #55, respectively. No correlation between their null activity and the phenotypes of the patients could be established since all but patient #94 presented with mild disease.

The other three mutations, displaying different degrees of enzyme activity, were p.P49L, p.P200L and p.A446S, present in patients #80, #79 and #90, respectively, all in the heterozygous state. In these cases, a correlation between their residual activity and the mild phenotype of the patients was observed.

We found the activity of p.P49L to be 75% of that in the wild type. While our work was ongoing, Kozich et al. (Kozich, et al., 2010) published an analysis of mutation p.P49L expressed in *E. coli*. They found it to be an active enzyme, indistinguishable from the wild type. Mutation p.P49L was found in two siblings, one with adult onset and the other asymptomatic. Genetic analysis of the proband (#80a in Table 2 and subject II.1 in Fig. 4), showed compound heterozygosity for p.P49L and p.R125Q (Table 2). Genotyping of his siblings revealed an asymptomatic sister also carrying both mutations (#80b; II.3 in Fig. 4). The proband had suffered a myocardial infarction at age 53. Blood testing revealed moderate hyperhomocysteinemia and hypermethioninemia (58 and 655 μM, respectively) and he responded well to PLP (tHcy: 28 μM after treatment). On the other hand, his asymptomatic sister, aged 54, presented with severe hyperhomocysteinemia (543 μM) and hypermethioninemia (1723 μM) but had no clinical sign of classical

homocystinuria and had given birth to three healthy offspring without complications. As the two *CBS* mutations were inherited separately by the offspring, we know that they were not in *cis* in the mother or in his affected brother. Mutation p.R125Q, the other mutation in family #80, was originally described as a null activity mutation (Sebastio, et al., 1995). In their recent publications, Kozich et al. (Kozich, et al., 2010) and Majtan et al. (Majtan, et al., 2010) expressed it in *E. coli.* In both studies the reduced activity was improved under permissive conditions or in the presence of chaperones. Taken together, these new results on p.P49L and p.R125Q help explain the mild or null affectation of patients #80a and #80b. In addition, the *MTHFR* 677C>T polymorphism may act as a phenotype modifier in this family. However, the discrepancy between the blood markers and the phenotype of patient #80b remains unexplained.

Mutation p.P200L was identified as the sole mutation in patient #79. The patient's symptoms are limited to hypertension and severe hyperhomocysteinemia (183 μM), which does not respond to vitamin B6 and folate treatment. A second *CBS* mutation may be deep intronic, located in a regulatory region far from the gene, or involved in a complex rearrangement not detected by our screening methods. Mutation p.A446S was found in a mildly affected patient (#90, Table 2), in compound heterozygosis with the null mutation p.D321V (Fig. 2). The activity of p.A446S was very similar to that of the wild type and this correlates with the mild phenotype of the patient, who only presented with lens dislocation at 45 years, despite a high tHcy level (105 μM, which was reduced to 40 μM after two months of treatment with folic acid and vitamins B6 and B12).

The poor genotype–phenotype correlation widely observed by us and others among the homocystinuric patients and the demonstration that some patients

carrying two *CBS* mutant alleles are asymptomatic, call for environmental and genetic modifiers involved in the pathophysiology of the disease. Some of these genetic factors may be involved in the folding and degradation of the mutant proteins.

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References:

Banerjee R, Zou C. 2005. Redox regulation and reaction mechanism of human cystathionine-β-synthase: a PLP-dependent hemesensor protein. Arch Biochem Biophys 433:144-156.

Bukovska G, Kery V, Kraus JP. 1994. Expression of human cystathionine beta-synthase in Escherichia coli: purification and characterization. Protein Expr Purif 5:442-8.

Chen X, Wang L, Fazlieva R, Kruger WD. 2006. Contrasting behaviors of mutant cystathionine beta-synthase enzymes associated with pyridoxine response. Hum Mutat 27:474-82.

de Franchis R, Kozich V, Mclinnes RR, Kraus JP. 1994. Identical genotipes in siblings with differnt homocystinuric phenotypes: identification of three mutations in cystationine B-synthase usin en improved bacterial expression system. Hum Mol Genet 3:1103-1108.

de Franchis R, Sperandeo MP, Sebastio G, Andria G. 1998. Clinical aspects of cystathionine beta-synthase deficiency: how wide is the spectrum? The Italian Collaborative Study Group on Homocystinuria. Eur J Pediatr 157 Suppl 2:S67-70.

Diaz-Font A, Santamaria R, Cozar M, Blanco M, Chamoles N, Coll MJ, Chabas A, Vilageliu L, Grinberg D. 2005. Clinical and mutational characterization of three patients with

multiple sulfatase deficiency: report of a new splicing mutation. Mol Genet Metab 86:206-11.

El-Said MF, Badii R, Bessisso MS, Shahbek N, El-Ali MG, El-Marikhie M, El-Zyoid M, Salem MS, Bener A, Hoffmann GF and others. 2006. A common mutation in the CBS gene explains a high incidence of homocystinuria in the Qatari population. Hum Mutat 27:719.

Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den HM, Kluijtmans LA, van dHL and others. 1995. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase [letter]. Nat Genet 10:111-3.

Gallagher PM, Ward P, Tan S, Naughten E, Kraus JP, Sellar GC, McConnell DJ, Graham I, Whitehead AS. 1995. High frequency (71%) of Cystathionine B-Synthase Mutation G307S in Irish Homocystinuria Patients. Hum Mutat 6:177-180.

Gaustadnes M, Ingerslev J, Rutiger N. 1999. Prevalence of congenital homocystinuria in Denmark. N Engl J Med 340:1513.

Janosik M, Meier M, Kery V, Oliveriusova J, Burkhard P, Kraus JP. 2001a. Crystallization and preliminary X-ray diffraction analysis of the active core of human recombinant cystathionine beta-synthase: an enzyme involved in vascular disease. Acta Crystallogr D Biol Crystallogr 57:289-91.

Janosik M, Oliveriusova J, Janosikova B, Sokolova J, Kraus E, Kraus JP, Kozich V. 2001b. Impaired heme binding and aggregation of mutant cystathionine beta-synthase subunits in homocystinuria. Am J Hum Genet 68:1506-1513.

Kabil O, Toaka S, LoBrutto R, Shoemaker R, Banerjee R. 2001. Pyridoxal phosphate binding sites are similar in human heme-dependent and yeast heme-independent cystathionine beta-synthases. Evidence from 31P NMR and pulsed EPR spectroscopy that heme and PLP cofactors are not proximal in the human enzyme. J Biol Chem 276:19350-5.

Katsushima F, Oliveriusova J, Sakamoto O, Ohura T, Kondo Y, Iinuma K, Kraus E, Stouracova R, Kraus JP. 2006. Expression study of mutant cystathionine beta-synthase found in Japanese patients with homocystinuria. Mol Genet Metab 87:323-8.

Kery V, Poneleit L, Meyer JD, Manning MC, Kraus JP. 1999. Binding of pyridoxal 5-phosphate to the heme protein human cystathionine beta-synthase. Biochemistry 38:2716-2724.

Kluijtmans LA, Boers GH, Stevens EM, Renier WO, Kraus JP, Trijbels FJ, van den Heuvel LPWJ, Blom HJ. 1996. Defective Cystathionine B-synthase regulation by S-Adenosylmethionine in a partially pyridoxine responsive homocystinuria patient. J Clin Invest 98:285-289.

Kopecka J, Krijt J, Rakova K, Kozich V. 2011. Restoring assembly and activity of cystathionine beta-synthase mutants by ligands and chemical chaperones. J Inherit Metab Dis. 34:39-48.

Kozich V, Kraus JP. 1992. Screening for mutations by expressing patient cDNA segments in E. coli: Homocystinuria due to Cystathionine β -Synthase Deficiency. Hum Mutat 1:113-123.

Kozich V, Sokolova J, Klatovska V, Krijt J, Janosik M, Jelinek K, Kraus JP. 2010. Cystathionine beta-synthase mutations: effect of mutation topology on folding and activity. Hum Mutat 31:809-19.

Kraus J. 1987. Cystathionine b-synthase. Methods in enzymology 143:388-394.

Kraus J, Janosik M, Kozich V, Mandell R, Shih V, Sperandeo MP, Sebastio G, de Franchis R, Andria G, Kluijtmans LAJ and others. 1999. Cystathionine beta-synthase mutations in homocystinuria. Hum Mutat 13:362-375.

Maclean KN, Gaustadnes M, Oliveriusova J, Janosik M, Kraus E, Kozich V, Kery V, Skovby F, Rudiger N, Ingerslev J and others. 2002. High homocysteine and thrombosis without connective tissue disorders are associated with a novel class of cystathionine beta-synthase (CBS) mutations. Hum Mutat 19:641-655.

Magner M, Krupkova L, Honzik T, Zeman J, Hyanek J, Kozich V. 2011. Vascular presentation of cystathionine beta-synthase deficiency in adulthood. J Inherit Metab Dis 34:33-7.

Majtan T, Liu L, Carpenter JF, Kraus JP. 2010. Rescue of cystathionine beta-synthase (CBS) mutants with chemical chaperones: purification and characterization of eight CBS mutant enzymes. J Biol Chem. 285:15866-15873.

Meier M, Janosik M, Kery V, Kraus JP, Burkhard P. 2001. Structure of human cystathionine beta-synthase: a unique pyridoxal 5 '-phosphate-dependent heme protein. EMBO J 20:3910-3916.

Moat SJ, Bao L, Fowler B, Bonham JR, Walter JH, Kraus JP. 2004. The molecular basis of cystathionine beta-synthase (CBS) deficiency in UK and US patients with homocystinuria. Hum Mutat 23:206.

Mudd S, Levy H, Tangerman A, Boujet C BN, Davidson-Mundt A, Hudgins L, Oyanagi K, Nagao M, Wilson W. 1995. Isolated persistent hypermethioninemia. Am J Hum Genet 57:882-92.

Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE, Andria G, Boers GH, Bromberg IL, Cerone R and others. 1985. The natural history of homocystinuria due to cystathionine beta-synthase deficiency. Am J Hum Genet 37:1-31.

Refsum H, Fredriksen A, Meyer K, Ueland PM, Kase BF. 2004. Birth prevalence of homocystinuria. J Paediatr 144:830-832.

Santamaria R, Vilageliu L, Grinberg D. 2008. SR proteins and the nonsense-mediated decay mechanism are involved in human GLB1 gene alternative splicing. BMC Res Notes 1:137.

Sebastio G, Sperandeo MP, Panico M, de Franchis R, Kraus JP, Andria G. 1995. The molecular basis of homocystinuria due to Cystathionina B-Sinthasa deficiency in italian families, and report of four novel mutations. Am J Hum Genet 56:1324-1333.

Singh LR, Chen X, Kozich V, Kruger WD. 2007. Chemical chaperone rescue of mutant human cystathionine beta-synthase. Mol Genet Metab 91:335-42.

Singh LR, Gupta S, Honig NH, Kraus JP, Kruger WD. 2010. Activation of mutant enzyme function in vivo by proteasome inhibitors and treatments that induce Hsp70. PLoS Genet 6:e1000807.

Skovby F, Gaustadnes M, Mudd SH. 2010. A revisit to the natural history of homocystinuria due to cystathionine beta-synthase deficiency. Mol Genet Metab 99:1-3.

Taoka S, Lepore BW, Kabil O, Ojha S, Ringe D, Banerjee R. 2002. Human cystathionine beta-synthase is a heme sensor protein. evidence that the redox sensor is heme and not the vicinal cysteines in the CXXC motif seen in the crystal structure of the truncated enzyme. Biochemistry 41:10454-10461.

Urreizti R, Asteggiano C, Bermudez M, Cordoba A, Szlago M, Grosso C, de Kremer RD, Vilarinho L, D'Almeida V, Martinez-Pardo M and others. 2006a. The p.T191M mutation of the CBS gene is highly prevalent among homocystinuric patients from Spain, Portugal and South America. J Hum Genet 51:305-13.

Urreizti R, Asteggiano C, Cozar M, Frank N, Vilaseca MA, Grinberg D, Balcells S. 2006b. Functional assays testing pathogenicity of 14 cystathionine-beta synthase mutations. Hum Mutat 27:211.

Urreizti R, Balcells S, Rodes M, Vilarinho L, Baldellou A, Couce ML, Munoz C, Campistol J, Pinto X, Vilaseca MA and others. 2003. Spectrum of CBS mutations in 16

homocystinuric patients from the Iberian Peninsula: high prevalence of T191M and absence of I278T or G307S. Hum Mutat 22:103.

Walter JH, Wraith JE, White FJ, Bridge C, Till J. 1998. Strategies for the treatment of cystathionine beta-synthase deficiency: the experience of the Willink Biochemical Genetics Unit over the past 30 years. Eur J Pediatr 157 Suppl 2:S71-6.

Wilcken DE, Wilcken B. 1997. The natural history of vascular disease in homocystinuria and the effects of treatment. J Inherit Metab Dis 20:295-300.

Yap S, Boers GHJ, Wilcken B, Wilcken DEL, Brenton DP, Lee PJ, Walter JH, Howard PM, Naughten ER. 2001. Vascular outcome in patients with homocystinuria due to cystathionine beta-synthase deficiency treated chronically - A multicenter observational study. Arterioscl Thromb Vas Biol 21:2080-2085.

Yap S, Naughten E. 1998. Homocystinuria due to cystathionine beta-synthase deficiency in Ireland: 25 years' experience of a newborn screened and treated population with reference to clinical outcome and biochemical control. J Inherited Metab Dis 21:738-747.

Figure Legends:

Figure 1. Southern Blot analysis of the *CBS* gene. ***A***: scheme of the human *CBS* gene. The *Drd*I (D) and *Afl*II (A) restriction sites and the corresponding restriction fragments (F1 to F4) are indicated below the scheme. The positions of the probes (A to D) are indicated above. ***B***: Southern Blot analysis of patient #87 and her mother (#87m) hybridized with the whole set of probes. Two wild-type DNAs were included in the analysis. M: Molecular weight marker. C: Southern Blot analysis hybridized with probe B. The arrow indicates the new band found in the patient and her mother. ***D***: PCR amplification of the fragment from intron 3 to intron 7. An extra band of reduced size was found in patient 87 and her mother, corresponding to a 794-bp deletion. The asterisk indicates the heteroduplex. ***E***: Chromatogram of patient 87 showing the deletion boundaries.

Figure 2. Heterologous expression and activity of seven *CBS* mutations. **A:** SDS-PAGE followed by Western blot immunodetection of wild-type and mutant CBS

proteins expressed in *E. coli* XL Gold. Ten µg of total protein extract was loaded in each lane**.** Six replicates were performed for each mutation. pKK: Empty vector used as negative control. **B:** *In vitro* enzyme activity of wild-type and mutant *CBS* alleles from *E. coli* XL Gold extracts in the absence of both PLP and SAM (black boxes), in the presence of PLP (grey boxes), and in the presence of both PLP and SAM (white boxes). Wild-type CBS activity in the presence of PLP was taken as the reference value (100%). Activity values greater than 4% are indicated in the figure. Values are the means of six replicates. Error bars represent SD.

Figure 3. Splicing analysis of mutation c.667-14_-7del8 included in a minigene construction. **A:** scheme of the minigene: grey boxes represent *GLB1* exons and white boxes are *CBS* exons. The thick horizontal line represents the *CBS* introns and the thin line the *GLB1* introns. PCMV: CMV promoter; BGHpA: BGH polyadenylation site. Wild-type and mutant splicing patterns are indicated above and below the gene respectively. The dot indicates the position of the mutation. The wild-type and mutant constructs were transiently transfected into HeLa cells. Total RNA was extracted 24 h after transfection, reverse-transcribed and PCR-amplified with primers T7 and SP6 (indicated by arrows)**. B**: PCR amplification of the wild-type (1) mutant (2) and the minigene construct without the *CBS* exons (3). The asterisk points to a minor band lacking exon 4 observed in the wild-type construct. **C**: Diagrams showing the three main PCR products.

Figure 4. Pedigree of family 80. Patient 80a (II.1), the proband, is marked with an arrow. Patient 80b is member II.3. *CBS* genotypes are indicated below the pedigree symbols; the *MTHFR* c.677C>T genotypes are also included in brackets. Total plasma homocysteine levels (µMol/L) are indicated when available. NA: not available.

Table 1. List Of The 22 Different Mutations Found In This Cohort.

DNA	Deduced Protein Change	Exon-Intron	Alleles	Restr. Enz.[1]	References
c.146C>T	p.P49L	Ex 1	**1**		de Franchis et al., 1998
c.253G>A	p.G85R	Ex 2	**1**		Maclean et al., 2002
c.361C>T	p.R121C	Ex 3	**1**		Katsushima et al., 2006
c.374G>A	p.R125Q	Ex 3	**1**		Marble et al., 1994
c.518delTGA	**p.M173del**	**Ex 4**	**2**		**This study**
c.532-37_736+438del794	**p.V178GfsX23**	**In 4-In 6**	**1**		**This study**
c.572C>T	p.T191M	Ex 5	**7**		Urreizti et al., 2003
c.599C>T	**p.P200L**	**Ex 5**	**1**	*Msp*I-	**This study**
c.667-14_667-7del8	**p.Y223GfsX23**	**In 5**	**1**		**This study**
c.676G>A	p.A226T	Ex 6	**2**		Shan et al., 1998
c.689delT	**p.L230RfsX39**	**Ex 6**	**1**	*Msp*I+	**This study**
c.824G>A	p.C275Y	Ex 7	**1**		Urreizti et al., 2003
c.833T>G	**p.I278S**	**Ex 8**	**1**	*Tsp*Ri+	**This study**
c.833T>C	p.I278T	Ex 8	**4**		Kozich et al., 1992
c.841C>T	**p.D281N**	**Ex 8**	**1**	*Bam*HI-	**This study**
c.862_866del5	**p.E289GfsX39**	**Ex 8**	**2**		**This study**
c.962A>T	**p.D321V**	**Ex 9**	**1**	*Hpy*8I-	**This study**
c.1085C>T	p.T353M	Ex 10	**2**		Dawson et al., 1997
c.1136G>A	p.R379Q	Ex 10	**1**		Urreizti et al., 2003
c.1330G>A	p.D444N	Ex 12	**4**		Kluijtmans et al., 1996
c.1336G>T	**p.A446S**	**Ex 12**	**1**	*Bst*NI+	**This study**
c.1566delG	p.K523SfsX18	Ex 16	**5**		Castro et al., 1999

Mutations not previously described are indicated in bold

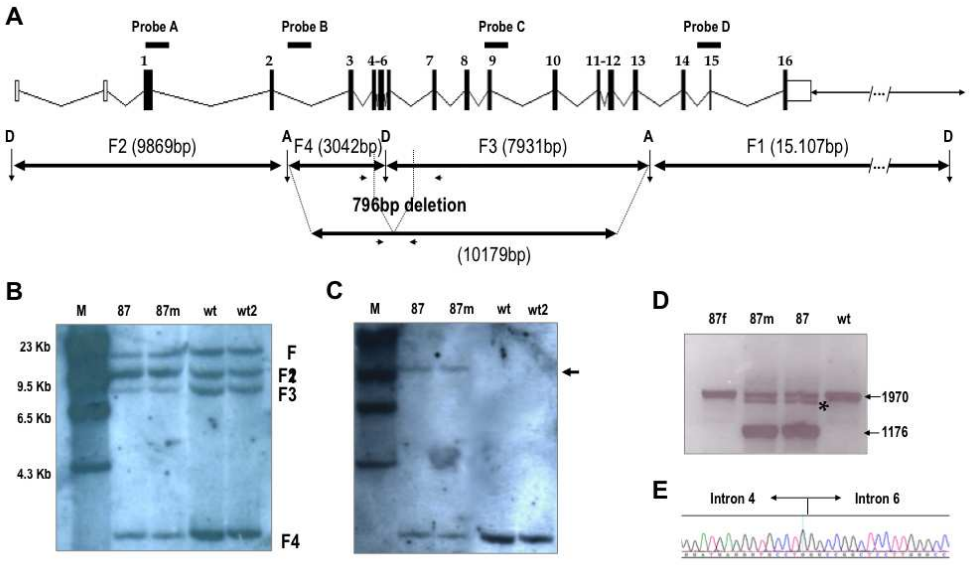
[1]Novel missense mutations were checked in control chromosomes using the indicated enzyme. +: the mutation creates a new site; -: the mutation destroys a site.

Reference Sequences: Genomic, GenBank NG_008938.1; cDNA, ENST00000352178. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Table 2. Patients, CBS And MTHFR C.667C>T Genotypes, Disease Severity And Geographical Origin

Patient	Genotype	MTHFR (c.677 C>T)	Severity	Country
74	p.[T353M]+[D444N]	TT	Severe	Spain
79	p.**[P200L]+[?]**	CT	Asymptomatic	Spain
80a	p.[R125Q]+[P49L]	TT	Mild	Spain
80b	p.[R125Q]+[P49L]	CT	Asymptomatic	
82a	p.[T191M]+[**D281N]**	CC	Mild	Spain
82b	p.[T191M]+[**D281N]**	CC	Mild	
82c	p.[T191M]+[**D281N]**	CC	Mild	
83	p.[T353M]+[D444N]	TT	Mild	Spain
84	p.[I278T]+[R121C]	CT	Mild	Spain
88	p.[D444N]+[D444N]	CT	Mild	Spain
92	p.[T191M]+[T191M]	CT	Mild	Spain
93	p.[T191M]+[T191M]	CT	Severe	Spain
94	p.[T191M]+[**I278S]**	CT	Severe	Spain
100	p.[T191M]+[R379Q]	CT	Mild	Spain
107	c.[1566delG]+[1566delG]	CT	NA	Spain
108	p.[I278T]+[C275Y]	TT	Mild	Spain
81	c.[1566delG]+[1566delG]	TT	Severe	Portugal
87	c.[1566delG]+[**532-37_736+438del794]**	CC	neonatal screening (asymptomatic)	Portugal
109	**p.[E289GfsX39]**+[**E289GfsX39]**	CT	Severe	Portugal
68a	p.[G85R]+**c.[667-14_667-7del8]**	CT	Severe	Argentina
68b	p.[G85R]+**c.[667-14_667-7del8]**	CT	Severe	
89	**c.[689delT]**+[**689delT]**	CT	Severe	Argentina
90	**p.[D321V]**+[**A446S]**	CT	Mild	Argentina
91	p.[A226T]+[A226T]	CT	Severe	Argentina
105	p.[I278T]+[I278T]	ND	NA	Norway
55	**p.[M173del]**+[**M173del]**	ND	Mild	Indian

ND: Not Determined.
NA: Not Available.



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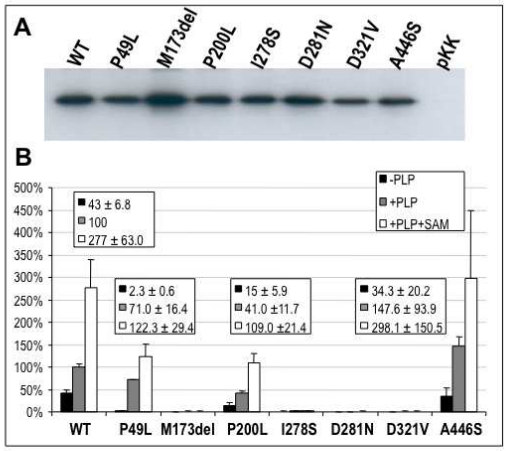

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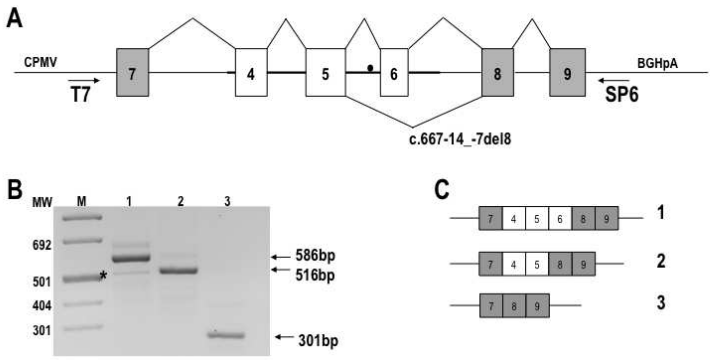

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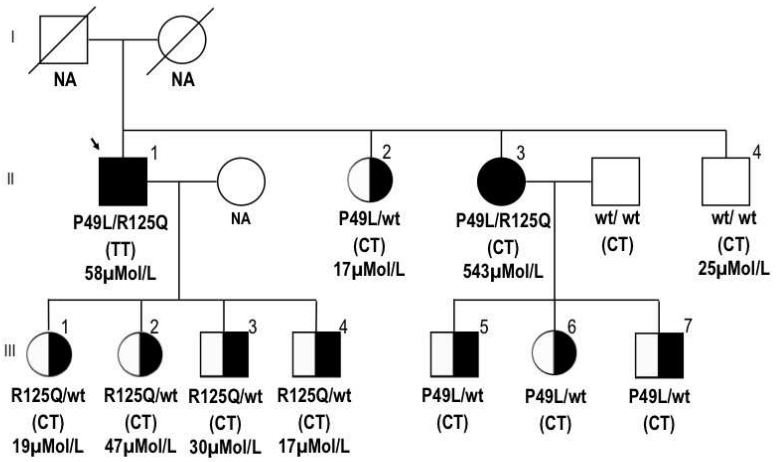

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