Mitochondrial Carbonic Anhydrase VA Deficiency Resulting from *CA5A* Alterations Presents with Hyperammonemia in Early Childhood

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Four children in three unrelated families (one consanguineous) presented with lethargy, hyperlactatemia, and hyperammonemia of unexplained origin during the neonatal period and early childhood. We identified and validated three different *CA5A* alterations, including a homozygous missense mutation (c.697T>C) in two siblings, a homozygous splice site mutation (c.555G>A) leading to skipping of exon 4, and a homozygous 4 kb deletion of exon 6. The deleterious nature of the homozygous mutation c.697T>C (p.Ser233Pro) was demonstrated by reduced enzymatic activity and increased temperature sensitivity. Carbonic anhydrase VA (CA-VA) was absent in liver in the child with the homozygous exon 6 deletion. The metabolite profiles in the affected individuals fit CA-VA deficiency, showing evidence of impaired provision of bicarbonate to the four enzymes that participate in key pathways in intermediary metabolism: carbamoylphosphate synthetase 1 (urea cycle), pyruvate carboxylase (anaplerosis, gluconeogenesis), propionyl-CoA carboxylase, and 3-methylcrotonyl-CoA carboxylase (branched chain amino acids catabolism). In the three children who were administered carglumic acid, hyperammonemia resolved. CA-VA deficiency should therefore be added to urea cycle defects, organic acidurias, and pyruvate carboxylase deficiency as a treatable condition in the differential diagnosis of hyperammonemia in the neonate and young child.

Hyperammonemia is a medical emergency that requires immediate and targeted treatment. Correct diagnosis is therefore essential, but it is challenging given heterogeneous etiologies, including genetic (inborn errors of metabolism), developmental (transient neonatal hyperammonemia), and environmental (infectious hepatitis, medication) causes.[1] We present four children from three unrelated families with infantile hyperammonemic encephalopathy and hyperlactatemia. The underlying cause in each of these children was deficiency of carbonic anhydrase VA (CA-VA) (*CA5A* [MIM 114671]), an inborn error of metabolism broadening the differential diagnosis for hyperammonemia.

This study was initiated as part of the Treatable Intellectual Disability Endeavor in British Columbia and approved by the institutional review boards of BC Children's Hospital and the University of British Columbia. Parents provided written informed consent.

In family 1, the female index (II-1 in Figure 1), her younger affected brother (II-2), and her unaffected sister (II-3) were born to healthy nonconsanguineous parents of Belgian-Scottish descent after uneventful pregnancies and deliveries. The index and her male sibling developed lethargy, tachypnea, hypoglycemia (2.2 and 2.9 µmol/l), hyperlactatemia (9.8 and 8.8 µmol/l), hypernatremia (Na 152 and 150 µmol/l), and hyperammonemia (780 and 238 µmol/l) with respiratory alkalosis (pH 7.48, pCO_2 10.8 mm Hg, HCO_3^- 10.4 mEq/l; and pH 7.46, pCO_2 21 mm Hg, HCO_3^- 23) within the first days of life. Liver transaminases, albumin, and clotting factors remained normal. Urine organic acids demonstrated high lactic, β-hydroxybutyric, and acetoacetic acid excretion, as well as increases in carboxylase substrates and related metabolites. Plasma amino acid analysis showed elevations of glutamine, alanine, and proline and reduction of citrulline and arginine. Further biochemical test results are shown in Table 1. Known urea cycle defects and primary causes of hyperlactatemia were excluded by sequencing and deletion/duplication analysis of N-acetylglutamate synthase (*NAGS* [MIM 608300]), carbamoylphosphate
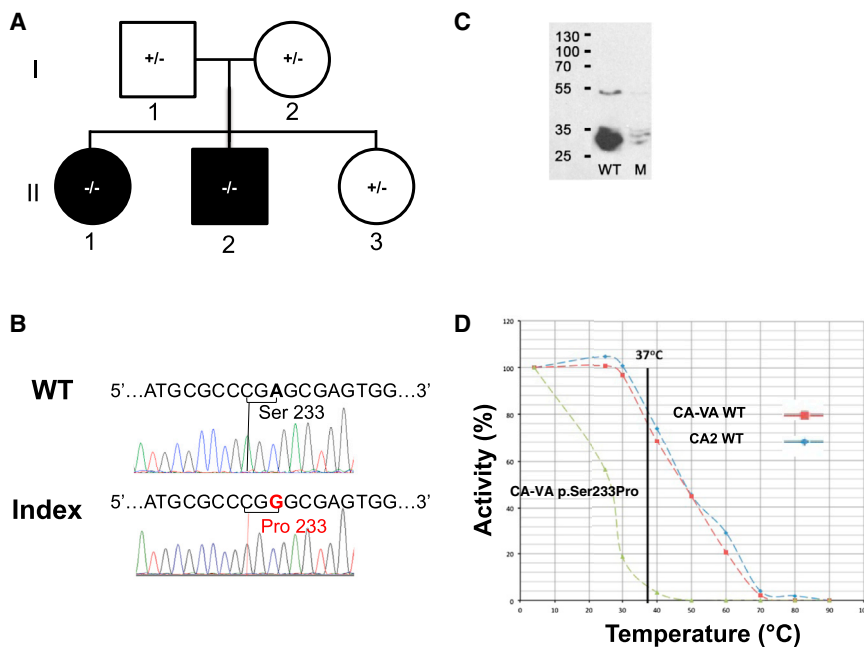
Figure 1. Family 1 with p.Ser233Pro Missense Variant

(A) Pedigree (black fill indicates clinically affected individuals, II-1 and II-2).

(B) Sanger sequence of *CA5A* from index (II-1) and control (wild-type sequence; WT) subjects; the variant nucleotide position and the corresponding codon alteration (p.Ser233Pro) are indicated.

(C) Immunoblot analyses by SDS-PAGE (ImageJ software) of WT and p.Ser233Pro (mutant; M) CA-VA protein levels in COS-7 cell lysates; the molecular weights (kDa) of protein standards are indicated on the left. Normal and mutant (c.697T>C) *CA5A* cDNAs, including the full mitochondrial targeting sequences, were synthesized via the NCBI reference sequence (NM_001739.1) by Genscript. The cDNAs were cloned into pUC57 at XhoI and BglII sites and verified by Sanger sequencing. Subsequently, the cDNA inserts were subcloned via the same restriction sites into the pCXN mammalian expression vector. COS-7 cells (ATCC CRL-1651) were transfected with wild-type or mutant *CA5A* plasmids with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, as previously described.[8] A β-glucuronidase expression plasmid was cotransfected as a marker of transfection efficiency.[24] COS-7 cells were harvested 48–72 hr after transfection in lysis buffer and then lysed by sonication on ice.

(D) Thermal stability profiles for WT (red) and p.Ser233Pro mutant (green) CA-VA enzymes. Carbonic anhydrase II (CA2; blue) was used as a control.

synthetase (*CPS1* [MIM 608307], and pyruvate carboxylase (*PC* [MIM 608786]), as well as measurement of PC and biotinidase activities in fibroblasts and serum. Hyperammonia-hyperornithinemia-homocitrullinuria syndrome (MIM 238790) and lysinuric protein intolerance (MIM 222710) were excluded on the basis of a normal urine amino acid profile, and pyruvate dehydrogenase complex deficiency was excluded on the basis of an elevated lactate: pyruvate ratio (5.7 μmol/l:0.036 μmol/l = 138 [reference interval 10–20.7]) in the male sibling. Blue native gel analysis for respiratory chain complexes I–V was normal. Molecular analysis of nuclear-encoded ATPase deficiency (*TMEM70* [MIM 612418]) did not reveal disease-causing mutations. Chromosomal microarray analysis (Affymetrix-Cytoscan HD) was unremarkable, and homozygosity analysis did not reveal evidence of consanguinity or uniparental disomy.

Clinical and metabolic findings normalized in both siblings with the administration of intravenous dextrose and bicarbonate, as well as enteral carglumic acid (Carbaglu). In our institution, carglumic acid is used to resolve hyperammonemia of unknown origin.[2] Brain MRI and MRS on day 5 of life revealed a small periventricular petechial focus near the trigone of the right lateral ventricle, with a small lactate peak on spectroscopy in the male sibling; in the female sibling, both MRI and EEG were unremarkable.

At 2.5 and 3.5 years of age, during intercurrent illness, the index presented with lethargy, hyperammonemia, and hyperlactatemia. Hyperammonemia resolved with a single dose of carglumic acid. She has remained clinically stable with L-carnitine, vitamin C, coenzyme Q10, and "sick-day management" with high-caloric and lipid-rich formula during illnesses. At the age of 4.5 years, she exhibited mild axial hypotonia and septal thickening on cardiac ultrasound. Developmental and behavioral assessment at age 4.5 confirmed average functioning in all domains except for below-average motor coordination (Beery Buktenica Developmental Test of Visual-Motor Integration). The male sibling at age 2.3 years had difficulty engaging in an assessment as a result of heightened activity and self-directed behavior; scores on Bayley Scales of Infant and Toddler Development (Third Edition) were below average. Biochemical profiles and somatic and psychomotor development are unremarkable in the unaffected younger sibling, now 1.5 years of age.

WES was performed for the two affected siblings and their unaffected parents via the Agilent SureSelect kit and Illumina HiSeq 2000 (Perkin-Elmer). Rare variants were assessed for their potential to disrupt protein function and screened under a series of genetic models—primarily the Mendelian recessive mode of inheritance given the rarity of the phenotype and the pattern of inheritance of most IEMs.

Approximately 99% of the observed variations were classified as common (Table S1 available online). Eight rare candidate variants fit the autosomal-recessive model of homozygous (*CA5A*, *ARSB* [MIM 611542], *CDKN2B* [MIM 600431], *CDKN2A* [MIM 600160], *OGG1* [MIM 601982]) or compound heterozygous (*GRK4* [MIM 137026], *SPG11* [MIM 610844], *EPHX2* [MIM 132811])

Table 1.   Overview of Biochemical Abnormalities Resulting from CA-VA Deficiency: In Theory and for the Index Cases for Families 1, 2, and 3

Theoretical Possibilities		Actual Results[a]	Family 1			Family 2		Family 3	
Possible Enzyme Deficiency	Predicted Secondary Biochemical Abnormalities	Metabolite	Female	Male	Normal[b]	Male	Normal	Male	Normal
carbamoyl phosphate synthetase	↑ ammonia	plasma ammonia (μM)	780*	238*	<40	422*	<50	258*	<40
	↓ citrulline	plasma citrulline (μM)	5*	18	8–47	17	3–36		10–45
	↓ arginine	plasma arginine (μM)	17*	NA	32–142	35	17–119	22	12–133
	↑ glutamine	plasma glutamine (μM)	1,051*	1,237*	457–746	2,606*	243–822	571	254–823
	N ornithine	plasma ornithine (μM)	15*	82	27–207	146	38–272	17	15–200
	N orotate	urine orotate	non-det	1.9	<4.3	2.2	<4.9	0.4	<3
pyruvate carboxylase	↓ gluconeogenesis (hypoglycemia)	serum glucose (mm)	2.2*	2.9*	3.3–7.0	2.9*	3.0–8.0	3.2*	3.5–6.0
		serum lactate (mm)	9.1*	8.8*	0.5–2.2	8.1*	1.0–1.8	5.6*	0.6–2.6
		plasma alanine (μm)	1,531*	609*	148–475	1,078*	132–455	603*	143–439
	redox imbalance (↑ lactate & dicarboxylic acids)	plasma proline (μm)	418*	371*	40–332	625*	78–523	283*	52–298
	↓ tricyclic acid cycle intermediates (cataplerosis)	urine lactate	3,737*	4,109*	<200	28,000*	<456	grossly increased*	
		urine 3-OH-butryric acid	7,902*	2,657*	<21	7,060*	<22	grossly increased*	
		urine aceto-acetic acid	584*	927*	non-det	++[c]		grossly increased*	
		urine fumaric acid	13.8*	38.5*	<10	8	<13	moderately increased	
		urine 2-α-ketoglutaric acid	143*	254.6*	<112	300*	<267	slightly increased*	
		urine adipic acid	55*	219.8*	<29	340	<25	normal	
		urine suberic acid	18.6*	48.6*	<13	29	<15	normal	
		urine sebacic acid	NA	18.7*	<10	NA		normal	
	↑ lysine	plasma lysine (μm)	87	161	71–272	306	71–272	94	71–272
proprionyl-CoA carboxylase	↑ 3-OH-propionic acid	urine 3-OH-propionic acid	79.38*	54.57*	<16	59*	<21	normal	
	↑ propionylglycine	urine proprionylglycine	1.29*	3.28*	non-det	5.6*	<2	trace*	
	↑ methylcitrate	urine methylcitrate	6.4*	non-det	non-det	normal[c]		trace*	
3-methylcrotonyl-CoA carboxylase	↑ 3methylcrotonylglycine	urine 3-methylcrotonylglycine	22.9*	non-det	<10	17*	<5	trace*	
	↑ 3OH-isovaleric acid	urine 3-OH-isovaleric acid	40.13*	55.50*	<38	327*	<55	increased*	

All values in urine are expressed as μmol/mmol of creatinine. Abbreviations are as follows: N, normal (within reference range); NA, not available; non-det, nondetectable.
[a]In each individual, the value with maximal deviation from normal during crisis is provided. Asterisks (*) indicate abnormal values.
[b]Normal values differ for each family studied because values were measured in different laboratories.
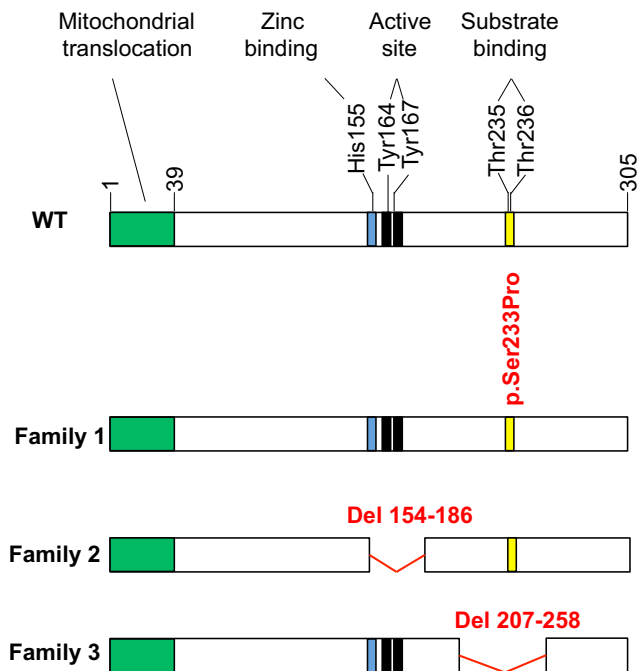[c]Qualitative assessment.

Figure 2. Effect of Genetic Variants Identified in CA-VA

Shown is a schematic diagram of the 305 amino acid wild-type (WT) CA-VA. Residues 1–39 encode a mitochondrial translocation signal (green). Homology predictions indicate that histidine 155 binds a zinc ion (blue), tyrosines 164 and 167 are active site residues (black), and threonines 235 and 236 comprise a substrate-binding region (yellow). Shown in red are the deduced CA-VA variants identified in this study. The index and affected brother of family 1 has a nonsynonymous Ser to Pro mutation at residue 233, adjacent to the substrate-binding region. The index of family 2 has a deletion of residues 154–186 (exon 4), thereby missing the metal-binding and active-site residues. The index of family 3 has a deletion of residues 207–258 (exon 6; the substrate-binding region), which results in absent protein.

variants in the affected siblings. The final set of rare variants (mean average frequency < 1%) was assessed for the potential to disrupt protein function via the Sift and PolyPhen2 software systems. Of these, only one variant (c.697T>C; RefSeq accession number NM_001739.1) in *CA5A* on chromosome 16 was considered a functional candidate; this variant was not reported in dbSNP (version 137), NHLBI ESP, or our in-house genome database (comprising 100 exomes and 10 whole genomes; anno December 2013). Integrative Genomics Viewer 2.0.34 was used to visualize the read alignment and assess variant quality prior to Sanger validation. Given the existence of a related pseudogene,[3] the *CA5A* variant was confirmed by targeted Sanger sequencing via carefully designed primers to avoid amplification of the pseudogene sequences; this was achieved by review of paired-end sequence data and selection via a BLAST search of appropriate regions with least similarity, especially close to the 3′ end. Affected siblings are confirmed homozygous, whereas unaffected parents and the unaffected youngest female are heterozygous carriers (Figures 1A and 1B). This variant corresponds to a Ser to Pro substitution at position 233 that is predicted

to disrupt structure around the conserved Thr235 residue that forms part of the substrate-binding region of the enzyme (Figure 2). Indeed, the p.Ser233 residue is highly conserved evolutionarily across species and in all 12 active human carbonic anhydrase isoforms. Studies of human CA-II have demonstrated that mutations in this hydrophobic patch in the active site destabilize the structure around the substrate-binding region and dramatically reduce the activity of the mutant CA-II.[4]

Tissues with CA-VA (liver, kidney, skeletal muscle) were not available for the affected siblings. Therefore, the effect of the p.Ser233Pro substitution on CA-VA was characterized in cultured mammalian cells (COS-7). A marked reduction was observed in the steady-state levels of CA-VA p.Ser233Pro compared with wild-type protein despite similar transfection efficiencies (Figure 1C). As summarized in Table 2, CA-VA p.Ser233Pro-specific activity in total cell lysates is reduced to 20% of wild-type protein activity, whereas activities of the cotransfected marker enzyme β-glucuronidase were comparable. There was a negligible change in the activity of mutant enzyme in the presence of added zinc (22% of WT), suggesting that both WT and mutant enzymes in COS-7 cells are saturated with zinc (data not shown). Thermal stability of mutant recombinant human CA-VA was compared with the WT CA-VA and recombinant human Carbonic Anhydrase II as an additional control (Figure 1D). After a 30 min preincubation, the mutant enzyme had lost 80% of its activity at 30°C and almost all its activity at 40°C. By contrast, both WT CA-VA and human CA-II were much more stable at 30°C and 40°C, retaining approximately 100% and 70% residual activity, respectively. In a separate experiment (Table 3), preincubation of the mutant enzyme at 37°C (normal human body temperature) for 30 min retained only 5% of mutant enzyme activity, whereas wild-type protein activity was 80% relative to activity after a 4°C preincubation. The quantity of immune-reactive CA-VA p.Ser233Pro was demonstrably lower than that of wild-type CA-VA (37% of wild-type) at 72 hr after transient transfection of COS-7 cells (data not shown). Similar temperature-sensitive mutants are associated with disease in cases of medium chain acyl-coA dehydrogenase deficiency (*ACADM* [MIM 607008]) and recessively inherited parkinsonism (*PINK1*, a putative mitochondrial kinase [MIM 608309]).[5,6]

In family 2, a male child (II-1 in Figure 3A) was born spontaneously at gestational age 36^{+2} weeks to nonconsanguineous Russian parents. On day 4 of life, he presented with lethargy, weight loss (15% below birth weight), jaundice, and tachypnea. Initial investigations showed hyperammonemia (316 and 422 μmol/l), hyperlactatemia (8.1 mmol/l), mild hypoglycaemia (2.9 mmol/l), metabolic acidosis (pH 7.16, pCO$_2$ 13 mm Hg, HCO$_3^-$ 5 mEq/l), and ketonuria. Despite fluid resuscitation, sodium bicarbonate infusion, and antibiotics, the neonate's clinical and biochemical status deteriorated; liver transaminases and synthetic function remained normal. Metabolic

Table 2. Steady-State Carbonic Anhydrase Activity in COS-7 Cells Cotransfected with Wild-Type and p.Ser233Pro Mutant CA-VA and β-Glucuronidase

Enzymes	Specific Activity (EU/mg Cell Protein)	Specific Activity Minus Vector (EU/mg Cell Protein)	Percent of WT CA-VA	BGUS Activity (EU/mg)	Transfection Efficiency
PCXN vector only	0.28	0.00	0	37.5	1
WT	1.88	1.60	100	7070	188.5
MT	0.60	0.32	20	8550	228

Values represent the average of two transfections; the enzyme activities were determined in duplicate and calculated as enzyme unit (EU) per milligram (mg) of enzyme protein. The specificity of the assay was verified with the CA-specific inhibitor acetazolamide (500 nM). Abbreviations are as follows: WT, wild-type; MT, mutant.

investigations are shown in Table 1; molecular analysis of *CPS1* and *NAGS* did not reveal disease-causing mutations. Carglumic acid and biotin were initiated, along with protein-free formula and intravenous lipids; 12 hr later, the metabolic acidosis and hyperammonemia resolved. He resumed breastfeeding with normal weight gain, ammonia levels, and urine metabolites. Carglumic acid was stopped at 4 months of age, and the infant exhibited normal psychomotor development at age 6 months with the use of sick-day formula during illness.

Sanger sequencing of all seven exons of *CA5A* in the index identified a synonymous c.555G>A transition (RefSeq NM_001739.1) at the final base of exon 4 (Figure 3B). Given that guanine is the most common nucleotide found at this end of an exon in vertebrate genes, RT-PCR was undertaken to demonstrate an effect on mRNA splicing.[7] RNA was purified from white blood cells of the index and a control subject. RT-PCR via primers designed to amplify exons 2–7 of *CA5A* generated distinctly different product sizes (approximately 550 bp and 650 bp, respectively; Figure 3B). Sanger sequencing of the ~550 bp band revealed an in-frame deletion of exon 4 from the index RNA (Figure 3C). We cannot exclude the presence of other transcripts in vivo, which were missed because of the limited sensitivity of the assay combined with low *CA5A* expression in white blood cells. Alternatively, this single transcript may be explained by preferential formation during the transcription/splicing processes. Homology with carbonic anhydrase isoforms identifies three critical residues in the deleted CA-VA transcript (residues 154–185): His155, which binds to a catalytically essential zinc molecule, and Tyr164 and Tyr167, which form part of the active site of the CA-VA enzyme.[8] Thus, this deletion is predicted to significantly impair CA-VA enzyme activity, if not lead to protein misfolding and degradation.

In family 3, a male child (II-5 in Figure 4A) was born at term by Caesarian section (because of placenta previa) as the youngest of five children to first-cousin consanguineous Pakistani parents. At 13 months of age, after unremarkable development, he presented with a 1-day history of visual unresponsiveness. At admission, he was encephalopathic with hyperammonemia (258 μmol/l) and hyperlactatemia (4.9 mmol/l), with a compensated metabolic acidosis (pH 7.43, pCO$_2$ 24.8 mm Hg, HCO$_3^-$ 14 mEq/l). His encephalopathy improved after 48 hr of intravenous fluids and antibiotics administered for presumed meningo-encephalitis (cultures were negative). At the age of 16 months, he had a similar crisis; there were no signs of liver injury. Further metabolic investigations are shown in Table 1. Sodium benzoate and L-arginine were initiated with improvement after 48 hr, and he was discharged on a protein-restricted diet. Urea cycle defects (OTC [MIM 311250], CPS1 [MIM 237300], NAGS [MIM 237310] deficiencies) and PC (MIM 266150), citrin (MIM 605814), and biotinidase (MIM 253260) deficiencies were excluded by molecular or enzymatic analyses. After these two crises, he has demonstrated good developmental progress with only minor learning difficulties (no formal testing was available). He continues to have infrequent episodes of vomiting and ketoacidosis without hyperammonemia or lactic acidosis; the frequency of these episodes has not increased since the withdrawal of sodium benzoate and arginine therapy at the age of 7 years.

Sanger sequencing of the *CA5A* exons for the index revealed a deletion of 4 kb encompassing exon 6 (Figure 4B). Relative copy number was assessed in the family via qPCR of exon 6 sequence, confirming the homozygous deletion in the two siblings and heterozygosity in the parents (Figure S1). Absence of CA-VA protein was confirmed by immunoblot in existing liver biopsy tissue (Figure 4C). Limited information is available on the neonatal and early childhood course in his four older siblings; parents reported no major health problems and normal learning ability. The oldest brother (II-2 in Figure 4) is homozygous for the *CA5A* deletion but declined further evaluation at age 17. Neonatal or childhood insults and a possible mild presentation cannot be excluded because of limited history. A benign clinical course after early childhood is consistent with the index cases of each of the three families after initial metabolic decompensations. The condition may exhibit intrafamilial variability of the phenotype, as reported for other inborn errors of metabolism.[9,10]

CA-VA deficiency is a human inborn error of metabolism presenting with hyperammonemic encephalopathy in early life. Initial evidence of causality for the identified *CA5A* alterations is provided by their similar biochemical phenotypes during metabolic crises. Findings are consistent with dysfunction of all four enzymes to which CA-VA provides bicarbonate as substrate in mitochondria

Table 3. CA-VA Wild-Type and p.Ser233Pro Mutant Enzyme Activities in COS-7 Cell Lysates, before and after Incubation at Human Body Temperature

Enzyme	EU/mg Cell Protein before Heating	EU/mg Cell Protein after Heating (30 min at 37°C)
WT	1.02 (100%)	1.25 (100%)
MT	0.15 (14.7%)	0.07 (5.4%)

The enzyme activities were determined in duplicate and calculated as enzyme unit (EU) per milligram (mg) of enzyme protein. Incubation was at 37°C for 30 min. Abbreviations are as follows: WT, wild-type; MT, mutant.

(CPS1 and three biotin-dependent carboxylases: propionyl-CoA [PCC], 3-methylcrotonyl-CoA [3MCC], and pyruvate carboxylase [PC]).

The carbonic anhydrase family of zinc-containing metallo-enzymes all catalyze the reversible conversion of carbon dioxide to bicarbonate ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$). Mitochondria are impermeable to HCO_3^-, and thus the two intramitochondrial carbonic anhydrases (CA-VA and CA-VB) are pivotal in providing HCO_3^- for multiple mitochondrial enzymes that catalyze the formation of essential metabolites of intermediary metabolism in the urea and Krebs cycles (Figure 5).[11,12]

These include CPS1, which catalyzes the synthesis of carbamoylphosphate from ammonia and bicarbonate, a rate-limiting step in the conversion of waste nitrogen into urea, and pyruvate carboxylase (PC), which stands at the interface between glycolysis and the Krebs cycle, facilitating the production of oxaloacetate from pyruvate, the former being a major anaplerotic substrate essential for the maintenance of the Krebs cycle.[13] Primary PC deficiency is biochemically characterized by lactic acidosis,

hyperammonemia, and hypoglycemia, as was observed in our CA-VA-deficient individuals with presumed secondary PC deficiency leading to hypoglycemia through impaired gluconeogenesis.[14]

CA-VA deficiency provides the unique opportunity to study the opposite consequences of CPS1 deficiency (hyperammonemia, elevated glutamine, and decreased citrulline and other amino acids distal to the block) and PC deficiency (hyperammonemia, normal to decreased glutamine, increased citrulline and lysine). Given that glutamine was elevated in three of the four affected individuals and that the citrulline was low to normal in all, it appears that an impaired urea cycle, rather than deficient glutamate, was the major cause of hyperammonemia. Combined with the normal lysine levels and relatively mild elevations of 2-α-ketoglutaric acid and other Krebs cycle intermediates, the biochemical profiles in our CA-VA deficiency individuals support a predominant effect of (secondary) CPS1 versus PC deficiency.

The observed moderate increase of urinary 3-OH propionic acid, propionyl glycine, and methylcitrate along with traces of 3-methylcrotonylglycine are consistent with the expected reduced activities of PCC and 3MCC in CA-VA deficiency. Although biotinidase and holocarboxylase synthetase (HCS [MIM 253270]) deficiencies share the metabolite profiles of secondary PC, PCC, and 3MCC deficiencies, there are three major differences relative to primary CA-VA deficiency: (1) the significantly higher level of PCC and 3MCC metabolites in (even well-controlled) individuals with the two former disorders compared to those with CA-VA deficiency during metabolic decompensation; (2) the presence of CPS1 deficiency as (likely) major cause of hyperammonemia in CA-VA
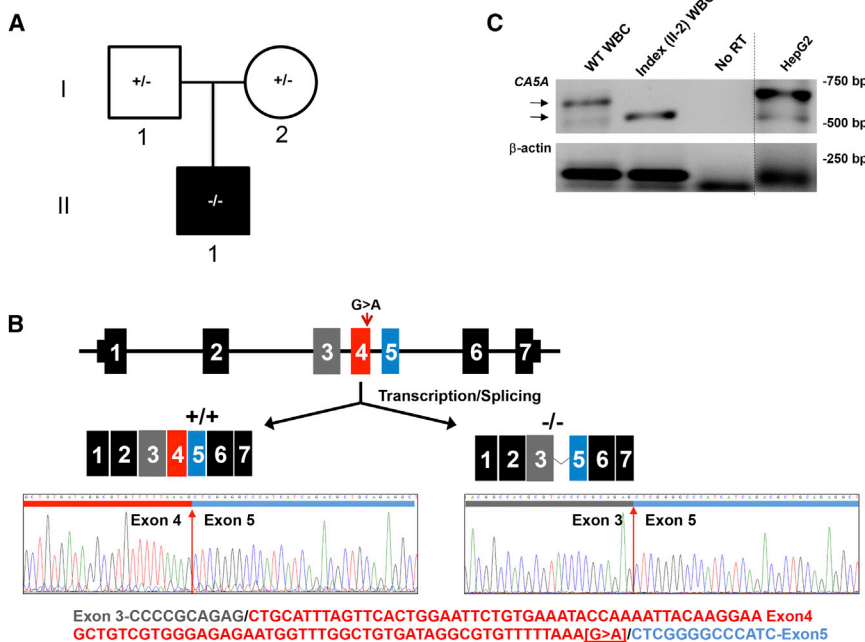

Figure 3. Family 2 with Exon 4 Splice Deletion
(A) Pedigree (black fill indicates clinically affected individual).
(B) Sanger sequencing of RT-PCR products generated in (B) with exons denoted by colors. Top: The *CA5A* structure (not to scale) and a schematic of the observed *CA5A* transcripts produced in a control subject and the index. Bottom: Sanger sequence of transcripts at exon 4 boundary in a control subject (+/+) and the index (−/−), along with WT *CA5A* cDNA sequence, color-coded as in the top panel.
(C) RT-PCR of *CA5A* mRNA from white blood cells (WBCs) or cultured liver cells (HepG2). Arrows indicate the products of differing size amplified from control subject (WT sequence) and index (II-1) WBCs. As controls, reverse transcriptase was omitted from the reaction (no RT) and a control gene (β-actin) was amplified in separate lane on a different cell type (denoted by the line).
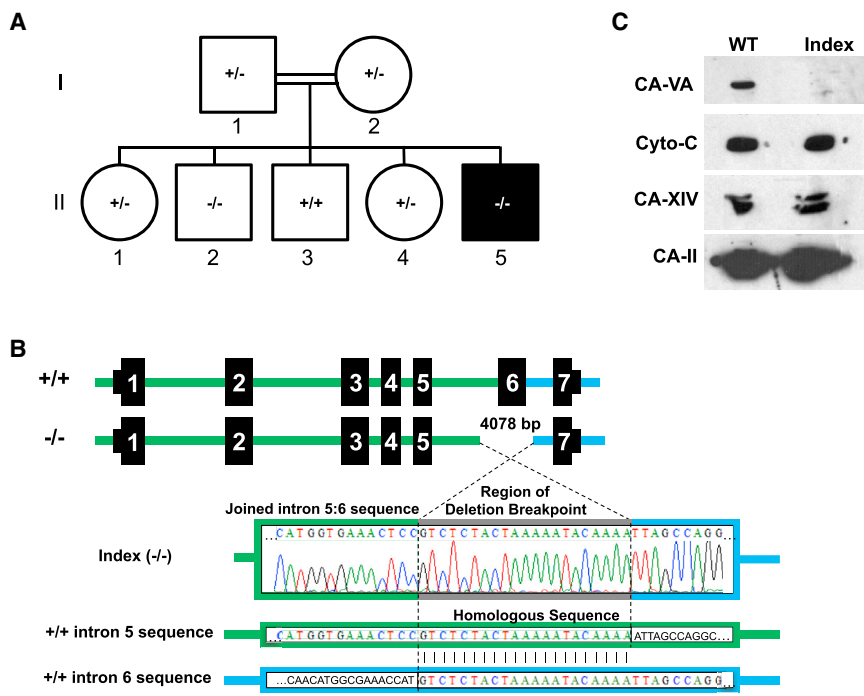
Exon 3-CCCCGCAGAG/CTGCATTTAGTTCACTGGAATTCTGTGAAATACCAAAATTACAAGGAA Exon4
GCTGTCGTGGGAGAGAATGGTTTGGCTGTGATAGGCGTGTTTTTAAA[G>A]/CTCGGGGCCCATC-Exon5

Figure 4. Family 3 with Exon 6 Deletion
(A) Pedigree (black fill indicates clinically affected individual, II-5).
(B) Top: Schematic representation of the 4,078 bp deletion that encompasses exon 6 of *CA5A*. Bottom: Sanger sequencing of PCR products generated from genomic DNA of the index (II-5) with a 21 bp repeated sequence (gray) at the breakpoint found in both intron 5 (green) and intron 6 (blue).
(C) Immunoblot analyses of control subject (WT) and index (II-5) liver homogenates. Samples were probed for CA-VA,[25] cytochrome c (Cyto-c), carbonic anhydrase XIV (CA 14),[26] and carbonic anhydrase II (CA 2)[27] via specific antibodies.

deficiency; and (3) the presence of acetyl-CoA carboxylase deficiency (MIM 613933) in HCS and biotinidase deficiency.[15,16] Our CA-VA-deficient individuals exhibited normal levels of free fatty acids and of total and free carnitine, as well as normal acylcarnitine profiles (data not shown), mostly probably as a result of the activity of the cytosolic acetyl-CoA carboxylase 2 isoform that is not affected by impaired provision of mitochondrial HCO_3^-.[17]

Although not approved for this indication, carglumic acid may be of benefit as synthetic treatment of partial CPS1 deficiency through its known ability to enhance the activity of this enzyme.[2] This could increase production of carbamoyl phosphate even at HCO_3^- levels below the Km for the enzyme. Although the hyperammonemia resulted from a reduction of substrate to CPS1, rather than a primary CPS1 deficiency, three individuals (families 1 and 2) showed a good response to carglumic acid (normalization of ammonia and for the female of family 1, a mild increase of citrulline), consistent with the suggestion that enhanced CPS1 activity can partially compensate for reduced HCO_3^- resulting from CA-VA deficiency. This hypothesis should be tested in the Car5A-null mouse model (Car5Adl1Sws),[18] especially given the high cost of this medication and uncontrolled circumstances in which these children were managed with multiple therapeutic modalities.

Newborn screening profiles (Tandem MS/MS), specifically C3 and C5OH levels, were unremarkable in all four cases, probably because of the relatively mild biochemical profile for the carboxylase-related metabolites along with the low sensitivity of acylcarnitine analyses compared to urine organic acids. Furthermore, outside of acute events, biochemical parameters remained normal in all affected children except for mildly elevated blood lactate and/or ketonuria. We propose several explanations for the relatively benign clinical course in these individuals and lack of apparent phenotype in the oldest male sibling in family 3. First, overlapping function of CA-VB may help prevent deleterious sequelae of reduced CA-VA activity.[19] In the mouse, Car5A is mainly localized in liver and its deficiency results in profound hyperammonemia. Car5B, though almost undetectable in liver, is predominant in mitochondria of many other tissues. Nonetheless, Car5B deficiency alone has no obvious phenotype. However, when superimposed on Car5A deficiency in the doubly deficient mouse (Car5Adl1Sws/Car5Bdl1Sws), Car5B deficiency aggravated the hyperammonemia and hypoglycemia and shortened survival.[18] Thus, Car5B does contribute to handling the metabolic load, though its action is evident only in the absence of Car5A. Second, although carbonic anhydrases accelerate the conversion of CO_2 to HCO_3^- by 1,000-fold or greater, some bicarbonate is produced via the nonenzymatic reaction, even in the absence of carbonic anhydrases.[20] Sufficiency for the product of this limited nonenzymatic source of bicarbonate may differ for the four different bicarbonate-requiring enzymes depending on their individual Kms.

Thus, CA-VA deficiency should be considered among urea cycle defects, organic acidurias, and PC deficiency in the differential diagnosis for hyperammonemia and hyperlactatemia in the neonate and young child. Whereas many of the biochemical markers are nonspecific, some metabolites such as propionylglycine and 3-methylcrotonylglycine are more specific for deficiencies of PCC and 3MCC deficiencies that occur secondary to CA-VA deficiency. However, the increase in these metabolites is subtle compared with individuals who have a primary defect of these enzymes. Furthermore, these abnormalities may be present only during symptomatic episodes. Therefore, diagnostic molecular analysis of *CA5A* should be considered in persons with suggestive biochemical findings.
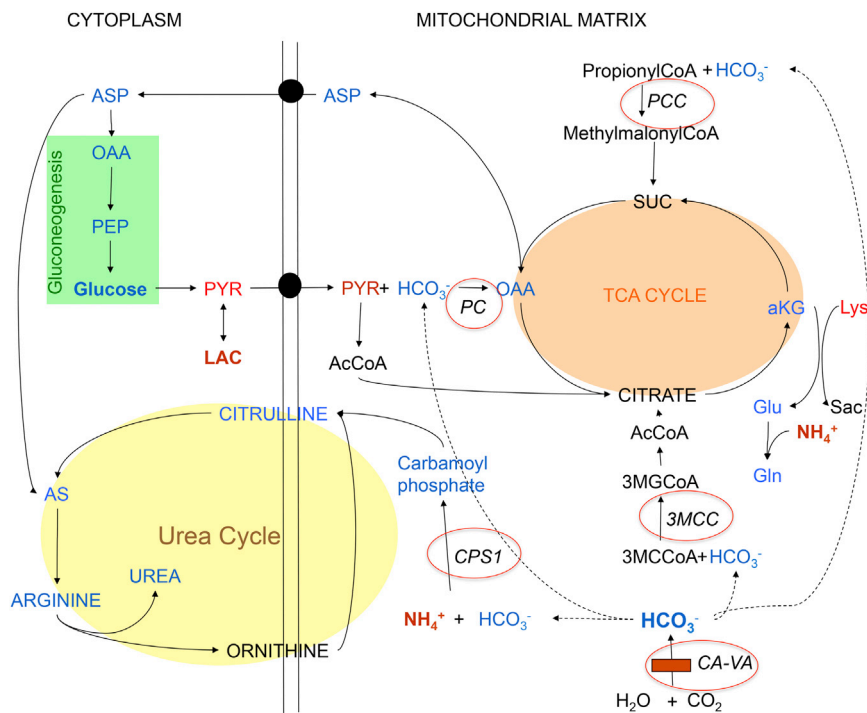
Figure 5. Biochemical Pathways Affected by CA-VA Deficiency

CA-VA deficiency is indicated by the horizontal red bar (bottom right). Dotted lines link CA-VA-mediated bicarbonate production and intramitochondrial donation to the bicarbonate-dependent carboxylases; the affected enzyme deficiencies are denoted with red circles. Enzymes: CPS1, carbamoylphosphate synthetase I; PC, pyruvate carboxylase; PCC, propionyl CoA carboxylase; 3MCC, 3-methylcrotonyl CoA carboxylase. Metabolites: SUC, succinyl CoA; αKG, α-ketoglutarate; OAA, oxaloacetate; Glu, glutamate; Gln, glutamine; Sac, saccharopine; Lys, lysine; ASP, aspartate; PEP, phosphoenolpyruvate; PYR, pyruvate; LAC, lactate; AcCoA, acetyl CoA; AS, argininosuccinate; 3MGCoA, 3-methylglutaconyl CoA; 3MCCoA, 3-methylcrotonyl CoA.

CA-VA deficiency expands the list of treatable inborn errors of metabolism potentially causing intellectual disability.[21] Effective therapy in the affected individuals comprised (1) preventive sick-day management during intercurrent illnesses, including a high-caloric, lipid-rich formula restricted in protein but normal in carbohydrates; and possibly (2) carglumic acid to enhance the activity of the first step in the urea cycle as a treatment for the hyperammonemia.

Other experimental therapies deserving exploration include oral zinc supplementation (a CA-VA cofactor that may increase residual activity in persons with mutations outside the zinc-binding site) and interventions to enhance or replace anaplerosis to alleviate metabolic symptoms of PC dysfunction, such as the administration of aspartate and citrate (but not triheptanoin, given the secondary PCC deficiency[22]), as demonstrated by improved growth and survival in CA-VA-null mice receiving citrate water.[18,23]

Supplemental Data

Supplemental Data include one figure and one table and can be found with this article online at http://www.cell.com/AJHG/.

Acknowledgments

Web Resources

The URLs for data presented herein are as follows:

BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq
TIDE-BC, http://www.tidebc.org
Treatable Intellectual Disability App, http://www.treatable-id.org
UniProt Sequence Annotation (Features), http://www.uniprot.org/uniprot/P35218#section_features

Accession Numbers

The Leiden Open Variation Databank (http://databases.lovd.nl/shared/genes/CA5A) accession number for the c.697T>C mutation reported in this paper is CA5A_000001; for the c.555G>A mutation, CA5A_000002; and for the 4,078 bp exon 6 deletion is CA5A_000003.

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