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MUTATION IN BRIEF

HUMAN MUTATION

Molecular Spectrum of *SLC22A5 (OCTN2)* Gene Mutations Detected in 143 Subjects Evaluated for Systemic Carnitine Deficiency

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ABSTRACT: Systemic primary carnitine deficiency (CDSP) is caused by recessive mutations in the *SLC22A5* (*OCTN2*) gene encoding the plasmalemmal carnitine transporter and characterized by hypoketotic hypoglycemia, and skeletal and cardiac myopathy. The entire coding regions of the *OCTN2* gene were sequenced in 143 unrelated subjects suspected of having CDSP. In 70 unrelated infants evaluated because of abnormal newborn screening (NBS) results, 48 were found to have at least 1 mutation/unclassified missense variant. Twenty-eight of 33 mothers whose infants had abnormal NBS results were found to carry at least 1 mutation/unclassified missense variant, including 11 asymptomatic mothers who had 2 mutations. Therefore, sequencing of the *OCTN2* gene is recommended for infants with abnormal NBS results and for their mothers. Conversely, 52 unrelated subjects were tested due to clinical indications other than abnormal NBS and only 14 of them were found to have at least one mutation/unclassified variant. Custom designed oligonucleotide array CGH analysis revealed a heterozygous ~1.6 Mb deletion encompassing the entire *OCTN2* gene in one subject who was apparently homozygous for the c.680G>A (p.R227H) mutation. Thus, copy number abnormalities at the *OCTN2* locus should be considered if by sequencing, an apparently homozygous mutation or only one mutant allele is identified. ©2010 Wiley-Liss, Inc.

KEY WORDS: systemic primary carnitine deficiency; *SLC22A5 (OCTN2)* mutations; newborn screening; large deletion

INTRODUCTION

The organic cation transporter (OCTN) family plays an important role to move organic cation compounds across cell membranes. One member of this family, OCTN2, transfers carnitine (3-hydroxy-4-

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trimethylaminobutyric acid) across cell membrane in a Na^+ dependent manner and other organic cations such as tetraethylammonium (TEA) in a Na^+-independent manner (Ohashi, et al., 2001; Scaglia and Longo, 1999; Scaglia, et al., 1999; Scaglia, et al., 1998). Carnitine is mostly derived from dietary intake. It can also be synthesized from lysine and methionine in liver and kidney (Crill and Helms, 2007; Krajcovicova-Kudlackova, et al., 2000; Stanley, 2004). An essential role for carnitine is to transport long-chain fatty acids from cytosol into mitochondria for beta-oxidation (Stanley, 2004). OCTN2 is a transmembrane protein consisting of 12 transmembrane domains and one ATP binding domain. Mutations in the *SLC22A5 (OCTN2)* gene have been reported in patients with systemic primary carnitine deficiency (CDSP; MIM# 212140). The severe form of CDSP was the originally known presentation of the disease and is characterized by progressive infantile-onset cardiomyopathy, weakness, peripheral neuropathy and recurrent hypoglycemic hypoketotic encephalopathy. However, the advent of expanded newborn screening has broadened the clinical spectrum of disease to include asymptomatic newborns and mothers (El-Hattab, et al., 2010; Schimmenti, et al., 2007; Spiekerkoetter, et al., 2003; Vijay, et al., 2006). The disease is highly responsive to L-carnitine supplementation. Plasma carnitine levels in patients with CDSP are low with high urinary excretion of carnitine. CDSP patients usually respond well to oral L-carnitine supplementation with significant improvement of their clinical symptoms (Cederbaum, et al., 2002). Thus, early diagnosis and medical intervention are critical. Molecular diagnosis provides an essential tool for this purpose. Sequence analysis of the *OCTN2* gene has only become clinically available in recent years. In this study, we present the molecular genetic spectrum of the *OCTN2* gene mutations obtained since August, 2007 when the sequence analysis was offered as a clinical test at the Medical Genetics Laboratory (MGL) at Baylor College Medicine (BCM).

MATERIALS AND METHODS

Subjects and DNA extraction

Blood samples from 143 unrelated subjects from several medical centers were sent to the Mitochondrial Diagnostic Laboratory at Baylor College of Medicine (Houston, Texas) for sequence analysis of the *OCTN2* gene during August, 2007 to November 2009. These clinical samples were submitted for the follow-up of abnormal newborn screening results consistent with a low free carnitine or plasma carnitine deficiency found as a result of the diagnostic work-up of symptomatic subjects. Blood specimens were collected from these subjects and DNA was extracted from leukocytes using commercially available DNA isolation kits (Gentra Systems Inc., Minneapolis, MN) according to the manufacturer's instructions. This retrospective study has been approved by the Baylor College of Medicine Internal Review Board.

DNA sequencing analysis

Sequence analysis of coding regions and 50 bp of their flanking intronic sequences of the *OCTN2* gene was performed on all 143 samples. Sequence-specific oligonucleotide primers linked to the M13 universal primer sequences at the 5'-ends were designed to amplify each coding exon. PCR products were generated using FastStart DNA polymerase (Roche, IN) and purified with ExcelaPure 96-well UF PCR purification plates (Edge BioSystems, Gaithersburg, MD). Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing kit (version 3.1) and analyzed on an ABI3730XL automated DNA sequencer with the Sequencing Analysis Software v5.1.1 (Applied Biosystems, CA). The sequencing results were compared to the GenBank *SLC22A5* sequence (NM_003060.3) by using the Mutation Surveyor version 3.24 (SoftGenetics, PA). Nucleotide numbering reflects cDNA numbering with c.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.

Oligonucleotide array CGH (aCGH)

A custom designed clinical oligonucleotide Comparative Genomic Hybridization (CGH) array (MitoMet) was manufactured using the Agilent microarray platform (Agilent Technologies, Santa Clara, CA). This is a clinically validated 44K oligonucleotide array with a complete coverage of the mitochondrial genome (6,400 probes for the 16.6-kb genome) and about 192 nuclear genes related to mitochondrial functions and metabolic diseases, including the *OCTN2* gene, at an average spacing of approximately 250 bp/ probe (Chinault, et al., 2009; Wong, et al., 2008).

Bioinformatics analysis

Two computer-based algorithms, PolyPhen (http://genetics.bwh.harvard.edu/pph/) and SIFT (http://sift.jcvi.org/), were used for the prediction of the pathogenicity of unpublished missense variants. In this study, we consider an unreported missense variant as a pathogenic mutation if it is predicted to be deleterious by both algorithms. A novel nucleotide change resulting in nonsense, frame-shift or abnormal splicing is also classified as a deleterious mutation. A novel missense variant, which is predicted to be benign or inconsistent by the PolyPhen and SIFT algorithms, is considered as an unclassified variant. Structural/functional domains of the OCTN2 protein are based on the information provided by the Universal Protein Resource (UniProt) (http://www.uniprot.org/).

RESULTS

Entire coding exons and 50bp of the flanking intronic regions of the *OCTN2* gene were sequence analyzed in 143 unrelated subjects suspected of CDSP. Within this group, there were 91 unrelated subjects ascertained through abnormal newborn screening (NBS) and 52 unrelated subjects tested due to their symptomatic clinical presentation and low plasma carnitine values unrelated to abnormal NBS. Among them, 79 (~53%) carried at least 1 mutation/unclassified missense variant (Table 1). The group of 91 subjects ascertained through NBS was comprised of 70 infants and 21 mothers whose infants failed the NBS but were not molecularly tested for *OCTN2* mutations. The 21 mothers in this group were used as a surrogate for their infants (Supp. Table S1). Eighteen infants were found to have 2 mutations, 4 had 1 mutation and 1 unclassified missense variant, 23 carried 1 mutation, and 3 had 1 unclassified missense variant. Thus, sequence analysis detects at least one mutation in 69% (48/70) in NBS positive infants. The *OCTN2* gene was sequenced in 33 mothers, including 12 whose infants were molecularly tested and 21 mothers whose infants were not tested for *OCTN2* mutations. The purpose of presenting this group was to determine the yield of *OCTN2* mutations in mothers of infants suspected of having CDSP due to abnormal NBS. Among them, 42% (14/33) were found to carry 2 mutations, 33% (11/33) harbored 1 mutation, and 3 subjects had 1 unclassified missense variant (~9%). Thus, sequence analysis detects at least one *OCTN2* mutation in about 85% (28/33) of mothers whose infants had carnitine deficiency ascertained by NBS. Interestingly, 11 of 14 mothers ascertained through abnormal NBS in their infants and with 2 mutations in the *OCTN2* gene, were clinically asymptomatic (Supp. Table S1).

Table 1. Genotypes of 79 OCTN2 sequencing positive individuals

Subject #	AMD (gender)	Allele 1	Allele 2	Ascertained
		2 Mutations		
72	26 y (F)	c.136C>T (p.P46S)	c.136C>T (p.P46S)	NBS
13	1 y (M)	***c.1267+3_+24del22***	c.51C>G (p.F17L)	NBS
1b	27 y (F)	c.424G>T (p.A142S)	c.424G>T (p.A142S)	NBS
15	1 y (M)	c.760C>T (p.R254X)	c.43G>T (p.G15W)	NBS
76	23 y (F)	c.43G>T (p.G15W)	c.43G>T (p.G15W)	NBS
18	8 m (M)	c.248G>T (p.R83L)	c.248G>T (p.R83L)	NBS
19	5 m (M)	c.136C>T (p.P46S)	c.695C>T (p.T232M)	NBS
104	30 y (F)	***c.1304_1313del10 (p.G435EfsX20)***	c.695C>T (p.T232M)	Others
29	5 m (F)	c.1400C>G (p.S467C)	c.51C>G (p.F17L)	NBS
31	1 m (M)	c.1319C>T (p.T440M)	c.1195C>T (p.R399W)	NBS
105	1.5y (F)	c.505C>T (p.R169W)	***c.1520T>C (p.L507S)***	Others
106	25y (M)	c.136C>T (p.P46S)	***c.1520T>C (p.L507S)***	Others
33	3 w (M)	c.505C>T (p.R169W)	c.760C>T (p.R254X)	NBS
4b	31y (F)	c.1195C>T (p.R399W)	***c.1324_1325GC>AT (p.A442I)***	NBS

Subject #	AMD (gender)	Allele 1	Allele 2	Ascertained
6a	6 w (F)	c.136C>T (p.P46S)	c.424G>T (p.A142S)	NBS
40	5 m (M)	c.136C>T (p.P46S)	c.424G>T (p.A142S)	NBS
112a	8 y (F)	c.806delT (p.L269HfsX27)	c.1319C>T (p.T440M)	Others
79	38 y (F)	c.1400C>G (p.S467C)	c.1400C>G (p.S467C)	NBS
81	38 y (F)	c.136C>T (p.P46S)	c.695C>T (p.T232M)	NBS
7a	3 y (F)	c.248G>T (p.R83L)	c.641C>T (p.A214V)	NBS
82	30 y (F)	***c.1064C>T (p.S355L)***	c.51C>G (p.F17L)	NBS
83	27 y (F)	c.1319C>T (p.T440M)	***c.769C>T (p.R257W)***	NBS
127	14 y (M)	***c.350G>A (p.W117X)***	***c.573delG (p.N192IfsX12)***	Others
8a	6 m (M)	c.95A>G (p.N32S)	c.95A>G (p.N32S)	NBS
85	25 y (F)	c.51C>G (p.F17L)	c.51C>G (p.F17L)	NBS
87	34 y (F)	c.43G>T (p.G15W)	c.43G>T (p.G15W)	NBS
12a	1 m (F)	***c.680G>A (p.R227H)***	***OCTN2* deletion**	NBS
50	5 m (M)	c.695C>T (p.T232M)	***c.680G>A (p.R227H)***	NBS
52	5 m (M)	***c.791C>G (p.T264R)***	***c.791C>G (p.T264R)***	NBS
11b	21 y (F)	c.136C>T (p.P46S)	c.424G>T (p.A142S)	NBS
3b	32 y (F)	c.136C>T (p.P46S)	c.1354G>A (p.E452K)	NBS
59	20 m (M)	c.95A>G (p.N32S)	c.641C>T (p.A214V)	NBS
10a	2 m (M)	c.136C>T (p.P46S)	c.424G>T (p.A142S)	NBS
136	30 y (F)	c.136C>T (p.P46S)	c.1193C>T (p.P398L)	Others
58	5 m (M)	c.43G>T (p.G15W),	c.43G>T (p.G15W)	NBS
59	15m (F)	c.136C>T (p.P46S)	c.695C>T (p.T232M)	NBS
140	8 y (F)	c.506G>A (p.R169Q)	c.506G>A (p.R169Q)	Others
69	2 m (F)	c.845G>A (p.R282Q)	c.424G>T (p.A142S)	NBS
91	43 y (F)	c.695C>T (p.T232M),	c.424G>T (p.A142S)	NBS
		1 Mutation + 1 unclassified missense variant		
32	5 w (F)	c.136C>T (p.P46S)	***c.688T>C (p.F230L)***	NBS
38	6 m (F)	c.1345T>G (p.Y449D)	***c.1072T>A (p.Y358N)***	NBS
42	3 m (F)	c.424G>T (p.A142S)	***c.224G>C (p.R75P)***	NBS
49	2 m (M)	c.95A>G (p.N32S)	***c.529A>G (p.M177V)***	NBS
		1 Mutation		
73	31 y (F)	***c.1327T>G (p.F443V)***		NBS
92	12 y (F)	c.396G>A (p.W132X)		Others
14	6 w (M)	***c.1364C>G (p.P455R)***		NBS
2a	4 y (M)	c.844C>T (p.R282X)		NBS
75	31 y (F)	c.844C>T (p.R282X)		NBS
20	2 m (F)	c.1400C>G (p.S467C)		NBS
25	118 m (M)	c.1462C>T (p.R488C)		NBS
26	7 m (M)	c.396G>A (p.W132X)		NBS
27	20m (F)	c.136C>T (p.P46S)		NBS
77	25 y (F)	c.424G>T (p.A142S)		NBS

Subject #	AMD (gender)	Allele 1	Allele 2	Ascertained
110	5 y (M)	***c.34G>A (p.G12S)***		Others
6a	5 w (F)	c.844C>T (p.R282X)		NBS
80	24 y (F)	c.1345T>G (p.Y449D)		NBS
124	5 y (M)	c.136C>T (p.P46S)		Others
45	1 m (F)	c.1319C>T (p.T440M)		NBS
125	15 y (F)	c.1195C>T (p.R399W)		Others
46	3.5 y (F)	c.136C>T (p.P46S)		NBS
48	6 w (F)	***c.557T>C (p.L186P)***		NBS
86	25 y (F)	c.424G>T (p.A142S)		NBS
129	19 y (F)	***c.364G>T (p.D122Y)***		Others
53	11 m (M)	c.695C>T (p.T232M)		NBS
55	7 m (M)	c.424G>T (p.A142S)		NBS
56	2 m (M)	c.844C>T (p.R282X)		NBS
57	5 m (F)	c.1193C>T (p.P398L)		NBS
90	24 y (F)	***c.955C>T (p.Q319X)***		NBS
61	2 m (F)	c.136C>T (p.P46S)		NBS
62	2 m (F)	c.136C>T (p.P46S)		NBS
64	5 m (F)	c.136C>T (p.P46S)		NBS
66	7 m (M)	c.1304delG (p.G435AfsX24)		NBS
		1 unclassified missense variant		
71	35 y (F)	***c.718G>A (p.A240T)***		NBS
30	15 m (F)	***c.287G>C (p.G96A)***		NBS
9a	2 m (F)	***c.428C>T (p.P143L)***		NBS
119	2 y (M)	***c.1645C>T (p.P549S)***		Others
88	38 y (F)	***c.368T>G (p.V123G)***		NBS
135	4 m (M)	***c.934A>G (p.I312V)***		Others
65	1 m (F)	***c.196A>C (p.T66P)***		NBS

AMD: Age at molecular diagnosis; Bold and italic: novel changes in this study; Gray: unclassified missense variants; NBS: Newborn screening; Others: ascertained not through NBS. Nucleotide numbering reflects cDNA numbering with c.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.

Based on the data available to us, the average free carnitine levels in infants ascertained through an abnormal newborn screening carrying two, one, or no mutations in the *OCTN2* gene did not achieve a statistically significant difference using one way analysis of variance (ANOVA) with a resulting P value = 0.879 (Table 2A).

Fifty-two unrelated subjects in this study were analyzed for *OCTN2* mutations due to low plasma carnitine levels obtained as a result of a diagnostic work-up for clinical indications other than abnormal NBS results such as cardiomyopathy, hypoglycemia, hypotonia, easy fatigability, development delay and epilepsy (Supp. Table S2). Seven (7/52=13%) of them have 2 mutations, 5 (5/52=10%) carried 1 mutation, 2 (2/52=4%) harbored 1 unclassified variant, and 38 (38/52=72%) had no mutations. Thus, sequence analysis detected at least one *OCTN2* mutation in 27% (14/52) of this group of subjects. Within this group and the group of mothers tested due to abnormal NBS in their infants who were not tested molecularly, the means of the free carnitine levels for the three subgroups with two, one and no mutations in the *OCTN2* locus were also statistically different when compared by using one way analysis of variance (ANOVA) with $P<0.05$ (Table 2B).

Table 2A. Free carnitine levels (μM) in infants ascertained through newborn screening

Subject #	2 *OCTN2* mutations	Subject #	1 OCTN2 mutation	Subject #	No mutations
5a	6	**6a**	14	**16**	9
15	2	**9a**	12	**17**	3
31	2	**11a**	2	**23**	7
52	1	**30**	7		
54	17	**48**	6		
59	7	**2a**	6		
32	6	**65**	3		
Mean ± 1SD	5.86 ± 5.46	**Mean ± 1SD**	7.14 ± 4.41	**Mean ± 1SD**	6.33 ± 3.06

*P= 0.879 (one way ANOVA)

Table 2B. Free carnitine levels (μM) in subjects other than neonates

Subject #	2 *OCTN2* mutations	Subject #	1 OCTN2 mutation	Subject #	No mutations
3b	0	**5b**	20	**102**	NA
7b	4	**6b**	6	**116**	NA
11b	2	**7c**	31	**117**	NA
79	4	**77**	NA	**123**	12
81	4	**90**	5	**133**	5
82	4	**9b**	16	**134**	2
105	9	**9c**	NA	**74**	16
106	12	**71**	6	**137**	NA
112a	0	**112b**	28	**142**	7
136	2				
Mean ± 1SD	***4.10 ± 3.78	**Mean ± 1SD**	16.00 ± 10.85	**Mean ± 1SD**	8.40 ± 5.59

*P<0.05 (one way ANOVA)

To investigate the possibility of heterozygous large deletions/duplications, which might be missed by sequence analysis, 25 subjects with one heterozygous mutation and 1 subject with an apparently homozygous mutation were analyzed using custom designed oligonucleotide targeted array CGH (aCGH) analysis (Chinault, et al., 2009; Wong, et al., 2008). The subject with an apparently homozygous c.680G>A (p.R227H) mutation by sequence analysis was a female infant with an abnormal NBS result (#12a in Table 1 and Supp. Table S1). Her mother was heterozygous for the point mutation but her father was negative for the mutation. The oligonucleotide aCGH analysis detected a ~1.6 Mb deletion encompassing the entire *OCTN2* gene in this infant. This deletion was not detected in her father's blood specimen, suggesting that the deletion is either a *de novo* event or reflects gonadal mosaicism in her father (Figure 1). Large deletions were not detected in the other 25 samples analyzed.

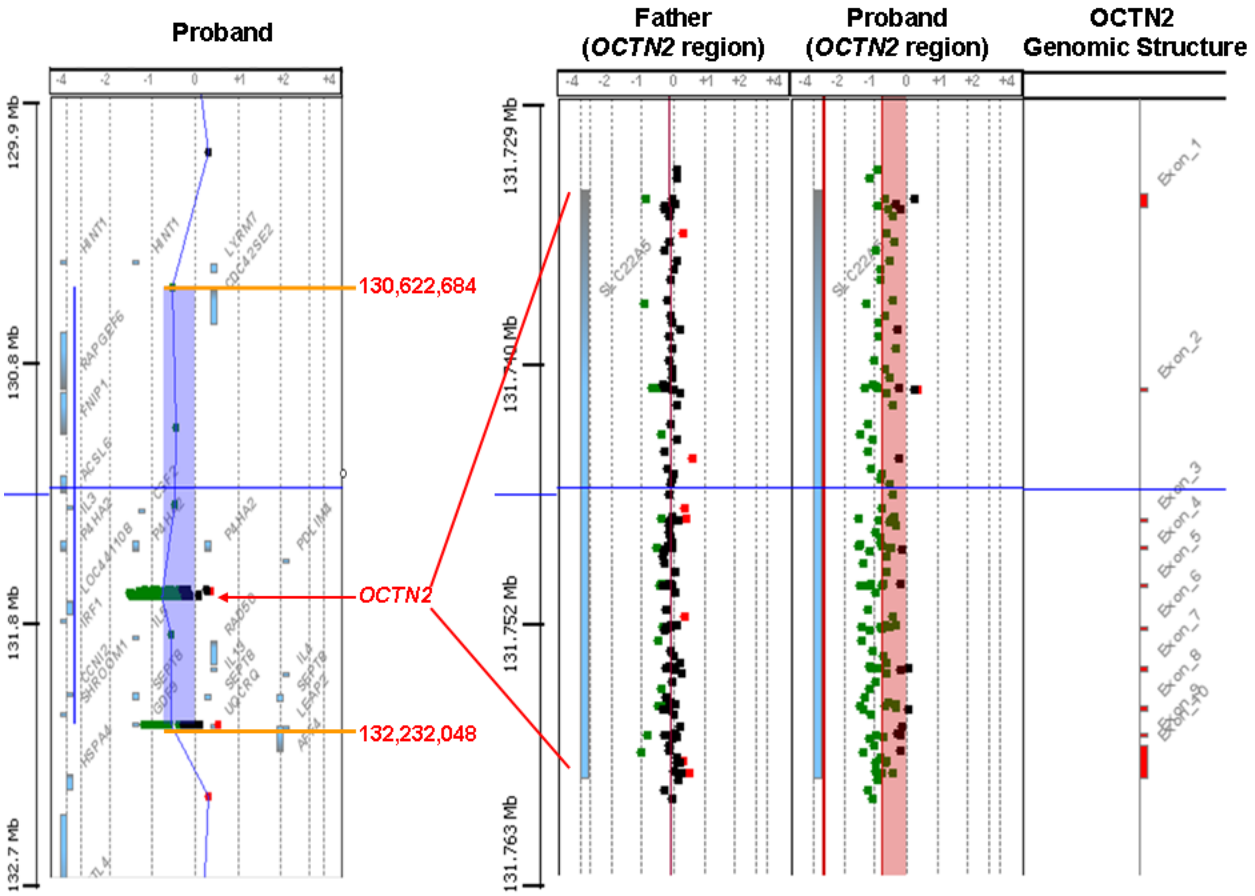

Figure 1. The result of aCGH analysis on the subject with an apparently homozygous c.1412G>C (p.471R>H) mutation. A deletion of at least 1.6 Mb (blue box, nucleotide position 130,622,684 to 132,232,048, Ensembl version 54) encompassing the entire *OCTN2* gene and other genes was detected (left). This deletion was not detected in the father. Each dot represents an oligonucleotide probe. Green dots represent copy number loss. Black dots indicate no copy number changes, and the red dots indicate copy number gain. The coordinates on the right of the left picture indicates the minimum deletion region. The 2 genes, *OCTN2* and *UQCRQ*, with dense probe coverage are targeted genes in this MitoMet array. The genes and their positions within this region are also indicated.

In this study, a total of 122 mutant alleles with 51 different mutations/unclassified missense variants (Table 3) and Figure 2 were identified in 79 subjects (Table 1). Twenty-three mutations have been reported and 28 have not been previously reported in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php) or the *OCTN2* Database at the ARUP Laboratories (http://www.arup.utah.edu/database/OCTN2/OCTN2_display.php) (Table 3). Among 28 novel changes, 17 of them are considered to be pathogenic either due to null mutation (frame-shift, nonsense or splicing) or based on deleterious prediction by both SIFT and PolyPhen algorithms. Eleven novel missense variants are considered as unclassified based on benign or inconsistent prediction by the SIFT and/or PolyPhen algorithms. Among the 51 different mutations identified, one is large deletion, 11 are null and the remainders are missense. The most frequently occurring mutation is the c.136C>T (p.P46S) with a frequency of ~16% (19/122). Eleven subjects were homozygous for 10 different mutations (Table 1). Thirty-nine subjects including 11 homozygous, had 2 mutations, 4 subjects carried 1 mutation and 1 unclassified missense variant, 29 subjects harbored 1 mutation and 7 subjects carried 1 unclassified variant (Table 1). Thus, at least one mutation was detected in 55% (79/143) of all subjects analyzed in this study. Mutations/unclassified missense variants identified in this study are located in all exons. However, ~37% of mutant alleles (44/122) are located in exon 1 while exon 1 accounts for ~23.5% of entire coding nucleotides (393/1674). The most frequently affected

domain in this study is transmembrane domain 10, which harbors 8.2% (10/122) of mutant alleles in a region of ~3.8% (21/557 amino acids) of the OCTN2 protein (Table 3).

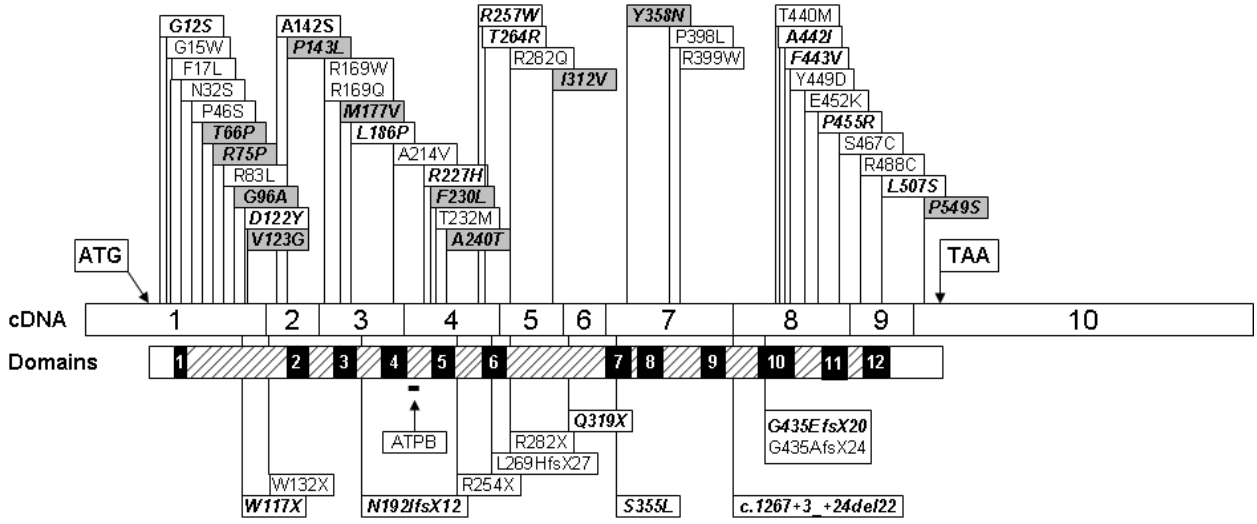

Figure 2. Graphic presentation of mutations/unclassified missense variants identified in this study. Entire cDNA structure with 10 exons is presented with open boxes. Missense mutations/unclassified missense variants are above and null mutations are below the cDNA structure. Each change is approximately placed according to its location. The functional domains, transmembrane domains in black boxes and loop regions in hatched boxes, are placed according to their corresponding mutation/variant positions. Domain positions of the OCTN2 protein are based on the information provided by the Universal Protein Resource (UniProt) (http://www.uniprot.org/). Bold and italic represent novel changes. Gray represents unclassified missense variants. ATPB: ATP binding domain.

Table 3. Detected mutations and unclassified variants, and their frequencies and locations

Mutations/unclassified missense variants	Freq	Exons	Domains
c.34G>A (p.G12S)	1	Exon 1	N-terminus
c.43G>T (p.G15W)	7		
c.51C>G (p.F17L)	5		
c.95A>G (p.N32S)	4		TM 1
c.136C>T (p.P46S)	19		L 1
c.196A>C (p.T66P)	1		
c.224G>C (p.R75P)	1		
c.248G>T (p.R83L)	3		
c.287G>C (p.G96A)	1		
c.350G>A (p.W117X)	1		
c.364G>T (p.D122Y)	1		
c.368T>G (p.V123G)	1		
c.396G>A (p.W132X)	2	Exon 2	
c.424G>T (p.A142S)	12		
c.428C>T (p.P143L)	1		TM 2
c.505C>T (p.R169W)	2	Exon 3	L 2
c.506G>A (p.R169Q)	2		
c.529A>G (p.M177V)	1		TM 3
c.557T>C (p.L186P)	1		
c.573delG (p.N192IfsX12)	1		

Mutations/unclassified missense variants	Freq	Exons	Domains
c.641C>T (p.A214V)	2		TM 4
c.680G>A (p.R227H)	2	Exon 4	L 4
c.688T>C (p.F230L)	1		
c.695C>T (p.T232M)	7		
c.718G>A (p.A240T)	1		TM 5
c.760C>T (p.R254X)	2		L 5
c.769C>T (p.R257W)	1		
c.791C>G (p.T264R)	2		TM 6
c.806delT (p.L269HfsX27)	1		
c.844C>T (p.R282X)	4	Exon 5	L 6
c.845G>A (p.R282Q)	1		
c.934A>G (p.I312V)	1		
c.955C>T (p.Q319X)	1	Exon 6	
c.1064C>T (p.S355L)	1	Exon 7	TM 7
c.1072T>A (p.Y358N)	1		
c.1193C>T (p.P398L)	2		L 8
c.1195C>T (p.R399W)	3		
c.1267+3_+24del22	1	Intron 7	TM 9
c.1304_1313del10 (p.G435EfsX20)	1	Exon 8	TM 10
c.1304delG (p.G435AfsX24)	1		
c.1319C>T (p.T440M)	4		
c.1324_1325GC>AT (p.A442I)	1		
c.1327T>G (p.F443V)	1		
c.1345T>G (p.Y449D)	2		
c.1354G>A (p.E452K)	1		L 10
c.1364C>G (p.P455R)	1		
c.1400C>G (p.S467C)	4		TM 11
c.1462C>T (p.R488C)	1	Exon 9	L 11
c.1520T>C (p.L507S)	2		TM 12
c.1645C>T (p.P549S)	1	Exon 10	C-terminus
~1.6 Mb deletion	1	Entire gene	Entire protein
total	**122**		

Bold and italic: novel changes in this study. Gray: unclassified missense variant. TM, transmembrane domain. L: inter-transmembrane loop. Nucleotide numbering reflects cDNA numbering with c.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.

DISCUSSION

CDSP is a pan-ethnic disorder (Koizumi, et al., 1999; Lamhonwah, et al., 2004; Melegh, et al., 2004; Tang, et al., 2002; Wilcken, et al., 2001). It can result in significant clinical consequences including death if left untreated. The clinical symptoms can be dramatically improved after the administration of carnitine supplementation, except those with irreversible consequences such as central nervous system involvement due to hypoglycemia (Cederbaum, et al., 2002). The clinical spectrum ranges from asymptomatic adults and newborn infants to infantile sudden death. Thus, it is a good example of the utility of newborn screening for early diagnosis and early medical intervention.-

A limiting factor in our study could be a bias in the diagnostic work-up for CDSP in symptomatic subjects due to the lack of uniform ascertainment criteria among referring clinicians. In addition, most of the samples were not from local subjects and therefore we do not have complete access to clinical information.

Patients with CDSP exhibit low plasma carnitine levels. Although there are reported familial cases where plasma carnitine levels were much lower in patients with 2 mutations than ~~that~~ in patients with only one mutant allele (Komlosi, et al., 2009; Tang, et al., 1999; Wang, et al., 2001), our results (Table 2A) did not show striking differences in free plasma carnitine levels among newborns with two *OCTN2* mutations, heterozygotes and those with no *OCTN2* mutations, demonstrating no clear relationship between free plasma carnitine levels and *OCTN2* genotype in newborns ascertained through abnormal NBS. These results suggest that other factors may account for the low free plasma carnitine levels observed in newborns with no mutations in the *OCTN2* gene. One of the potential limitations in the analysis of these data is that plasma carnitine levels in newborns will reflect maternal carnitine levels (Novak, et al., 1981). In addition, carnitine values in newborns will vary most likely based on whether babies are breast fed or formula fed and the maternal carnitine status during and after pregnancy in breast fed infants. It is known that in pregnancy there is a decrease in plasma free carnitine levels and more recent studies in pregnant women have revealed an increase in urinary losses of carnitine which could certainly impact the carnitine status of breast fed infants (Cho and Cha, 2005; Crill and Helms, 2007; Koumantakis, et al., 2001; Schoderbeck, et al., 1995). In neonates, carnitine could be considered a conditionally essential nutrient due to a reduced biosynthetic ability observed in this population. Neonates and infants lack sufficient activity of the biosynthetic enzyme gamma-butyrobetaine hydroxylase which is approximately 12% of that seen in adults (Crill and Helms, 2007). In particular, premature neonates may be at risk for carnitine deficiency due to a lack of placental transfer in the third trimester and decreased tissue stores. Moreover, the lack of maturity of renal tubular function in premature neonates could lead to increased renal elimination (Crill, et al., 2006). Plasma carnitine levels in premature newborns rapidly decrease during the first 3 days after birth if no carnitine supplementation is implemented (Novak, et al., 1981). Conditions such as respiratory distress syndrome (RDS) may result in low plasma carnitine levels in preterm newborns. Since carnitine is an essential component of the membrane phospholipid fatty acid turnover process in human cells, carnitine may cause lung maturation via membrane phospholipid repair activity. Thus, the low carnitine levels observed in RDS in preterm newborns could be explained by increased use of carnitine in fetal lung tissue for surfactant synthesis (Korkmaz, et al., 2005). Furthermore, genetic conditions such as maternal type 1 glutaric acidemia could also be ascertained as a result of abnormal NBS suggestive of CDSP (Crombez, et al., 2008). Unfortunately, detailed clinical information to determine whether any of these modifying factors were present is not available to us.

Conversely, there was statistical significance (Table 2B) when the free plasma carnitine levels were compared among the three different genotypes in the group of symptomatic subjects and mothers ascertained through the abnormal NBS of their infants. Although it is not surprising to find a difference between affected subjects and heterozygotes, the mean of the plasma free carnitine levels in those subjects with no *OCTN2* mutations was lower than in those with one *OCTN2* mutation. These results should not be considered definite as the volume of our sample is not large and we were only able to obtain few free values especially among those subjects with no mutations. Moreover as discussed above, there are many other genetic and environmental factors that could be responsible for secondary carnitine deficiency (Scaglia and Longo, 1999).

The abnormal newborn screening results may uncover maternal CDSP cases harboring *OCTN2* mutations. Some of these mothers may be asymptomatic (El-Hattab, et al., 2010; Schimmenti, et al., 2007; Vijay, et al., 2006). In this study, we identified 11 clinically asymptomatic mothers carrying two *OCTN2* mutations through abnormal newborn screening in their infants. This finding is consistent with our previous report that an expanding clinical spectrum for CDSP includes asymptomatic individuals (El-Hattab, et al., 2010). Asymptomatic cases have also been reported in a family with a homozygous *OCTN2* mutation, in which the father and 2 sons all carried the homozygous c.1412G>C (p.471R>H) mutation but only one son had clinical symptoms consistent with Reye-like syndrome episodes of hepatopathy and encephalopathy (Spiekerkoetter, et al., 2003). Thus, we could hypothesize that there may be undetected asymptomatic fathers with CDSP whose heterozygote offspring are ascertained through an abnormal NBS. These data clearly suggest that other genetic or environmental factors may play a role in modulating the clinical manifestations of CDSP. Therefore, it is possible that a certain percentage of asymptomatic individuals carrying 2 *OCTN2* mutations may be in the general population. There are polymorphisms in the *OCTN2* promoter region such as c.-207C/G that may affect OCTN2 activity. The c.-207G/G has been reported to be associated with higher activity of the carnitine transporter (Urban, et al., 2006). However, c.-207G/G has been found in all three symptomatic mothers who carried two mutations, suggesting that this particular polymorphism alone may not play a critical role in the expression of a clinical phenotype.

Various types of mutations in *OCTN2* gene, including missense, nonsense, frame-shift and splice site mutations were found as reported in other studies (Lamhonwah, et al., 2002; Longo, et al., 2006; Wang, et al., 2000a). There seems to be no clear relationship between genotype and clinical phenotype, in particular among symptomatic subjects suspected of having CDSP. Our findings are consistent with the results of previous studies (Lamhonwah, et al., 2002; Longo, et al., 2006; Wang, et al., 2000b). The observed clinical variability may be due to other environmental factors such as infections, fasting, decreased or absent intake of foods rich in carnitine as observed in vegetarian or vegan diets, or the use of medications that would lead to carnitine deficiency such as valproic acid and pivalic acid (Holme, et al., 1989; Tein, et al., 1993).

Although mutations were found in all exonic coding regions, they occur most frequently in the coding region of exon 1, as it has been similarly reported (Longo, et al., 2006). Exon 1 contains the N-terminus, the first transmembrane domain, and the majority of the first extracellular loop (Figure 2). *In vitro* subcellular localization studies of mutant molecules revealed that this region is very important for OCTN2 trafficking from the endoplasmic reticulum to the cell membrane (Maekawa, et al., 2007; Urban, et al., 2006). Therefore, mutations in this region may cause protein structural changes affecting the localization of OCTN2.

The *OCTN2* gene encodes a protein of 557 amino acids with 12 putative transmembrane domains. Of interest, the region most frequently affected by mutations is transmembrane domain 10 (TM10). It accounts for only ~3.8% (21/557) of the OCTN2 protein, but 8.2% of the times the mutations occur in this transmembrane domain. Biochemical assay of the TM10 p.Y449D mutant showed that the mutant selectively reduces the transport of L-carnitine but increases TEA transport (Urban, et al., 2006), suggesting that this domain might be involved in substrate binding specificity. However, this hypothesis requires further investigation.

Using a sequencing method alone to evaluate molecular defects in the *OCTN2* gene has its limitations. Since only coding exons and about 50 bp of their flanking intronic regions were sequenced, mutations in the promoter regions or deep in introns are not analyzed. In addition, sequence analysis does not detect large heterozygous deletions or duplications. In this study, a large deletion encompassing the entire *OCTN2* gene was detected by using a targeted oligonucleotide array CGH, suggesting that large deletions could also account for the molecular alterations in CDSP. To our knowledge, this is the first reported large deletion involving the *OCTN2* gene. However, the large deletion was only found in one of 26 subjects tested for copy number variants on the *OCTN2* locus. Although small deletions and insertions have been previously reported in patients with CDSP (Lamhonwah, et al., 2002; Lamhonwah and Tein, 1998; Nezu, et al., 1999; Wang, et al., 1999), large deletions may not constitute a common molecular alteration in the *OCTN2* locus. More studies are required to support this finding.

In conclusion, sequence analysis of *OCTN2* gene in infants and their mothers with abnormal newborn screening results suggestive of CDSP yields high mutation detection rates (69% and 85% respectively) while only 27% of subjects with clinical indications suggestive of CDSP harbored mutations in the *OCTN2* gene. These results suggest that sequence analysis of the *OCTN2* gene in infants suspected of having CDSP and their mothers should be considered as a molecular follow-up study of abnormal newborn screening results. Large deletions at the *OCTN2* locus may also be considered if clinically indicated when sequence analysis does not detect two pathogenic mutations and the clinical phenotype is compelling for CDSP.

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Supp. Table S1. OCTN2 sequencing results for NBS abnormal infants and and/or their immediate relatives

Subject #	Age (Gender)	Clinical presentation	Plasma carnitine		*SLC22A5* gene sequencing	
			T	F	Allele 1	Allele 2
1a	3 w (M)	Abnormal NBS	NA	NA	c.424G>T (p.A142S)	---
1b	27 y (F)	Mother (asymptomatic)	NA	NA	c.424G>T (p.A142S)	c.424G>T (p.A142S
2a	4 y (M)	Abnormal NBS	9	6	c.844C>T (p.R282X)	---
2b	42 y (F)	Mother (asymptomatic)	NA	NA	---	---
3a	3 m (M)	Abnormal NBS	NA	NA	c.136C>T (p.P46S)	---
3b	32 y (F)	Mother (easy fatigability)	0	0	c.136C>T (p.P46S)	c.1354G>A (p.E452K)
4a	14 m (F)	Abnormal NBS	NA	NA	c.1195C>T(p.R399W)	---
4b	31 y (F)	Mother (asymptomatic)	NA	NA	c.1195C>T(p.R399W)	***c.1324-1325GC>AT (p.A442I)***
4c	4 y (M)	Brother (asymptomatic)	NA	NA	c.1195C>T(p.R399W)	---
4d	13 y (F)	Sister (asymptomatic)	NA	NA	***c.1324-1325GC>AT (p.A442I)***	---
5a	6 w (F)	Abnormal NBS	7	6	c.424G>T (p.A142S)	c.136C>T (p.P46S)
5b	30 y (F)	Mother (asymptomatic)	21	20	c.424G>T (p.A142S)	---
5c	31y (M)	Father (asymptomatic)	NA	NA	c.136C>T (p.P46S)	---
6a	5 w (F)	Abnormal NBS	19	14	c.844C>T (p.R282X)	---
6b	38 y (F)	Mother (asymptomatic)	9	6	c.844C>T (p.R282X)	---
7a	3 y (F)	Abnormal NBS	NA	NA	c.248G>T (p.R83L)	c.641C>T (p.A214V)
7b	29 y (F)	Mother (easy fatigability)	5	4	c.248G>T (p.R83L)	c.641C>T (p.A214V)
7c	32 y (M)	Father (asymptomatic)	43	31	c.248G>T (p.R83L)	---
8a	6 m (M)	Abnormal NBS	NA	NA	c.95A>G (p.N32S)	c.95A>G (p.N32S)
8b	28 y (F)	Mother (asymptomatic)	NA	NA	c.95A>G (p.N32S)	---
9a	2 m (F)	Abnormal NBS	16	12	***c.428C>T (p.P143L)***	---
9b	42 y (F)	Mother (asymptomatic)	18	16	***c.428C>T (p.P143L)***	---
9c	14 y (F)	Sister (asymptomatic)	21	NA	***c.428C>T (p.P143L)***	---
10a	2 m (M)	Abnormal NBS	NA	NA	c.136C>T (p.P46S)	c.424G>T (p.A142S)

Subject #	Age (Gender)	Clinical presentation	Plasma carnitine		*SLC22A5* gene sequencing	
			T	F	Allele 1	Allele 2
10b	24y (F)	Mother (asymptomatic)	NA	NA	c.424G>T (p.A142S)	---
11a	4m (F)	Abnormal NBS	2	2	c.424G>T (p.A142S)	---
11b	21 y (F)	Mother (asymptomatic)	3	2	c.136C>T (p.P46S)	c.424G>T (p.A142S)
12a	1 m (F)	Abnormal NBS	NA	NA	***c.680G>A (p.R227H)***	*OCTN2* deletion
12b	35y (F)	Mother (asymptomatic)	NA	NA	***c.680G>A (p.R227H)***	
12c	35y (M)	Father (asymptomatic)	NA	NA	**-**	**-**
13	1 y (M)	Abnormal NBS	NA	NA	***c.1267+3-+24del22***	c.51C>G (p.F17L)
14	6 w (M)	Abnormal NBS	NA	NA	***c.1364C>G (p.P455R)***	---
15	1 y (M)	Abnormal NBS	5	2	c.760C>T (p.R254X)	c.43G>T (p.G15W)
16	2 m (M)	Abnormal NBS	12	9	---	---
17	3 m (F)	Abnormal NBS	14	3	---	---
18	8 m (M)	Abnormal NBS	NA	NA	c.248G>T (p.R83L)	c.248G>T (p.R83L)
19	4 m (M)	Abnormal NBS	NA	NA	c.136C>T (p.P46S)	c.695C>T (p.T232M)
20	4 w (F)	Abnormal NBS	NA	NA	c.1400C>G (p.S467C)	---
21	6 m (M)	Abnormal NBS	NA	NA	---	---
22	5 m (F)	Abnormal NBS	NA	NA	---	---
23	1 y (M)	Abnormal NBS	NA	7	---	---
24	2 y (M)	Abnormal NBS	NA	NA	---	---
25	18 m (M)	Abnormal NBS	NA	NA	c.1462C>T (p.R488C)	---
26	7 m (M)	Abnormal NBS	NA	NA	c.396G>A (p.W132X)	---
27	20 m (F)	Abnormal NBS	NA	NA	c.136C>T (p.P46S)	---
28	3 y (F)	Abnormal NBS	NA	NA	---	---
29	5 m (F)	Abnormal NBS	NA	NA	c.1400C>G (p.S467C)	c.51C>G (p.F17L)
30	15 m (F)	Abnormal NBS	12	7	***c.287G>C (p.G96A)***	---
31	1 y (M)	Abnormal NBS	5	2	c.1319C>T(p.T440M)	c.1195C>T (p.R399W)
32	5 w (F)	Abnormal NBS	8	6	c.136C>T (p.P46S)	***c.688T>C (p.F230L)***
33	3 w (M)	Abnormal NBS	NA	NA	c.505C>T (p.R169W)	c.760C>T (p.R254X)
34	2 m (F)	Abnormal NBS	NA	NA	---	---
35	10 m (F)	Abnormal NBS	NA	NA	---	---
36	2 m (F)	Abnormal NBS	NA	NA	---	---
37	1 y (M)	Abnormal NBS	NA	NA	---	---
38	6 m (F)	Abnormal NBS	NA	NA	c.1345T>G (p.Y449D)	***c.1072T>A (p.Y358N)***
39	5 w (M)	Abnormal NBS	NA	NA	---	---
40	5 m (M)	Abnormal NBS	NA	NA	c.136C>T (p.P46S)	c.424G>T (p.A142S)
41	2 y (F)	Abnormal NBS	NA	NA	---	---

Subject #	Age (Gender)	Clinical presentation	Plasma carnitine		*SLC22A5* gene sequencing	
			T	F	Allele 1	Allele 2
42	3 m (F)	Abnormal NBS	NA	NA	c.424G>T(p.A142S)	***c.224G>C (p.R75P)***
43	5 w (M)	Abnormal NBS	NA	NA	---	---
44	8 m (F)	Abnormal NBS	NA	NA	---	---
45	4 w (F)	Abnormal NBS	NA	NA	c.1319C>T (p.T440M)	---
46	3.5 y (F)	Abnormal NBS	NA	NA	c.136C>T (p.P46S)	---
47	4 m (F)	Abnormal NBS	NA	NA	---	---
48	6 w (F)	Abnormal NBS	9	6	***c.557T>C (p.L186P)***	---
49	2 m (M)	Abnormal NBS	NA	NA	c.95A>G (p.N32S)	***c.529A>G (p.M177V)***
50	5 m (M)	Abnormal NBS	NA	NA	c.695C>T (p.T232M)	***c.680G>A (p.R227H)***
51	8 m (M)	Abnormal NBS	NA	NA	---	---
52	6 m (M)	Abnormal NBS	5	1	***c.791C>G (p.T264R)***	***c.791C>G (p.T264R)***
53	1 y (M)	Abnormal NBS	NA	NA	c.695C>T (p.T232M)	---
54	20 m (M)	Abnormal NBS	18	17	c.95A>G (p.N32S)	c.641C>T (p.A214V)
55	7 m (M)	Abnormal NBS	NA	NA	c.424G>T (p.A142S)	---
56	2 m (M)	Abnormal NBS	NA	NA	c.844C>T (p.R282X)	---
57	5 m (F)	Abnormal NBS	NA	NA	c.1193C>T (p.P398L)	---
58	5 m (M)	Abnormal NBS	NA	NA	c.43G>T (p.G15W)	c.43G>T (p.G15W)
59	15 m (F)	Abnormal NBS	10	7	c.136C>T (p.P46S)	c.695C>T (p.T232M)
60	4 w (M)	Abnormal NBS	NA	NA	---	---
61	2 m (F)	Abnormal NBS	NA	NA	c.136C>T (p.P46S)	---
62	2 m (F)	Abnormal NBS	NA	NA	c.136C>T (p.P46S)	---
63	2 m (M)	Abnormal NBS	NA	NA	---	---
64	5 m (F)	Abnormal NBS	NA	NA	c.136C>T (p.P46S)	---
65	1 m (F)	Abnormal NBS	NA	3	***c.196A>C (p.T66P)***	---
66	7 m (M)	Abnormal NBS	NA	NA	c.1304-delG (p.G435AfsX24)	
67	2 m (F)	Abnormal NBS	NA	NA	---	---
68	5 w (F)	Abnormal NBS	NA	NA	---	---
69	2 m (F)	Abnormal NBS	NA	NA	c.424G>T (p.A142S)	c.845G>A (p.R282Q)
70	6 w (F)	Abnormal NBS	NA	NA	---	---
71	35 y (F)	Mother (fasting intolerance)	7	6	***c.718G>A (p.A240T)***	---
72	26 y (F)	Mother (asymptomatic)	NA	NA	c.136C>T (p.P46S)	c.136C>T (p.P46S)
73	31 y (F)	Mother (asymptomatic)	NA	NA	***c.1327T>G (p.F443V)***	
74	28 y (F)	Mother (asymptomatic)	18	16	---	---

Subject #	Age (Gender)	Clinical presentation	Plasma carnitine		*SLC22A5* gene sequencing	
			T	F	Allele 1	Allele 2
75	31 y (F)	Mother (asymptomatic)	NA	NA	c.844C>T (p.R282X)	---
76	33 y (F)	Mother (asymptomatic)	NA	NA	c.43G>T (p.G15W)	c.43G>T (p.G15W)
77	25 y (F)	Mother (asymptomatic)	4	NA	c.424G>T (p.A142S)	---
78	31 y (F)	Mother (asymptomatic)	NA	NA	---	---
79	38 y (F)	Mother (asymptomatic)	5	4	c.1400 (p.S467C)	c.1400 (p.S467C)
80	24 y (F)	Mother (asymptomatic)	NA	NA	c.1345T>G (p.Y449D)	---
81	38 y (F)	Mother (fatigability during pregnancy)	5	4	c.136C>T (p.P46S)	c.695C>T (p.T232M)
82	30 y (F)	Mother (asymptomatic)	6	4	***c.1064C>T (p.S355L)***	c.51C>G (p.F17L)
83	27 y (F)	Mother (asymptomatic)	NA	NA	c.1319C>T (p.T440M)	***c.769C>T (p.R257W)***
84	29 y (F)	Mother (asymptomatic)	NA	NA	---	---
85	25 y (F)	Mother (asymptomatic)	NA	NA	c.51C>G (p.F17L)	c.51C>G (p.F17L)
86	25 y (F)	Mother (asymptomatic)	NA	NA	c.424G>T (p.A142S)	---
87	34 y (F)	Mother (asymptomatic)	NA	NA	c.43G>T (p.G15W)	c.43G>T (p.G15W)
88	38 y (F)	Mother (asymptomatic)	NA	NA	***c.368T>G (p.V123G)***	---
89	34 y (F)	Mother (asymptomatic)	NA	NA	---	---
90	24 y (F)	Mother (asymptomatic)	6	5	***c.955C>T (p.Q319X)***	---
91	43 y (F)	Mother (asymptomatic)	NA	NA	c.424G>T (p.A142S)	c.695C>T (p.T232M)

* The infants with abnormal NBS are almost always asymptomatic at the time of evaluation.

** Normal levels for total plasma carnitine: 25-70 μmol/L. Normal levels for free plasma carnitine: 20-50 μmol/L. T: total plasma carnitine; F: free plasma carnitine; M: male; F: female; y: year; m: month; w: week; NBS: newborn screen; NA: not available, ---: no mutations found; Bold and italic: novel changes in this study; gray: unclassified missense variants.

*** Nucleotide numbering reflects cDNA numbering with c.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.

Supp. Table S2. ***OCTN2*** **sequencing results for subjects not ascertained through NBS**

Subject #	Age (Gender)	Clinical presentation	Plasma carnitine		*SLC22A5* gene sequencing	
			T	F	Allele 1	Allele 2
92	12 y (M)	NA	NA	NA	c.396G>A (p.W132X)	---
93	8 y (M)	DD, hypotonia	NA	NA	---	---
94	8 y (M)	NA	NA	NA	---	---
95	2 y (F)	DD, hypotonia, ataxia, lactic acidosis	NA	NA	---	---
96	8 y (F)	Myopathy, short stature, arrhythmia	NA	NA	---	---
97	2 y (F)	DD, seizures, microcephaly, FTT, myopathy, lactic acidosis	NA	NA	---	---
98	2 y (M)	DD, seizures, myopathy, arrhythmia	NA	NA	---	---
99	24 y (F)	Exercise intolerance, easy fatigability, headache	NA	NA	---	---
100	39 y (M)	NA	NA	NA	---	---
101	5 m (M)	Cardiomyopathy	NA	NA	---	---
102	4 y (M)	Hypotonia	13	NA	---	---
103	8 y (F)	NA	NA	NA	---	---
104	30 y (F)	Easy fatigability	NA	NA	***c.1304-1313del10 (p.G435EfsX20)***	c.695C>T(p.T232M)
105	1.5 y (F)	NA	10	9	c.505C>T (p.R169W)	***c.1520T>C (p.L507S)***
106	25 y (M)	NA, father of case 14	14	12	c.136C>T (p.P46S)	***c.1520T>C (p.L507S)***
107	17 y (F)	Myopathy, FTT, headache,	NA	NA	---	---
108	12 y (F)	NA	NA	NA	---	---
109	6 y (M)	DD, FTT, myopathy	NA	NA	---	---
110	5 y (M)	SIDS-like episode	NA	NA	***c.34G>A (p.G12S)***	---
111	39 y (F)	Easy fatigability	NA	NA	---	---
112a	8 y (F)	Cardiomyopathy	<1	NA	c.806delT (p.L269HfsX27)	c.1319C>T (p.T440M)
112b	12 y (F)	Headache, FH	36	28	c.806delT (p.L269HfsX27)	---
113	7 y (M)	DD, ataxia, lactic acidosis	NA	NA	---	---
114	8 y (F)	Hypoglycemia, seizure	NA	NA	---	---
115	27 y (F)	NA	NA	NA	---	---

Subject #	Age (Gender)	Clinical presentation	Plasma carnitine		*SLC22A5* gene sequencing	
			T	F	Allele 1	Allele 2
116	4 y (F)	DD, seizure	5	NA	---	---
117	17 y (F)	Seizure, muscle weakness, spastic paraplegia	9	NA	---	---
118	12 y (F)	DD, seizures	NA	NA	---	---
119	2 y (M)	DD, FTT, myopathy	NA	NA	***c.1645C>T (p.P549S)***	---
120	7 y (M)	DD, hypotonia	NA	NA	---	---
121	3 y (M)	Hypoglycemia	NA	NA	---	---
122	19 y (M)	Myopathy	NA	NA	---	---
123	18 y (F)	Asymptomatic, FH	17	12	---	---
124	5 y (M)	DD, hypotonia	NA	NA	c.136C>T (p.P46S)	---
125	15 y (F)	NA	NA	NA	c.1195C>T (p.R399W)	---
126	31 y (F)	NA	NA	NA	---	---
127	14 y (M)	Cardiomyopathy	NA	NA	***c.350G>A (p.W117X)***	***c.573delG (p.N192IfsX12)***
128	6 y (M)	NA	NA	NA	---	---
129	19 y (F)	Cardiomyopathy, myopathy	NA	NA	***c.364G>T (p.D122Y)***	---
130	6 y (M)	Hypotonia, hypoglycemia	NA	NA	---	---
130	27 y (F)	NA	NA	NA	---	---
132	7 y (F)	Hypoglycemia	NA	NA	---	---
133	9 y (M)	Cardiomyopathy	13	5	---	---
134	5 y (F)	DD, autism, liver dysfunction	3	2	---	---
135	4 m (M)	Cardiomyopathy	NA	NA	***c.934A>G (p.I312V)***	---
136	30 y (F)	Exercise intolerance, diabetes	3	2	c.136C>T (p.P46S)	c.1193C>T (p.P398L)
137	28 y (F)	Easy fatigabiulity	0	NA	---	---
138	2 y (F)	Hypoglycemia	NA	NA	---	---
139	6 y (M)	Hypoglycemia	NA	NA	---	---
140	8 y (F)	DD, FTT, anemia	NA	NA	c.506G>A (p.R169Q)	c.506G>A (p.R169Q)
141	3 y (F)	Hypoglycemia	NA	NA	---	---
	5 y (F)	Hypoglycemia	NA	NA	---	---
142	35 y (F)	Recurrent episodes of hyperammonemia	16	7	---	---
143	28 y (F)	NA	NA	NA	---	---

* Normal levels for total plasma carnitine: 25-70 μmol/L. Normal levels for free plasma carnitine: 20-50 μmol/L. T: total plasma carnitine. F: free plasma carnitine. M: male. F: female. y: year. m: month. w: week. DD: Developmental delay. FTT:

failure-to-thrive. FH: family history of carnitine deficiency. NA: not available. ---: no mutations found. Bold and italic: novel changes in this study. gray: unclassified missense variants.

** Fifty-four individuals in 53 unrelated families and subject 105 and 106 were counted as 2 families since they carried 2 mutations with different genotypes.

***Nucleotide numbering reflects cDNA numbering with c.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.