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RESEARCH REPORT

Identification of Mutations and Evaluation of Cardiomyopathy in Turkish Patients with Primary Carnitine Deficiency

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Abstract Primary systemic carnitine deficiency (SCD) is an autosomal recessive disorder caused by defective cellular carnitine transport. Patients usually present with predominant metabolic or cardiac manifestations. SCD is caused by mutations in the organic cation/carnitine transporter *OCTN2* (SLC22A5) gene. Mutation analysis of *SLC22A5* gene was carried out in eight Turkish patients from six families. Six patients presented with signs and symptoms of heart failure, cardiomyopathy, and low plasma carnitine levels, five of them with concurrent anemia. A patient with dilated cardiomyopathy had also facial dysmorphia, microcephaly, and developmental delay. Tandem MS analyses in siblings of the patients revealed two more cases with low plasma carnitine levels. SCD diagnosis was confirmed in these two cases by mutation screening. These two cases were asymptomatic but echocardiography revealed left ventricular dilatation in one of them. Carnitine treatment was started before the systemic signs and symptoms developed in these patients. Mean value of serum carnitine levels of the patients was 2.63 ± 1.92 μmol/L at the time of diagnosis. After 1 year of treatment, carnitine values increased to 16.62 ± 5.11 ($p < 0.001$) and all responded to carnitine supplementation clinically. Mutation screening of the *OCTN2* gene study in the patients revealed two novel (p.G411V, p.G152R), and four previously identified mutations (p.R254X, p.R282X, p.R289X, p.T337Pfs12X). Early recognition and carnitine supplementation can be lifesaving in this inborn error of fatty acid oxidation.

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Abbreviations

BNP	Brain natriuretic peptide
DC	Dilated cardiomyopathy
EF	Ejection fraction
HC	Hypertrophic cardiomyopathy
OCTN2	Organic cation/carnitine transporter
SCD	Primary systemic carnitine deficiency
SLC22A5	Solute carrier family 22

Introduction

Primary systemic carnitine deficiency (SCD; MIM# 212140) is a potentially lethal, autosomal recessive disorder characterized by progressive cardiomyopathy, skeletal myopathy, hypoketotic hypoglycemia, and hyperammonemia

(Karpati et al. 1975; Mayatepek et al. 2000; Treem et al. 1988; Roe and Coates 1995). Although most of the fatty acid oxidation disorders affect heart and skeletal muscles and liver, cardiac failure is seen as the major presenting manifestation only in carnitine transporter deficiency (Stanley et al. 2006). The disease frequency is ranging from 1:40,000 to 1:120,000 newborns in different parts of the world and is possibly the second most frequent disorder of fatty oxidation after medium chain acyl CoA dehydrogenase deficiency (Koizumi et al. 1999; Wilcken et al. 2001, 2003). Studies in cultured fibroblasts from affected patients established that the primary defect in SCD involves the sodium-dependent high-affinity transporter situated in the plasmalemmal membrane (Tein et al. 1996; Treem et al. 1988). The same transporter is also involved in the renal reabsorption of carnitine, thus explaining the excessive renal waste of carnitine in SCD patients.

Primary carnitine deficiency is caused by mutations in *OCTN2* gene (SLC22A5), which encodes a plasma membrane carnitine transporter. *OCTN2*, transfers carnitine (3-hydroxy-4-trimethylaminobutyric acid) across cell membrane as Na+ dependent and other organic cations such as tetraethylammonium (TEA) as Na+− independent (Ohashi et al. 2001; Scaglia and Longo 1999; Scaglia et al. 1998, 1999). *OCTN2* gene is located on chromosome 5q31 with ten exons encoding a 557-amino acid transmembrane protein consisting of 12 transmembrane domains and one ATP-binding domain and predicted molecular mass of 63 kDa (Saito et al. 2002; Wu et al. 1999). More than 90 mutations have been identified up to date (Amat di San Filippo C et al. 2006a, b, 2008; Burwinkel et al. 1999; Cederbaum et al. 2002; Dobrowolski et al. 2005; Koizumi et al. 1999; Lamhonwah et al. 2002; Li et al. 2010; Makhseed et al. 2004; Mayatepek et al. 2000; Nezu et al. 1999; Rahbeeni et al. 2002; Spiekerkoetter et al. 2003; Tang et al. 1999; Vaz et al. 1999; Wang et al. 1999, 2000a, b, 2001).

In patients with SCD, the key to the diagnosis is the measurement of plasma carnitine levels that are extremely decreased (free carnitine <5 μM, controls 25–50 μM), and low renal uptake of carnitine (Cano et al. 2008; Scaglia et al. 1998; Treem et al. 1988). Diagnosis can be confirmed by demonstrating reduced carnitine transport in skin fibroblasts from the patient or by mutation analyses of the *SLC22A5* gene (Cano et al. 2008).

Since oral L-carnitine supplementation makes a significant improvement in clinical symptoms in patients, early diagnosis is of utmost importance. Therefore, molecular genetic analyses are essential and useful diagnostic tool for patients with asymptomatic SCD.

This study discusses the clinical and molecular features of patients who were being followed with a diagnosis of SCD.

Materials and Methods

Six patients presented with signs and symptoms of heart failure, cardiomyopathy and low plasma carnitine levels, and two siblings of the patients were enrolled in the study. The research protocol for genetic analysis was approved by the Hacettepe University, Ethical Board. Informed consent was obtained from all patients or their parents prior to genetic analyses. Genomic DNA was isolated from peripheral blood. Mutation screening of ten exons and their flanking intronic sequences of the *OCTN2* gene was performed in eight Turkish patients with SCD. PCRs were performed for 32 cycles, with each cycle consisting of denaturation at 95 °C for 4 min, annealing at 56–60 °C for 30 s, and extension at 72 °C for 30 s and then a final extension at 72 °C for 5 min. PCR products were purified with Qiagen MinElute 96 UF PCR purification plates. Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing kit (version 3.1) and analyzed on an ABI3130 automated DNA sequencer (Applied Biosystems, CA). A novel nucleotide changes resulting in missense changes were also analyzed in 100 control chromosomes and computer-based algorithms, PolyPhen (http://genetics.bwh.harvard.edu/pph/) was used for the prediction of the pathogenicity of novel missense variants.

Results

Mutation screening of the *SLC22A5* gene was performed in eight Turkish patients from six unrelated families. Six patients presented with symptoms of heart failure, cardiomyopathy by echocardiography, and low plasma carnitine levels, five of them were diagnosed with concurrent anemia. A patient (patient 4) with dilated cardiomyopathy had also facial dysmorphia, microcephaly, and developmental delay. Tandem MS analyses in siblings of the patients revealed two more patients with a low plasma carnitine levels and diagnosis was confirmed in these patients by mutation screening (patient 6, 8). These two patients were asymptomatic but echocardiography revealed left ventricular dilatation in one of them (patient 6). Treatment was started before the systemic signs and symptoms developed in these patients. We did not see early onset hypoketotic hypoglycemic encephalopathy in our cases. All of the affected patients had later age of presentation with progressive cardiomyopathy. The median age of the patients was 91.5 (57–145) months. Median age at onset was 30 (1–96) months whereas age at diagnosis was 71 (9–125) months. M/F ratio was 3/5 and all had consanguinity. Six patients had dilated cardiomyopathy, one had hypertrophic cardiomyopathy, and one was normal. In five patients, brain natriuretic peptide (BNP) levels were

higher than 100 pg/ml and in six patients EF was detected lower than 65% before carnitine treatment. Mean value of serum carnitine levels of the patients was 2.63 ± 1.92 μmol/L (N: 10–60) at the time of diagnosis. After 1 year of carnitine treatment, carnitine plasma values increased to 16.62 ± 5.11 (N: 10–60) ($p < 0.001$) and all responded to carnitine supplementation clinically. In four of the cases, the urinary carnitine levels were found to be raised. With carnitine supplementation, six of the cases were found to have an increase in the urinary carnitine levels (Table 1).

Three different types of pathogenic mutations (two missense, three nonsense, and one deletion) were detected in *OCTN2* gene. Mutation analysis has harbored for two novel mutations including p.G411V, p.G152R and four known mutations p.R254X, p.R282X, p.R289X, p.T337Pfs12X (Table 1). Two novel missense mutations (p.G411V, p.G152R) were screened in 100 control chromosomes and not detected. The locations of the five non-synonymous variants in the predicted secondary structure of OCTN2 protein are shown in Fig. 1. Carnitine transporter activity was measured only in one case and carnitine uptake: 0.0 (controls: 1.05 ± 0.27) was found (patient 3).

Discussion

Primary carnitine deficiency is caused by defective activity of the organic cation/carnitine transporter *OCTN2*, resulting in urinary carnitine wasting, low serum carnitine levels, and decreased intracellular carnitine accumulation (Scaglia et al. 1998; Tamai et al. 1998; Wu et al. 1998). Carnitine is essential for the transfer of long-chain fatty acids from the cytosol to mitochondria for subsequent β-oxidation and lack of it impairs the ability to use fat as fuel during periods of fasting or stress. This can result in an acute metabolic decompensation, most often early in life, with hepatic encephalopathy, hypoketotic hypoglycemia, Reye syndrome, and sudden infant death, or in a more insidious presentation, which may be very early in childhood but is more often of later onset, with cardiomyopathy (Scaglia and Longo 1999; Longo et al. 2006).

Primary carnitine deficiency impairs the accumulation of carnitine within organs and tissues (Longo et al. 2006). In the heart, carnitine is essential for normal fatty acid β-oxidation and even partial deficiency could lead to organ dysfunction (Koizumi et al. 1999). In this study, six patients with the symptoms of heart failure, five had dilated cardiomyopathy, whereas one had hypertrophic cardiomyopathy. Our study showed that the incidence of dilated cardiomyopathy was higher than hypertrophic cardiomyopathy as has been indicated in the literature

Table 1 Clinical, biochemical, and molecular analysis of the patients with *OCTN2* gene defect

Patient no	Age (months)/ sex	Cardiomyopathy type	Age at onset/age at diagnosis (months)	Hemoglobin levels (before and after treatment) (gr/dl)	Plasma free carnitine levels (before and after treatment) (μmol/L)	Urine carnitine levels (before and after treatment) (μmol/l)	BNP levels (before and after treatment) (pg/ml)	EF % (before and after treatment)	[a]Nucleotide change	Protein effect	Consanguinity	Mental retardation (mild)
1	69/F	DC	54/59	7,4/11,7	1,36/10,17	941/535	986/50	37/69	c.1009delA	p.T337Pfs12X	+	–
2	57/M	HC	1/9	10,3/12,2	3,96/9,26	400/726	–/–	49/79	**c.1232G > T**	**p.G411V**	+	+
3	77/F	DC	6/10	10,9/13,8	1,19/19,89	–/849	175/33.5	46/85	c.844C > T	p.R282X	+	–
4	130/M	DC	15/119	11/14.6	2,22/24,81	223/223	134/60	34/72	c.865C > T	p.R289X	+	+
[b]5	145/F	DC	4/124	5,6/10,9	1,37/15,74	614/2820	340/12,6	22/67	**c.454G > C**	**p.G152R**	+	–
[b]6	101/F	DC	75/75	9,8/10,4	2,75/19,12	190/735	21,7/35	63/63	**c.454G > C**	**p.G152R**	+	–
[c]7	104/F	DC	24/89	10,8/12,7	1,45/16	31,7/1386	986/13,5	37/71	c.760C > T	p.R254X	+	–
[c]8	82/M	N	66/67	–/–	6,76/17,94	24,6/94	28,3/90	76/76	c.760C > T	p.R254X	+	+

DC dilated cardiomyopathy, *HC* hypertrophic cardiomyopathy, *N* normal, *BNP* brain natriuretic peptide, *EF%* ejection fraction

[a] All patients are homozygous for the detected mutations

[b,c] Sibling

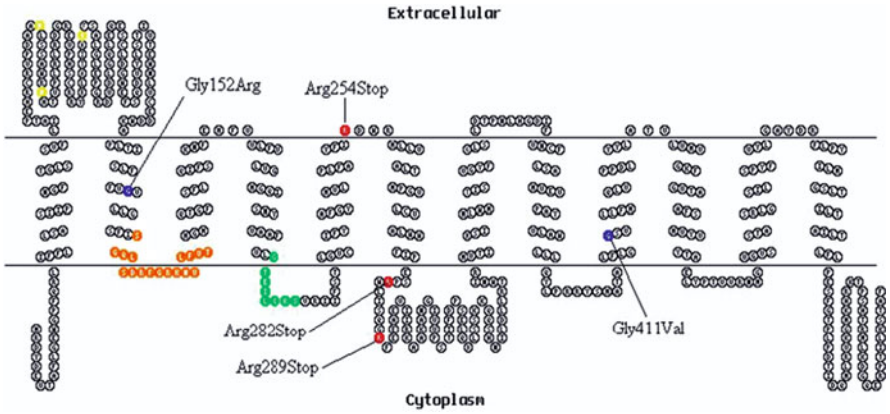

Fig. 1 Location of the five nonsynonymous variants in the predicted secondary structure of OCTN2 protein. The transmembrane topology diagram was rendered using TOPO2 transmembrane protein display software (http://www.sacs.ucsf.edu/TOPO/topo.html). Novel amino-acid substitutions are shown as *blue circles*, previously reported stop mutations that were also found in our patients are shown as *red circles*. Putative N-glycosylation sites are shown in *yellow*, ATP-binding motif is circled in *orange* and "sugar transporter sequence signature" is shown as *green circles*

(Longo et al. 2006; Wang et al. 2000a, b). In the literature, no correlation between the types of mutation and cardiomyopathy was found. In our case with a diagnosis of hypertrophic cardiomyopathy, the clinical findings were observed earlier (patient 2). A new homozygous mutation of c.1232G > T was identified in this patient. As in other previous studies, no genotype–phenotype relationship was observed. (Garavaglia et al. 1991; Lamhonwah et al. 2002; Li et al. 2010; Longo et al. 2006; Stanley et al. 1991; Wang et al. 2000a, b, 2001). Even siblings with the same mutation have different ages of onset and different progressions of disease pointing to the presence of clinical heterogeneity (Table 1). It was observed that the sibling with minor clinical symptoms had higher plasma levels of carnitine. This therefore suggests that the wide variability in phenotypic expression in carnitine transporter defect is most likely related to exogenous stressors that exacerbate the carnitine deficiency. These could include decreased intake due to dietary carnitine deficiency (vegetarian diets), drugs that increase the elimination of carnitine (valproic acid, pivalic acid) or inhibitors of carnitine transport (verapamil, pyrilamine, β-lactam antibiotics), and conditions such as fasting or infection, which would increase the demands on carnitine-dependent fatty acid oxidation (Holme et al. 1989; Lamhonwah et al. 2002; Li et al. 2010; Spiekerkoetter et al. 2003; Tein et al. 1993; Toh et al. 2010). Lack of genotype/phenotype correlation could also be influenced by polygenic factors.

Since newborn screening with tandem mass spectrometry is not a common practise in our country, the cases are usually diagnosed late. The median (min–max) age of disease onset findings was 30 (1–96) months, and age at diagnosis is 71 (9–125) months. The cases presented with cardinal symptoms of malaise, easy fatigability, and anorexia along with findings of heart failure and anemia. Physical examination revealed tachycardia, tachypnea, and palor. Telecardiography revealed increased cardiothoracic ratio and by echocardiography five dilated and one hypertrophic type of cardiomyopathy were detected. The patient's siblings were screened by tandem mass spectrometry revealed two asymptomatic cases and echocardiography revealed one case of cardiomyopathy (patient 6). The echocardiography of other case was normal (patient 8). This case's serum carnitine level was higher than other cases (6.76 μmol/L). Identification of mutations in siblings is critical because of the progressive and lethal nature of this disorder and the high incidence of sudden unexpected infant deaths unless there is early diagnosis and prompt therapeutic intervention (Stanley et al. 1991; Tein et al. 1990). Free carnitine levels in the plasma before initiation of therapy were 2.63 ± 1.92 μmol/L (N: 10–60), after a year of therapy free carnitine levels in the plasma raised to 16.62 ± 5.11 (N: 10–60) ($p < 0.001$). The increase in free carnitine levels was slow and never reached the upper limit. Almost in all cases urinary carnitine levels increased and they increased furthermore with carnitine supplementation. Carnitine supplementation with a daily dosage of 100 mg/kg was started per oral along with cardiac inotropic and diuretics agents. During the follow-ups, carnitine supplementations were adjusted according to serum carnitine levels. Only in one case, a dosage of 300 mg/kg/day was given. Adverse reactions to therapy were seen only in one patient as a putrefied fish smell of the feces and body of the patient and carnitine dosage was lowered subsequently. No side effect was seen in other cases. All cases showed clinical improvement. BNP levels which reflect the degree of heart failure decreased and ejection fractions were increased. Also five cases had moderate to mild anemia

and after therapy hemoglobin levels returned to normal. Three cases had iron deficiency (patient 1,5,6) and one case had both iron and vitamin B_{12} deficiencies (patient 2), these cases were given necessary supplements. The other case had no underlying cause for his mild anemia (patient 4). Carnitine deficiency could be the cause for his anemia. After therapy all cases had increase in their hemoglobin levels. In the literature, anemia was noted in some cases of primary carnitine transporter deficiency (Cano et al. 2008; Komlósi et al. 2009; Lamhonwah et al. 2002; Melegh et al. 2004; Tein and Di Mauro 1992). Carnitine is known to have a role in red blood cell metabolism: it stabilizes the cellular membrane and raises the red blood cell osmotic resistance (Evangeliou and Vlassopoulos 2003). Iron metabolism is also linked with carnitine because various authors showed low serum carnitine concentration in healthy children with iron deficiency anemia (Cemeroglu et al. 2001; Tanzer et al. 2001). Thus, anemia may be present in carnitine transporter deficiency and an iron deficiency may worsen anemia in this context. Moreover, iron deficiency may be a cause of secondary carnitine deficiency (Cano et al. 2008). One of the our cases had facial dysmorphia, microcephaly, mild mental retardation (patient 4). The chromosomal analysis of this case was normal and this case could not be related to another dysmorphic syndrome. The additional features observed in this patient may not be caused by this defect. Two other cases also had mild mental retardation (patient 2 and 8). No such relation between carnitine deficiency and mild mental retardation has been reported in the literature. Deficiency of carnitine and iron might have contributed to the development of mild mental retardation in these two patients. One of the cases identified by mass spectrometry screening of siblings revealed a history of recent story of muscle weakness such as difficulty in walking, climbing stairs (patient 8). Two other cases had similar myopathic findings (patient 2 and 5).

Using the PolyPhen program (http://genetics.bwh.harvard.edu/pph/), the potential functional effects of the novel p.G411V and p.G152R mutations were evaluated. The Gly411Val and Gly152Arg missense mutations were predicted to be possibly damaging according to position-specific independent count (PSIC) score differences derived from multiple alignment around substitution position. In this improper substitution, G411, in the nineth transmembrane domain (TMD9), the nonpolar glycine residue is replaced by a more nonpolar hydrophobic valine amino acid. In G152R residue in TMD2, the polar hydrophilic glycine residue is replaced by a nonpolar hydrophobic asparagine. Because of the localization of these two novel missense mutations in the transmembrane domain of the protein, they are functionally crucial. These variants may affect substrate specificity of OCTN2 protein.

Other known mutations namely p.R254X, p.R282X, p.R289X, p.T337Pfs12X detected by present study resulted in a premature insertion of a stop codon. The premature insertion of a stop codon can produce a truncated protein, result in unstable RNA, or cause exon skipping (Wang et al. 1999). These mutations would result in the production of a truncated membrane transporter with 5 (R254X), 6 (R282X, R289X, T337Pfs12X) transmembrane domains instead of the normal 12. The lack of several transmembrane domains may cause degraded or nonfunctional protein product. R254X, previously described mutation, was reported in Chinese and Saudi Arabians, whereas R282X was a common mutation in Caucasians (Burwinkel et al. 1999; Lamhonwah et al. 2004; Tang et al. 2002; Vaz et al. 1999; Wang et al. 1999). Other 1-bp deletion c.1009delA (T337Pfs12X) results in a frameshift with Thr337Pro leading to a predicted truncated protein of 347 aa length (Lamhonwah et al. 2002).

Conclusion

In conclusion, a total of six genetic mutations in the *OCTN2* gene were identified in this study, which of two were described as novel. Carnitine membrane transporter deficiency is one of the rare treatable etiologies of metabolic cardiomyopathies. It should be suspected and searched for by measuring the levels of free and total carnitine in plasma and urine from such patients. The clinical phenotype of OCTN2 deficiency may include anemia and mild mental retardation when other causes have been searched for and eliminated. Like biotinidase deficiency, primary carnitine deficiency is one of the metabolic disorders that is easy to treat disease with very good prognosis in early diagnosis.

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Synopsis

The article describes novel and previously detected mutations in *OCTN2* gene and their clinical consequences in patients with primary carnitine deficiency.

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