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RESEARCH ARTICLE

Functional Analysis of Mutations in the OCTN2 Transporter Causing Primary Carnitine Deficiency: Lack of Genotype–Phenotype Correlation

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Primary carnitine deficiency is an autosomal recessive disorder of fatty acid oxidation caused by defective carnitine transport. This disease is caused by mutations in the novel organic cation transporter OCTN2 (*SLC22A5* gene). The disease can present early in life with hypoketotic hypoglycemia or later in life with skeletal myopathy or cardiomyopathy. To determine whether the variation in phenotypic severity is due to mutations retaining residual function, we extended mutational analysis of OCTN2 to four additional European families with primary carnitine deficiency. Three patients were homozygous for novel missense mutations (R169W, G242V, A301D). The fourth patient was compound heterozygous for R169W and W351R substitutions. Stable expression of all the mutations in CHO cells confirmed that all mutations abolished carnitine transport, with the exception of the A301D mutation in which residual carnitine transport was 2–3% of the value measured in cells expressing the normal OCTN2 cDNA. Analysis of the patients characterized in molecular detail by our laboratory failed to indicate a correlation between residual carnitine transport and severity of the phenotype or age at presentation. Hum Mutat 16:401–407, 2000. © 2000 Wiley-Liss, Inc.

KEY WORDS: carnitine transport; carnitine deficiency; fatty acid oxidation; cardiomyopathy; SLC22A5; OCTN2

DATABASES:
SLC22A5 – OMIM:603377, 212140 (CDSP); GDB:9863277; GenBank:AB016625, AC004628, cDNA AB015050, AF057164; HGMD:SLC22A5

INTRODUCTION

Primary carnitine deficiency (MIM# 212140) is an autosomal recessive disorder of fatty acid oxidation caused by defective carnitine transport [Roe and Coates, 1995; Scaglia and Longo, 1999]. Carnitine is essential for the transfer of long-chain fatty acids from the cytosol to mitochondria for subsequent beta oxidation. The lack of carnitine impairs the ability to use fat as fuel during periods of fasting or stress. This can result in an acute metabolic presentation early in life with hypoketotic hypoglycemia, Reye syndrome, and sudden infant death or, in a more insidious presentation, later in life with skeletal or heart myopathy.

The gene for primary carnitine deficiency encodes a novel organic cation transporter OCTN2 (SLC22A5; MIM# 603377) [Wu et al., 1998; Tamai et al., 1998]. In patients with primary carnitine deficiency, mutations in the *SLC22A5* gene abolish or severely impair carnitine transport when expressed in heterologous cells [Nezu et al., 1999; Wang et al., 1999; Tang et al., 1999; Burwinkel et al., 1999; Vaz et al., 1999; Mayatepek et al., 2000; Wang et al., 2000]. Only one of the mutations iden-

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tified to date was found to retain residual carnitine transport activity [Wang et al., 2000] and it is unclear whether "leaky" mutations are associated with milder phenotypes.

In a few families, with presumably the same identical mutation in related individuals, both an acute metabolic and myopathic presentations have been described [Stanley et al., 1991], suggesting that environmental factors such as the supply of carnitine with the diet or infections and fasting, which increase the requirements for fatty acid oxidation, may play a major role in determining the timing of phenotypic expression. However, the majority of patients in whom a mutation has been identified had an acute metabolic presentation early in life [Tang et al., 1999; Wang et al., 1999; Nezu et al., 1999; Burwinkel et al., 1999] and only a few presented with cardiomyopathy. In addition, there is a paucity of "leaky" mutations reported to date.

Here we extend mutational analysis of OCTN2 to additional families and report four new missense mutations, one of which (A301D) retained some residual carnitine transport when expressed in Chinese hamster ovary cells. The presence of residual carnitine transport activity, however, did not result in a milder phenotype or late-onset disease.

MATERIALS AND METHODS

Patients

Patient 255 is an Italian boy who presented at three years of age with vomiting, hypoketotic hypoglycemia, and coma (patient two in Garavaglia et al., 1991]. His parents are first cousins. Two of his brothers died at 10 months of age with a similar illness and had liver steatosis and cardiac hypertrophy on autopsy.

Patient 430, an Italian boy, was diagnosed at 31 months of age after presenting with respiratory distress due to cardiomyopathy [patient one of Garavaglia et al., 1991]. His parents are unrelated.

Patient 669 is a Spanish boy who presented at three years nine months of age with heart failure due to cardiomyopathy [Briones et al., 1995]. The parents are unrelated.

Patient 1003 presented at five years of age with acute metabolic decompensation. His parents are unrelated.

All patients responded well to carnitine therapy with reversal of metabolic abnormalities and/or cardiac symptoms.

Cell Strains and Carnitine Transport

Fibroblasts from patients 255, 430, 669, and 1003 with primary carnitine deficiency were obtained by skin biopsy for diagnostic purposes [Garavaglia et al., 1991; Briones et al., 1995]. Fibroblasts were grown in Dulbecco-modified MEM supplemented with 15% fetal bovine serum. Chinese hamster ovary (CHO) cells were grown in Ham F12 medium supplemented with 6% fetal bovine serum.

Carnitine (0.5 μM) transport was measured at 37 C as described previously [Wang et al., 2000]. Nonsaturable carnitine transport was measured in the presence of 2 mM cold carnitine and was subtracted from total transport to obtain saturable transport. Values are reported as means ± SE of 3–6 independent determinations.

DNA Analysis and Molecular Techniques

Genomic DNA was extracted from fibroblasts by standard methods and amplified using PCR and primers flanking each of the 10 exons [Wang et al., 2000]. Mutations were confirmed by restriction analysis of independent PCR products.

The OCTN2 expression vector was generated by inserting the OCTN2 cDNA [Wu et al., 1998] in pcDNA3 as previously described [Wang et al., 1999]. Mutations were introduced by site-directed mutagenesis using the Quik Change™ system (Stratagene, La Jolla, CA) following the manufacturer's instructions. The final clones were sequenced to confirm the presence of the mutation and the absence of PCR artifacts. The clones were transfected into CHO cells using lipofectamine [Wang et al., 1999, 2000]. Cells were selected for 2 wk in 0.8 mg/ml of G418 and then used for the transport assay. Northern blot analysis and mRNA quantitation were performed on transfected CHO cells as previously described [Wang et al., 2000].

RESULTS

Mutations in the *SLC22A5* Gene

Carnitine transport was reduced to less than 5% of controls in fibroblasts from patients 255, 430, 669, and 1003 with primary carnitine deficiency (Fig. 1). DNA was isolated from fibroblast cultures and the 10 exons of the *SLC22A5* gene were sequenced. Patient 430 was homozygous for a 505C>T transition in exon 2 converting the codon for Arg 169 to Trp (R169W, Fig. 2A). This mutation abolished a MspI restriction site. Patient 1003 was heterozygous for this mutation. Since the family of patient 430 was not consanguineous, we tested whether he was truly homozygous for the R169W substitution or a compound heterozygote for this mutation and a deletion of the other allele. By restriction analysis, both his parents and

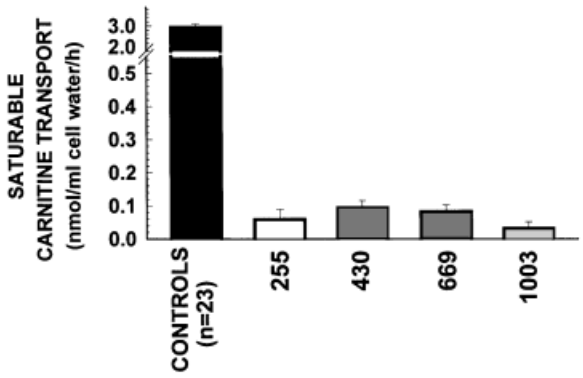

FIGURE 1. **Carnitine transport by fibroblasts obtained from patients with primary carnitine deficiency. Carnitine (0.5 μM) transport was measured for 4 hr at 37°C. Nonsaturable transport, measured in the presence of 2 mM cold carnitine, was subtracted from total transport to obtain saturable carnitine transport. Data are means± SD of 6 observations. Carnitine transport by the patients' cells was always significantly ($p<0.01$) different from that of the control group (note the broken scale on the Y axis).**

two siblings were heterozygous for the R169W mutation, confirming that patient 430 was homozygous for this mutation.

Patient 669 was homozygous for a 725G>T transversion in exon 4, converting the codon for Gly 242 to Val (G242V, Fig. 2B). The G242V mutation created a new AccI restriction site in exon 4. After digestion with AccI, the PCR-amplified exon 4 of patient 669 had bands of 105 and 143 bp, but missed the undigested 248 bp band seen in controls, consistent with homozygosity for the G242V mutation.

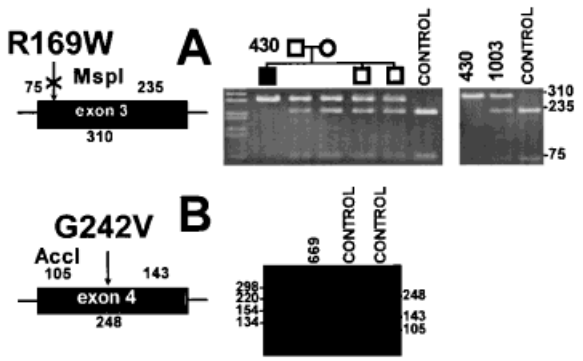

FIGURE 2. **R169W and G242V mutations in the *SLC22A5* gene in patients with primary carnitine deficiency. The R169W mutation (A) abolished a MspI restriction site, resulting in an undigested fragment of 310 bp. Patient 430 was homozygous and patient 1003 heterozygous for this mutation. Both parents and the two siblings of patient 430 were also heterozygous for this mutation. The G242V mutation created a novel AccI site (B), resulting in the formation of two novel DNA fragments of 143 and 105 bp. Patient 669 was homozygous for this mutation.**

Patient 255 was homozygous for a 902C>A transversion in exon 5, converting the codon for Ala 301 to Asp (A301D, Fig. 3A). This mutation abolished a Fnu4H1 restriction site. Fnu4H1 digestion of PCR-amplified exon 5 from patient 255 generated a single band of 256 bp, as compared to normal bands of 102 and 154 bp.

Patient 1003 was compound heterozygous for a 1051T>C transition in exon 6, causing a W351R substitution (Fig. 3B) and the 505C>T transition in exon 2 already presented for patient 430, and causing a R169W substitution (Fig. 2A). Analysis of intragenic polymorphisms indicated that the R169W mutation in patient 1003 occurred on the same allelic background of the mutations identified in patient 430 (not shown). The W351R substitution created a novel Fnu4H1 site in exon 6, generating additional bands of 72 bp and 40 bp (this latter not visible on the gel). Patient 1003 was heterozygous for the W351R mutation.

DNA from the parents of patients 669, 255, and 1003 was not available for analysis. Southern blot analysis of DNA from patients 669, 255, and 1003 failed to identify abnormal bands (not shown).

Expression of Mutant OCTN2 cDNAs in Mammalian Cells

To confirm their causative role, the missense mutations identified were created by site-directed mutagenesis in a pcDNA3-based expression vector and expressed in CHO cells (Fig. 4). Expression of the transfected cDNA was verified by Northern blot analysis. All the mutations identi-

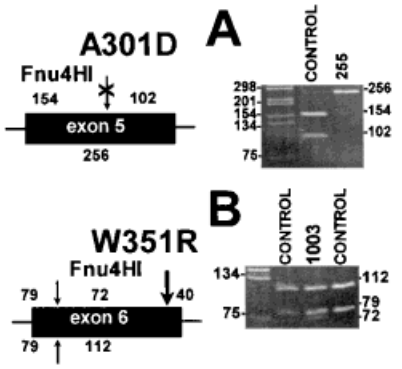

FIGURE 3. **A301D and W351R mutations in the *SLC22A5* gene in patients with primary carnitine deficiency. The A301D mutation abolished a Fnu4HI restriction site, causing the presence of an undigested fragment of 256 bp. Patient 255 was homozygous for this mutation. The W351R mutation created an additional Fnu4HI site, resulting in the appearance of a novel 72 bp band after restriction analysis. Patient 1003 was heterozygous for this mutation.**

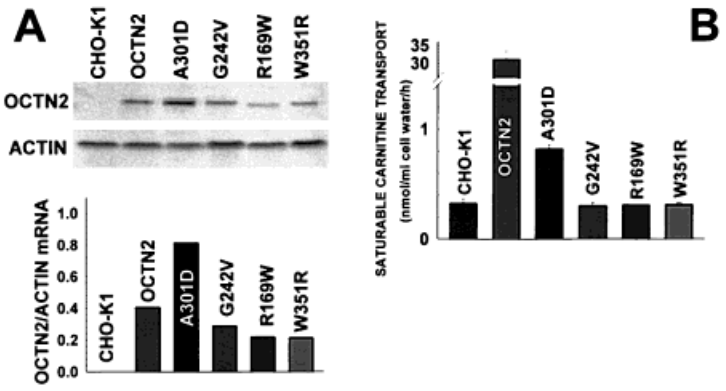

FIGURE 4. Expression of normal and mutant OCTN2 cDNA in CHO cells. CHO cells were transfected with the OCTN2 cDNA cloned in pcDNA3. After selection for resistance to G418 (0.8 mg/ml), expression of the *transgene* was verified by Northern blot analysis (Panel **A**) and quantified by CPM counting on an Instant Imager™ (Packard, Meriden, CT). Carnitine (0.5 μM) transport was measured for 1 hr and corrected for nonsaturable uptake (measured in the presence of 2 mM cold carnitine). (Panel **B**) Points are averages ± SD of triplicates.

fied markedly impaired carnitine transport when stably transfected into CHO cells. Analysis of multiple clones indicated that the R169W, G242V, and W351R mutations failed to increase carnitine transport above the levels measured in untransfected or mock-transfected (not shown) CHO cells. By contrast, the A301D mutation conserved residual carnitine transport activity which, in multiple clones, was about 2% of that of the normal OCTN2 cDNA.

Genotype–Phenotype Correlation

Tables 1 and 2 summarize the clinical presentation and mutational analysis of patients with primary carnitine deficiency whose mutations have been defined. All types of clinical presentations were observed in case of missense mutations (Table 1) or mutations resulting in the premature insertion of STOP codons (Table 2). The age at diagnosis of index cases was on average 2.7 ± 2.1 years (95% confidence interval: 1.5–3.9 years) in patients with at least one missense mutations and 3.9 ± 2.8 years (3.0–4.8 years for the 95% confidence interval) for patients whose mutations resulted in premature STOP codons. The two mutations retaining some residual carnitine transport activity (A301D and E452K) had two different types of presentation and did not result in any marked increase of the age at diagnosis.

DISCUSSION

Primary carnitine deficiency is an autosomal recessive disorder of fatty acid oxidation that can present at different ages and with involvement of different organs. Different types of presentations can be observed even within the same family. Here we report four novel missense mutations in the OCTN2 carnitine transporter (Figs. 2,3). All these mutations markedly impaired carnitine transport by the patients' fibroblasts (Fig. 1) and completely abolished carnitine transport when expressed in CHO cells, with the exception of A301D which had minimal, but significant, residual carnitine transport activity, at least when expressed in CHO cells (Fig. 4). These missense mutations affected different portions of the OCTN2 carnitine trans-

TABLE 1. Missense Mutations in Families With Primary Carnitine Deficiency

Patient	Diagnosis age (years)	Presentation weakness, W cardiac C, metabolic M	Consanguinity	Affected siblings	Mutations	Residual carnitine transport	Reference
430	2.5	C,W	–	+	R169W/R169W	0	Garavaglia et al. [1991]; this study
1003	5	M	–	–	R169W/W351R	0	This study
SGG	2	C	–	–	R169Q/R282X	?/0	Burwinkel et al. [1999]
Pt 1	1	C	?	–	Y211C/Y211C	?	Vaz et al. [1999]; Rodrigues Pereira et al. [1988]
Pt 2	1.7	C,M	–	–	Y211C/Y211C	?	Vaz et al. [1999]
669	3.9	C,W	–	–	G242V/G242V	0	Briones et al. [1995]; this study
	0.5	M	–	–	W283R/V446F	<2%	Mayatepek et al. [2000]
255	3	W,M	+	+	A301D/A301D	2	Garavaglia et al. [1991]; this study
Balt-1	7	C	+	–	E452K/E452K	3	Wang et al. [2000]
T1	0.5	M	–	+	P478L/W132X	<0.5%	Tang et al. [1999]

TABLE 2. Mutations Resulting in Premature STOP Codons in Families With Primary Carnitine Deficiency

Patient	Diagnosis age (years)	Presentation weakness, W cardiac C, metabolic M	Consanguinity	Affected siblings	Mutations	Residual carnitine transport	Reference
KR	5.9	C,W	–	+	1-176del 1-176del	0	Nezu et al. [1991];
AK	8	W,M	–	–	405insC/W132X	0	Nezu et al. [1999]
10665	2	M	–	–	R282X/R282X	0	Stanley et al. [1991]; Wang et al. [1999]
Pt 3, DKH	2	C,W	–	–	R282X/R282X	0	Vaz et al. [1999]; Burwinkel et al. [1999]
2996	0.5	M	–	–	Y401X/458X	0	Wang et al. [1999]; Scaglia et al. [1998]
TH	5	M	–	–	IVS8-1G>A IVS8-1G>A	0(?)	Nezu et al. [1999]

porter (Fig. 5). The R169W substitution affected a conserved residue in the glucose transporter (GLUT) signature motif of the carnitine transporter located in the intracellular loop between transmembrane domains 2 and 3 [Wu et al., 1998; Tamai et al., 1998]. The GLUT signature is conserved between members of the facilitative glucose transporter family and is present in two copies in symmetrical regions of GLUT1–GLUT5, suggesting early gene duplication [Mueckler, 1994]. This site may be a target of cAMP- and cGMP-dependent protein phosphorylation [Borson et al., 1996]. More importantly, this region is probably important for substrate-induced conformational change of the carrier during transport, since substitution of R92 in GLUT4 (the amino acid homologous to R169 in OCTN2) with Leu (R92L) decreases glucose transport, without affecting cytochalasin B or ATP-BMPA (a bulky ligand that binds to the extracellular surface of the transporter) binding [Schurman et al., 1997]. It has been proposed that the transient and alternating contact between the positive charges of Arg and the negative charges of Glu residues favors the progression of the substrate through the membrane by allowing the correct arrangement of the helices formed by the transmembrane domains [Schurman et al., 1997]. Substitution of R169 with additional amino acids may reveal whether the GLUT signature motif of OCTN2 functions in a manner similar to that of facilitative glucose transporters and defines the mechanism by which substitutions completely abolish activity.

This C->T transition occurred in a CpG region and it is not surprising that another patient has a different mutation (R169Q) in the same residue [Burwinkel et al., 1999]. The effect of this latter substitution on membrane transport has not yet been confirmed by expression studies [Burwinkel et al., 1999]. Two of our patients (430 and 1003) had the same R169W mutation (Fig. 2). In both cases, the mutation occurred on the same haplotypic background suggesting that, in our cases, it may represent the same ancestral allele.

The G242V and W351R mutations affect residues located in putative transmembrane domains 5 and 7, respectively (Fig. 5). In other transporters, mutations in transmembrane domains can have a severe effect on function, usually by impairing membrane insertion of the mature trans-

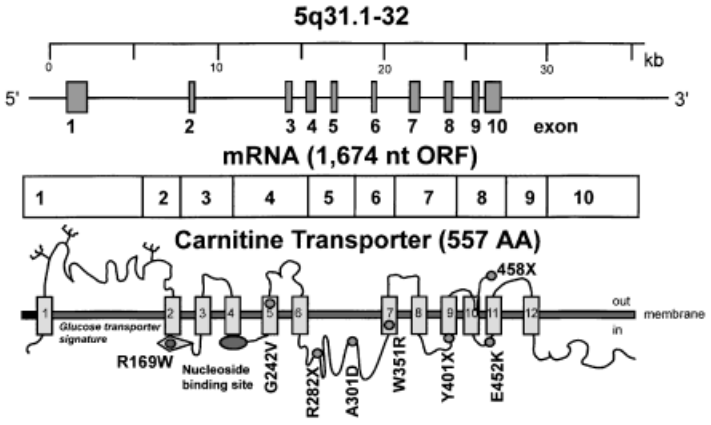

FIGURE 5. Mutations in the OCTN2 carnitine transporter in primary carnitine deficiency. The *SLC22A5* gene is composed by 10 exons spanning about 30 kb on 5q31.1-32. The open reading frame of the resulting mRNA is 1674 nucleotides. The encoded membrane protein is composed of 557 amino acids. Hydropathy analysis suggests that the transporter forms 12 transmembrane spanning domains (rectangles) with both the N- and C-termini facing the cytoplasm. Putative glycosylation sites are indicated by branching, the glucose transporter signature by a rhomboid, and the nucleoside binding site by an oval. Mutations identified in our patients with primary carnitine deficiency are indicated by circles.

porters [Martin et al., 1996]. Our finding of a complete lack of mediated carnitine transport by the mutant cDNA when expressed in CHO cells (Fig. 4) is compatible with this hypothesis. In other patients, the Y211C and P478L substitutions also affect transmembrane domains [Tang et al., 1999; Vaz et al., 1999]. While the Y211C mutation has not yet been expressed in heterologous cells, the P478L substitution impaired carnitine transport, but retained organic cation transport activity [Tang et al., 1999; Seth et al., 1999], suggesting that at least some of the P478L mutant transporters reach the plasma membrane.

The A301D mutations identified in patient 255 resemble, in many aspects, the E452K mutation previously described in patient Balt-1 with primary carnitine deficiency [Wang et al., 2000]. The A301D substitution affects an intracellular loop between transmembrane domains 6 and 7 (Fig. 5), while E452K modifies the intracellular loop between transmembrane domains 10 and 11 [Wang et al., 2000]. In contrast to the R169W, G242V, and W351R substitutions, the A301D-mutant OCTN2 retained small, but significant carnitine transport when expressed in CHO cells. Analysis of multiple clones indicated that cells expressing the A301D-mutant OCTN2 retained 1–3% of the normal carnitine transport activity of the wild type OCTN2. This residual activity is similar to that measured with E452K-mutant OCTN2 transporters [Wang et al., 2000]. Despite the similar in vitro results, patient 255 and Balt-1 presented very differently. Patient 255, homozygous for the A301D mutation, presented at three years of age with acute metabolic decompensation, while two of his brothers died at 10 months of age with a similar illness and pathologic evidence of abnormal fatty acid oxidation [Garavaglia et al., 1991]. By contrast, patient Balt-1, homozygous for the E452K mutation, had a slower course and presented at seven years of age with cardiomyopathy [Wang et al., 2000].

The cumulative analysis of the patients characterized in molecular details by others and us (Tables 1 and 2) indicates that the age at diagnosis (i.e. the survival before carnitine supplementation) ranged from 0.5 to eight years of age. There was no statistical difference in the age at diagnosis between the group of children with missense as compared to nonsense mutations. In addition, all types of presentation (metabolic, cardiomyopathic, and myopathic) were observed in the two groups. Homozygosity for the same null allele (R282X) was identified in two unrelated patients, one presenting with acute metabolic decompensation and one with cardiomyopathy [Wang et al., 1999; Burwinkel et al., 1999], indicating that the same primary genetic defects may be associated with different phenotypic expression. Here we find that two patients (255 and Balt-1) with similar levels of residual carnitine transport activity also had different timing and type of clinical presentation. These data support the fact that environmental factors, such as infections and the supply of carnitine in the diet, rather than the degree of impairment of carnitine transport, affect the time and type of phenotypic expression.

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