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Report

X-Linked Creatine-Transporter Gene (*SLC6A8*) Defect: A New Creatine-Deficiency Syndrome

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We report the first X-linked creatine-deficiency syndrome caused by a defective creatine transporter. The male index patient presented with developmental delay and hypotonia. Proton magnetic-resonance spectroscopy of his brain revealed absence of the creatine signal. However, creatine in urine and plasma was increased, and guanidinoacetate levels were normal. In three female relatives of the index patient, mild biochemical abnormalities and learning disabilities were present, to various extents. Fibroblasts from the index patient contained a hemizygous nonsense mutation in the gene *SLC6A8* and were defective in creatine uptake. The three female relatives were heterozygous for this mutation in *SLC6A8*, which has been mapped to Xq28.

Creatine and phosphocreatine play essential roles in the storage and transmission of phosphate-bound energy (Walker 1979; Wyss and Kaddurah-Daouk 2000). Humans maintain their creatine pool by biosynthesis and nutritional uptake. The enzymes involved in the biosynthesis of creatine are arginine:glycine amidinotransferase and guanidinoacetate methyltransferase (GAMT) (Walker 1979). Creatine is synthesized primarily in liver and also in kidney and pancreas. Cellular transport is of fundamental importance for creatine homeostasis in tissues void of creatine biosynthesis. Two creatine transporters have been identified that are members of solute-carrier family 6 (neurotransmitter transporters). The creatine-transporter gene, now called "*SLC6A8*" (MIM 300036), also known as "*CT1*" or "*CRTR*," has been mapped to Xq28 (Gregor et al. 1995). This gene is expressed in most tissues, with highest levels in skeletal muscle and kidney and somewhat lower levels in colon, brain, heart, testis, and prostate (Nash et al. 1994; Sora et al. 1994). The creatine-transporter gene *SLC6A10*, also known as "*CT2*," has been mapped to 16p11 and is expressed in testis only (Iyer et al. 1996; Xu et al. 1997). Recently, an inborn error of creatine biosynthesis was identified as a result of GAMT deficiency (Stöckler et al. 1996*a*, 1996*b*; Schulze et al. 1997; van der Knaap et al. 2000).

Our index patient, a white male child, was diagnosed with mild mental retardation at age 6 years and showed severe delay both in speech and in expressive-language function. Proton magnetic-resonance spectroscopy (MRS) of the brain revealed an almost complete absence of the creatine signal (Cecil et al. 2001), as is observed in patients with GAMT deficiency (Stöckler et al. 1996*a*, 1996*b*; Schulze et al. 1997; van der Knaap et al. 2000). However, guanidinoacetate (GUAC) in urine and plasma was normal (table 1). Thus, GAMT deficiency was ruled out. Creatine levels were increased in urine and plasma. Supplementation of creatine monohydrate, at 340 mg/kg body wt/d for 3 mo, did not result in either improved clinical condition or increased creatine signal in MRS of the brain (Cecil et al. 2001). The index patient is the only child of healthy parents, although both his mother and his maternal grandmother have a history of learning disability. The mother has two siblings, a healthy sister and a severely retarded brother; the mother's brother was not available for further study. Metabolic workup of the three female relatives of the index patient (his mother, his grandmother, and his aunt) showed normal GUAC in plasma and urine (table 1). In plasma, creatine levels were found to be normal for the maternal grand-

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Table 1

Metabolic Workup of Family with Creatine Deficiency

	CREATINE[a]		GUAC[a]	
SUBJECT(S)	Plasma (μM)	Urine (mM)	Plasma (μM)	Urine (mmol/mol creatinine)
Index patient	75	2.2	1.1	74
Mother	176[b]	>6[b]	1.4[b]	40[b]
Aunt	143	1.6	2.1	45
Grandmother	32	1.1	2.1	30
Controls				
Males	15–44[c]	<.35[c]		
Females	30–80[c]	<.70[c]		
Children			.7–1.4[d]	10–99[d]
Adults			.7–3.5[e]	

[a] Quantified by stable isotope–dilution GCMS, as described elsewhere (Struys et al. 1998); except for creatine measurements, $^2H_3$-creatine (CDN Isotopes) was used as the internal standard.
[b] During creatine treatment.
[c] From Kaplan and Pesce (1989).
[d] Age 0–10 years (4 females and 6 males) (Struys et al. 1998).
[e] Age 20–55 years (12 females and 13 males).

mother and increased for both the aunt and the mother. In urine, creatine levels were increased in all three female relatives. Urine and plasma of the mother were collected during creatine treatment. Compared with those of age-matched controls, MRS of the brains of both the mother and the maternal aunt demonstrated a reduced, but detectable, creatine signal.

The clinical presentation of the pedigree suggested an X-linked disorder (fig. 1*a*). Our hypothesis therefore is that this family suffers from a defect in the creatine-transporter gene *SLC6A8,* located at Xq28. The gene consists of 13 exons and spans ~8.4 kb (GenBank accession number Z66539) (Sandoval et al. 1996). The *SLC6A8* mRNA is ~3.9 kb, and its predicted open-reading frame encodes a protein of 635 amino acids, with a molecular mass of ~70 kD. Sequence analysis of amplified cDNA of *SLC6A8* from fibroblasts of the index patient revealed a hemizygous nonsense mutation. (Forward primer 5′-TTATTCCCTACGTCCTGATC and reverse primer 5′-GCTGGTGATGTGAGCTGAGT were used to amplify the translated region of the mRNA, which lacked the CG-rich exon 1, and separate primers were designed to amplify exon 1.) A 1539C→T transition in *SLC6A8* (GenBank accession number NM_005629) resulted in the substitution of an arginine codon by a termination codon (R514→X). Amplification and direct sequence analysis of exon 11 (by primers 5′-GTGGCCTGGGTGTACGGTAG and 5′-ATGAAGATGCCCTACGGACC) from genomic DNA from fibroblasts of the index patient confirmed this nonsense mutation (fig. 1*b*). All three female relatives of the index patient were heterozygous for this mutation, as determined in genomic DNA. Sequence analysis of cDNA from lymphoblasts of the mother showed, almost equally, expression of both the wild-type and the mutant allele, indicating stability of the nonsense allele; no expression of the mutant alleles was detected in fibroblasts of the three female carriers, which suggests X inactivation resulting in a mosaic expression of the mutant and wild-type alleles. In contrast to the grandmother and the mother of the index patient, the aunt of the index patient is asymptomatic. Skewed X inactivation may explain this phenotypic difference from the other two female carriers.

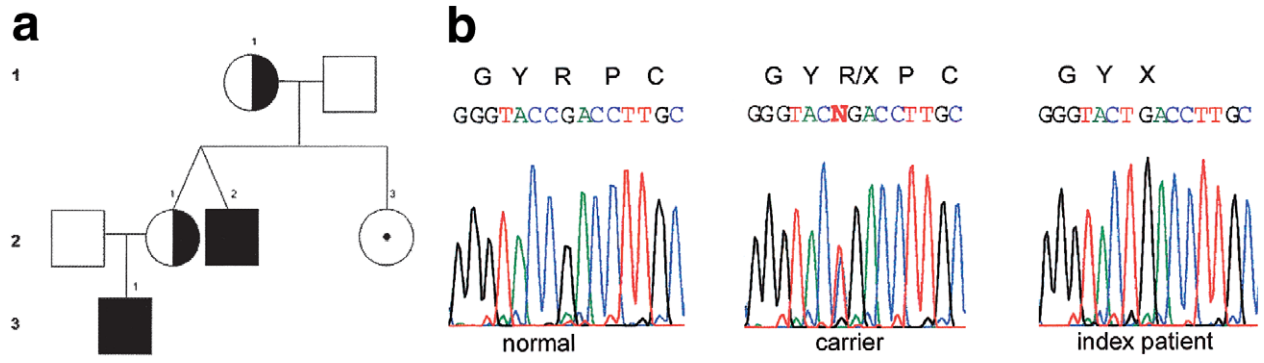

Figure 1 Identification of *SLC6A8* nonsense mutations in a white family with creatine-transporter deficiency. *a,* Pedigree. Affected individuals are indicated by completely blackened symbols, symptomatic carriers by half-blackened symbols, and the asymptomatic carrier by the dot within a circle. *b,* Sequence analysis of DNA of the normal control, the mother of the index patient (individual "2-1" in panel *a*), and the index patient (individual "3-1" in panel *a*). DNA was isolated from fibroblasts by DNAzol (GibcoBRL). PCR reactions were performed with Hotstar*Taq* (Qiagen), in a PE Applied Biosystems model 9700. PCR products were purified with PCR cleanup columns (Millipore). Subsequently, these purified PCR products were sequenced by capillary electrophoresis, on an ABI PRISM 310 with the Big Dye terminator-cycle sequencing kit (Perkin Elmer). Sequence analysis of part of exon 11—codons 512–516 (GenBank accession number NM_005629)—shows that the C→T transition, 15239C→T, results in a 514R→X replacement. The index patient (individual "3-1" in panel *a*) is hemizygous for this nonsense mutation, and the three females (individuals "1-1," "2-1," and "2-3" in panel *a*) are heterozygous for this nonsense mutation.

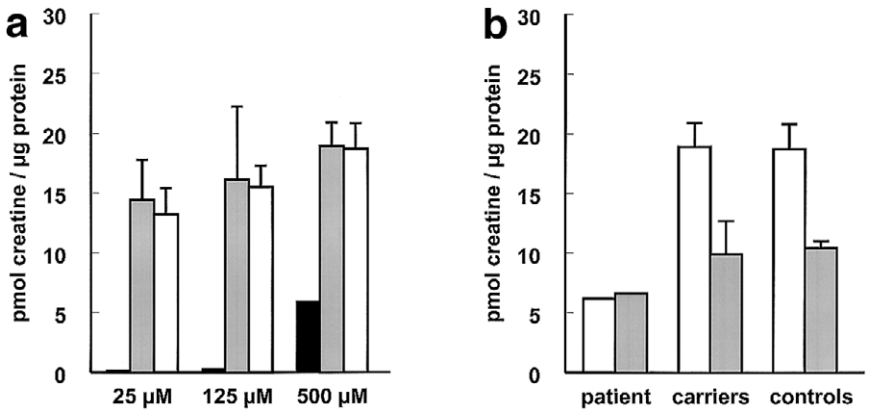

Figure 2 Creatine uptake in fibroblasts of the index patient, in fibroblasts of two carriers (expressing only the wild-type *SLC6A8*) and in cells of controls. *a,* Fibroblasts from the index patient (*black*), two carriers (*gray*), and two controls (*white*), cultured for 24 h in medium (HAM/F10 supplemented with 10% fetal bovine serum, penicillin, and streptavidin) that contains 25 μM creatine. Creatine was added to obtain final concentrations of 25, 125, and 500 μM, as indicated. *b,* Cells from the index patient, as well as cells from two carriers and from two controls, were incubated with 500 μM creatine for 24 h, in either the absence (*white*) or the presence (*gray*) of 500 μM 3-guanidinopropionate, an inhibitor of the creatine transporter. Prior to creatine measurement, the cells were washed twice with Hanks' balanced salt solution. Cell pellets were frozen until used. Protein concentrations were measured in 10% of the lysates, by the method used by Lowry. The lysates were used to measure creatine concentrations after the addition of stable isotope–labeled creatine as the internal standard. Each value represents the mean $\pm$ SD of duplicate incubations from the index patient, from two carriers, or from two controls.

The nonsense mutation predicts a truncated protein of 513 amino acids that lacks 122 amino acids of the C-terminus, including the putative transmembrane-spanning domains XI and XII of SLC6A8, which include 21 conserved amino acids (Nash et al. 1994). Domains IX–XI are thought to be responsible for substrate-specificity differences among the neurotransmitter-transporter subfamily, because these domains vary most among this subfamily of proteins (Nash et al. 1994). However, the nonsense mutation most likely results in an unstable and/or inappropriately folded protein that is completely inactive. To study creatine uptake in fibroblasts expressing the *SLC6A8*-null allele only, fibroblasts of the index patient were incubated for 24 h in the presence of different concentrations of creatine. Fibroblasts from a group of controls—comprising the mother and the grandmother of the index patient, who both express only the wild-type *SLC6A8* allele, and two female patients with an unrelated disease—were analyzed. Creatine uptake was measured in total cell lysates, by gas chromatography–mass spectrometry (GCMS) with stable isotope–labeled creatine as the internal standard. When cells from the index patient were incubated at a physiological-creatine concentration of 25 μM, creatine uptake was negligible (fig. 2*a*) (Wyss and Kaddurah-Daouk 2000). Even at 125 μM creatine, no uptake could be measured, confirming the absence of a functional creatine transporter in cells from the index patient. At higher creatine concentrations (500 μM), creatine uptake in the index patient's cells was approximately one-third of that in the control cells. This most likely represents either passive diffusion across the plasma membrane or, alternatively, transport of creatine via other neurotransmitter transporters; indeed, 24-h incubations with 500 μM creatine in the presence of 500 μM guanidinopropionate, an inhibitor of the creatine transporter, showed a twofold decrease in uptake in the cells of both carriers and controls but not in the cells of the index patient, supporting both assumptions (fig. 2*b*). No significant creatine-uptake difference was observed between cells of the carriers and those of the controls.

Recently, two sisters with reversible creatine deficiency in the brain, a syndrome presumed to be the result of a creatine-transporter defect in the brain, have been described elsewhere (Bianchi et al. 2000). Mutation detection in *SLC6A8* was not reported. In contrast to our index patient, the sisters clinically improved through creatine supplementation. That the patients are females and that they respond to creatine supplementation suggests yet another creatine-deficiency syndrome. Alternatively, the sisters, in comparison with their healthy mother, may have a milder creatine-transporter defect that is the result of a less-severe transporter mutation combined with unfavorable X-linked mosaicism.

Thus far, five patients with GAMT deficiency have been reported (Stöckler et al. 1996*a,* 1996*b;* Schulze et al. 1997; van der Knaap et al. 2000) who presented with developmental delay (severe speech and language delay), extrapyramidal movement abnormalities, hypotonia, and the absence of creatine and phosphocreatine in MRS of the brain. Furthermore, all patients showed increased concentrations of GUAC in their bodily fluids. The clin-

ical features, as well as the striking absence of creatine in MRS of the brain, are also encountered in our index patient, who suffered from a creatine-transporter defect. In contrast to what has been reported in patients with GAMT deficiency, GUAC levels in bodily fluids were not increased in our index patient. The similarity of clinical symptoms observed in both creatine-deficiency syndromes suggests that the disturbed creatine pool, rather than the increased GUAC levels, is important in the pathogenesis of GAMT deficiency.

In summary, we have described a new X-linked creatine-deficiency syndrome, which is confirmed by a hemizygous nonsense mutation in *SLC6A8* of a male index patient. Moreover, three female relatives of the index patient are heterozygous for this mutation. The laboratory hallmarks of this disease are the absence of creatine in the MRS of the brain, an increase of creatine in urine, and the presence of a severe mutation in the X-mapped *SLC6A8*. Clinically, the severe language delay, mild retardation, and X-linked inheritance in the family are the most prominent characteristics of this disease. As yet, no clinical improvement has been observed during creatine supplementation. Patients with less-severe mutations (i.e., missense mutations) may have milder clinical signs. It may prove worthwhile to screen patients with behavioral and/or cognitive syndromes, for decreased creatine signals in MRS, increased levels of creatine in bodily fluids, and/or mutational analysis of the creatine-transporter gene.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *SLC6A8* [accession numbers Z66539 and NM_005629])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *SLC6A8* [MIM 300036])

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