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Detection of a novel intragenic rearrangement in the creatine transporter gene by next generation sequencing

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ABSTRACT

Deficiency caused by mutations in the creatine transporter gene (*SLC6A8/CT1*) is an X-linked form of intellectual disability. The presence of highly homologous pseudogenes and high GC content of *SLC6A8* genomic sequence complicates the molecular diagnosis of this disorder. To minimize the pseudogene interference, exons 2 to 13 of *SLC6A8* were amplified as a single PCR product using gene-specific long-range PCR (LR-PCR) primers. The GC-rich exon 1 and its flanking intronic sequences were amplified separately in a short fragment under GC-rich conditions and a touchdown PCR program. Traditional Sanger sequence analysis of all coding exons of *SLC6A8* from a 3-year-old boy with creatine transporter deficiency did not detect deleterious mutations. The long-range PCR product was used as template followed by massively parallel sequencing (MPS) on HiSeq2000. We were able to detect a tandem duplication involving part of exons 11 and 12 in the *SLC6A8* gene. The deduced c.1592_1639dup133 mutation was confirmed to be a hemizygous insertion by targeted genomic DNA and cDNA Sanger sequencing. Combination of deep sequencing technology with long-range PCR revealed a novel intragenic duplication in the *SLC6A8* gene, providing a definitive molecular diagnosis of creatine transporter deficiency in a male patient.

1. Introduction

Deficiency of the creatine transporter (SLC6A8/CT1; OMIM: 300352) accounts for 1–5% of X-linked intellectual disability (XLID), which is previously referred to as X-linked mental retardation (XLMR) syndrome [1–6]. The creatine/phosphocreatine shuttle functions as a high-energy phosphate buffer system to maintain the energy supply in tissues that have high and fluctuating energy demands, such as muscle and brain. In mammals, creatine can be synthesized from arginine endogenously, mostly in liver, kidney and pancreas. In addition to endogenous synthesis, creatine can be obtained through diet. Synthesized or absorbed creatine is distributed to tissues via bloodstream. The creatine transporter SLC6A8 plays a pivotal role in the creatine uptake, especially for transport of creatine through the blood–brain barrier to the brain [7–10]. Expression of the *SLC6A8* gene is high in kidney, heart, skeletal muscle, and brain [11–13]. Creatine transporter knockout mice lack creatine in the brain and have learning and memory deficits, which recapitulate the phenotype of human creatine transporter deficiency [14].

Clinical diagnosis of creatine transporter deficiency relies on clinical features, abnormal metabolites, and proton magnetic resonance spectroscopy (MRS) [15–17]. While an increased urinary creatine:creatinine ratio is useful for screening purposes, reduction of intra-cerebral creatine as measured by MRS is most reliable and is the hallmark of this disease. Identification of a mutation in the X-linked creatine transporter gene confirms the diagnosis [18–20].

The gene encoding the creatine transporter, *SLC6A8*, consists of 13 coding exons spanning approximately 8.3 kb at Xq28 [21,22]. To date, more than 90 mutations in the *SLC6A8* gene have been identified in patients with creatine transporter deficiency [23]. The majority are single nucleotide substitutions or small insertion/deletions (in/dels). Six gross deletion mutations are reported in the literature [16,23–26]. Four of them are the large deletions involving a loss of multiple exons or the whole *SLC6A8* gene. In addition, analysis of X-chromosome copy number variation (CNV) by array comparative genomic hybridization (aCGH) detected large duplications at Xq28 including entire *SLC6A8* [27,28]. However, intragenic duplications involving exons have not been reported to date.

Here, we report a novel *SLC6A8* intragenic duplication uncovered in a male patient by the application of deep sequencing technology in

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combination with a long-range PCR strategy. The insertion of a 16 amino acid transmembrane hydrophobic helix is expected to disrupt the protein structure and functions, consistent with the diagnosis of creatine transporter deficiency in this patient.

2. Materials and methods

2.1. Specimens and sample preparation

Total genomic DNA was extracted from blood using a commercially available DNA isolation kit (Gentra Systems Inc., Minneapolis, MN) according to the manufacturer's instructions. Total RNA was isolated from the patient's blood sample using the RiboPure™-Blood Kit (Life technologies, Carlsbad, CA) following manufacturer's instructions. The cDNA was synthesized using ABI High Capacity RNA-to-cDNA Master Mix according to the manufacturer's protocols. The target cDNA regions were amplified using primers located at exons or exon–exon boundaries.

2.2. SLC6A8 pseudogene sequences and primer design

The genomic sequences of *SLC6A8* (NG_012016.1) and two pseudogenes, *SLC6A10P* (NC_000016.9, complement) and *LOC653562* (NC_000016.9), were obtained from GenBank. The 8.3 kb of *SLC6A8* (NM_005629.3, corresponding to the longest isoform 1) plus 1 kb upstream and downstream genomic sequences and its corresponding pseudogene sequences were aligned using the EBI ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The nucleotide positions unique to the *SCL6A8* gene were used for selections of gene-specific long-range PCR primers encompassing exons 2 to 13, and allele specific primers for exon 1. All primers listed in Table 1 were determined by the Primer3 software (version 0.4.0) and were carefully inspected to exclude the sequences containing SNPs. The 5′-end of LR-PCR forward primer was biotin-labeled to facilitate the purification of PCR products by streptavidin-conjugated magnetic beads.

2.3. Exon 1-specific PCR amplification

Due to high GC content, exon 1 of *SLC6A8* was amplified using a Roche GC-Rich PCR system containing 0.2 volume of GC-RICH Resolution Solution (Roche Diagnostics, Indianapolis, IN) and 100 ng of total genomic DNA in a final volume of 25 μl. The PCR reaction was carried out with an Eppendorf Mastercycler thermal cycler (Eppendorf North America, Hauppauge, NY) under a touchdown cycling condition: Step 1) 5 min denaturation at 95 °C, Step 2) 30 s denaturation at 95 °C, Step 3) 30 s annealing with temperature declining from 67 °C to 62 °C at a rate of 0.5 °C per cycle, Step 4) 60 s extension at 72 °C, repeat Steps 2–4 10 times, Step 5) 30 s at 95 °C, Step 6) 30 s at 61 °C, Step 7) 60 s at 72 °C, repeat Steps 5–7 35 times, Step 8) 10 min final extension at 72 °C. The PCR products of *SLC6A8* exon 1 were purified by the ExcelaPure UF PCR Purification system (Edge Biosystems, Gaithersburg, MD).

2.4. Long-range and nested PCR amplification of exons 2 to 13

The LR-PCR was performed using Takara LA Taq DNA polymerase Hotstart Version with 50 ng of total genomic DNA in a final volume of 25 μl. The thermal cycling protocol for *SLC6A8* LR-PCR is as follows: Step 1) 5 min denaturation at 95 °C, Step 2) 1 min denaturation at 95 °C, Step 3) 7.5 min at 68 °C for combined annealing and extension, Steps 2–3 were repeated 35 times, Step 4) 15 min final extension at 68 °C. After the removal of excess primers, dNTP, and salts using the ExcelaPure UF PCR Purification system, the LR-PCR products of *SLC6A8* were further purified by the Invitrogen Dynabeads® m-280 Streptavidin. The nested PCR of individual exon (from exon 2 to exon 13) was performed using Roche Faststart Taq DNA polymerase with bead-purified LR-PCR products as the template. A touchdown program similar to that for exon 1 amplification was used for nested PCR with one modification: after the 10 cycles of touchdown steps, there are only 25 addition cycles at 61 °C annealing temperature.

2.5. Sequence analysis

Purified PCR products of *SLC6A8* exons and at least 50 nucleotides of their flanking intronic regions were sequenced using BigDye Terminator chemistry on an ABI3730XL automated DNA sequencer. The Sanger sequencing results were compared and analyzed to the *SLC6A8* reference sequence (NG_012016) using the Mutation Surveyor software (SoftGenetics, State College, PA).

Table 1
SLC6A8 primers for sequencing analysis.

Primer name	Size (bp)	Forward primer	Reverse primer
a. Long range PCR primers			
*SLC6A8*_E2–13LR	6913	5′-Bio-CTGCTGGCCTCAGGAGATAGGC-3′	5′-CTCCTGTACCCCGACCCCTTC-3′
b. E1-specific primers[a]			
*SLC6A8*_E1a	405	5′-TTCTGACTGCGCCGCCCGC-3′	5′-AGCCCACGGCGAAGCCCACG-3′
*SLC6A8*_E1b	451	5′-CTATAGCGTGTCCGGCGACGA-3′	5′-GCCCCCTCCCCCCACCATG-3′
*SLC6A8*_E1c	474	5′-CTGACCGCCGCCCCCCGTGA-3′	5′-AGCGGCAGCGGCGGACGA-3′
c. E2–13 nested-PCR primers[a]			
*SLC6A8*_E2a	300	5′-ATGGGGCCTTTGGAGTCT-3′	5′-CACGACAGGAAAAGATTTCTCA-3′
*SLC6A8*_E2b	376	5′-GGCCACCCCTACACTGACT-3′	5′-AGACTGGGACGCCATCAC-3′
*SLC6A8*_E3a	486	5′-CACAAACAAGGCTCCAGGAG-3′	5′-GATGGACTGCCAGGCAAC-3′
*SLC6A8*_E3b	450	5′-CAGGGGAGTCAGGAGCAC-3′	5′-GGGCAACATGAGGGAGAC-3′
*SLC6A8*_E4a	376	5′-CTGGGCTGTGGGAGAGAAG-3′	5′-AGCATTGCTTGGCTTTCAGT-3′
*SLC6A8*_E4b	298	5′-GGACCCTGTGGCTCCATC-3′	5′-GCTAGACTCTGTCTGGTCACCTC-3′
*SLC6A8*_E5–6	457	5′-GGTGTGAGGGAGGTGGTG-3′	5′-GCCAGAGTGGATGGGTTAGG-3′
*SLC6A8*_E7	221	5′-AGCCTAACCCATCCACTCTG-3′	5′-CATCATGCATCTGGGTAGCA-3′
*SLC6A8*_E5–7	780	5′-TGGGGACCTCTGAACATACC-3′	5′-GGCCCACAATCTGACAAGTT-3′
*SLC6A8*_E6–7	589	5′-GAGGTGGAGGTGGAGAGG-3′	5′-TGGGAGGTCCTTTCTCTGTC-3′
*SLC6A8*_E8–9a	467	5′-CAGGACATCGGCTACAAGGT-3′	5′-AGTCAGGGAGGATGGCAGAC-3′
*SLC6A8*_E8–9b	623	5′-GGTTGACAGCGCCTCTGA-3′	5′-CTGCCAGAGCAGGGTGGT-3′
*SLC6A8*_E10–11a	390	5′-GTCTGCCATCCTCCCTGA-3′	5′-TTAATGGGGCCCCTACCC-3′
*SLC6A8*_E10–11b	432	5′-GGGTCTGCCTGTGACCTCT-3′	5′-GACCAGAATGCTGCGGTT-3′
*SLC6A8*_E12–13	584	5′-GGTAGGGGCCCCATTAAC-3′	5′-CAGGGGCTGCTATGGCTAC-3′
*SLC6A8*_E12	355	5′-TAAGGGCTGGGGGAGGT-3′	5′-CTGCCTGCCACCCTAGTC-3′
*SLC6A8*_E13	332	5′-CGGAGAGAGGCAGAGGAAGT-3′	5′-CCAGGCAGACCCCAAAAG-3′

[a] To facilitate sequencing analysis, E1-specific and E2–13 nested-PCR primers are all linked with universal M13 sequencing primers at 5′ end.