Chapter 3

Detection of low-level somatic and germline mosaicism by denaturing high-performance liquid chromatography in a EURO-MRX family with SLC6A8 deficiency.

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Detection of low-level somatic and germline mosaicism by denaturing high-performance liquid chromatography in a EURO-MRX family with SLC6A8 deficiency

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Abstract Creatine transporter deficiency is an X-linked mental retardation disorder caused by mutations in the creatine transporter gene, SLC6A8. In a European Mental Retardation Consortium panel of 66 patients, we identified a male with mental retardation, caused by a c.1059_1061delCTT; p.Phe354del mutation in the SLC6A8 gene. With the use of direct DNA sequencing, the mutation was also found in the brother of the proband, but not in their mother. However, by analyzing EDTA blood of the mother with denaturing high-performance liquid chromatography (DHPLC), we could show that the mother displays low-level somatic mosaicism for the three base-pair deletion. This study indicates DHPLC as an important tool in the detection of low-level mosaicism, as does it illustrate the importance of considering somatic and germline mosaicism in the case of apparent de novo mutation.

Keywords Creatine transporter deficiency · SLC6A8 · X-linked mental retardation · DHPLC · Low-level somatic mosaicism · Germline mosaicism

Introduction

Creatine transporter deficiency is an X-linked mental retardation (XLMR) disorder that was first described in 2001 [2, 13]. Clinical hallmarks have been identified as mental retardation, severe speech and language development delay, autistic features, epilepsy, low weight, and poor muscle build. Clinical manifestations of later onset include intestinal and neurological problems. Laboratory hallmarks are increased urinary creatine/creatinine ratio in affected males, marked reduction of creatine signal on proton magnetic resonance spectroscopy (MRS) of the brain and mutations in the SLC6A8 gene. Clinical features have been reviewed for 17 SLC6A8-deficient patients [4]. A previous-
ly reported study of a European XLMR (EURO-MRX) panel of 288 patients showed a prevalence of ~2.1% (CI: 0.43 to 3.73) of SLC6A8 deficiency in male patients with non-syndromic X-linked mental retardation [11]. In the present study, we investigated 66 additional male probands from EURO-MRX families and identified in one family a novel mutation in the SLC6A8 gene. The proband of this family and his affected brother were diagnosed with creatine transporter deficiency (Fig. 1a). Surprisingly, in DNA isolated from ethylenediaminetetraacetic acid (EDTA) blood of the mother, an obligate carrier, the mutation could not be detected by direct DNA sequencing (Fig. 1b). In a different approach, using denaturing high-performance liquid chromatography (DHPLC), we could prove that the mother was somatic mosaic for the mutation (Fig. 1c).

In this paper, we show that direct DNA sequencing, the current benchmark of patient diagnostics in SLC6A8 deficiency, has limited sensitivity and specificity concerning
the detection of low-level somatic mosaicism, in contrast to DHPLC. Therefore, we propose DHPLC to supersede direct DNA sequencing as the method of screening on low-level somatic mosaicism.

Materials and methods

Direct DNA sequencing and analysis

Direct DNA sequence reactions were performed using BigDye v3.1 terminator and an ABI 3100 machine (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). The obtained sequences were analyzed using the Mutation Surveyor (Softgenetics, State College, PA, USA) software package.

Amplification of SLC6A8 exon 7

From the SLC6A8 gene, exon 7, and flanking intronic sequences of the proband, the mother of the proband and a control male DNA were amplified using Optimase Polymerase according to the instructions of the manufacturer (Transgenomic, Omaha, NE, USA). In a total reaction volume of 20 μl, 10 pmol of both sense (5’-GCTACAAG TAAGCACCAGCGG-3’) and antisense primer (5’-CAGCA TTTCTATTGACATGTTCC-3’) were used. Amplification consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s, finished off with a final extension step of 72°C for 5 min.

Concentration of the polymerase chain reaction (PCR) products was measured by DHPLC in fully matured conditions (50°C). PCR products of the proband and the control male were mixed to obtain wild type to mutant ratios ranging from 0–100% to 50–50%. Samples were then either directly used for heteroduplex formation or stored at 4°C until further use.

Analysis of exon 7 by DHPLC

For DHPLC analysis, optimal heteroduplex formation was realized by denaturing PCR products at 95°C for 5 min and gradual cooling to 25°C over a period of 60 min. Subsequently, 5 μl of the heteroduplex/homoduplex mixture was loaded on a WAVE 3500HT DNA fragment analysis system (Transgenomic, Omaha, NE). Elution of the PS–DVB DNAsep column (Transgenomic, Omaha, NE) was performed in high-throughput mode with a linear gradient of increasing acetonitrile (ACN) concentration with a runtime of 3 min and a constant flow rate of 1.5 ml/min. Gradient was realized with the use of buffer A (0.1 M triethylammonium acetate [TEAA] and 0.025% ACN) and buffer B (0.1 M TEAA and 25% ACN). WAVEmaker 4.1 software (Transgenomic, Omaha, NE) was used to calculate the ratio of buffer A and B during the gradient, as well as the optimal partially denaturing temperature (T_m) for the amplicon. The T_m of this amplicon was determined as 63.2°C.

Amplification refractory mutation system

For the amplification refractory mutation system (ARMS) [7], primers were designed to specifically detect the mutated or the wild-type sequence. DNA from the proband, his mother and a male control was amplified using an Applied Biosystems 9800 fast thermocycler as follows: 20 s at 95°C, 35 cycles of 94°C for 1 s and 68°C for 25 s, followed by a final extension step of 10 min at 72°C. PCR reactions contained 10 μl Mastermix Polymerase (Applied Biosystems), 0.5 μl of 10 μM forward (5’-ATCA ACAGTGGGACCACCCCTTC-3’ [wild type]; 5’-ATCA ACAGTGGGACCACCTGTTC-3’ [mutation]) and reverse primer (5’-CAGCATTTGTTTGTGATTCC-3’) and 25 ng genomic DNA, in a total volume of 20 μl. Products were subsequently mixed with GelStar (BioWhittaker Molecular Applications, Rockland, ME) and loaded onto a 1% agarose gel in 0.5x TBE buffer. Amplified DNA fragments were made visible with the use of an Imago Imager (B&L Systems, Maarssen, The Netherlands).

Transient transfection of pEGFP-SLC6A8-Phe354del

To confirm the pathogenicity of the c.1059_1061delCTT; p. Phe354del variant, this variant was introduced in the SLC6A8 open reading frame by site-directed mutagenesis and subcloned in the pEGFP-N1 vector (pEGFP-SLC6A8-Phe354del). Subsequently, SLC6A8-deficient primary fibroblasts were transient transfected with pEGFP-SLC6A8 in triplicate, pEGFP-SLC6A8-Phe354del in triplicate and pEGFP-N1 in duplicate and tested for creatine uptake capacity when incubated with physiological creatine concentration of 25 μM as previously described by Rosenberg et al. in 2007. Cells were harvested by trypsinization, divided in aliquots for creatine measurement and Western blotting and stored as dry pellets at ~80°C until further use. Intracellular creatine content was measured by stable-isotope dilution GC-MS [14]. The presence of the creatine transporter was determined with the use of Western blotting. To prove SLC6A8-EGFP fusion protein expression, cell pellets were lysed in urea lysis buffer (8 M urea/100 mM NaCl/10 mM TrisHCl, pH 8.0). Protein content was measured using a Bicinchoninic acid protein assay. All the samples were normalized to the same protein concentration with additional lysis buffer and blue sample buffer. Cell lysates (25 μg) were size-separated by 12% sodium
dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-page) and transferred to a Hybond™ membrane by standard procedures. Immunodetection was performed using antibodies directed against the EGFP tag. Immune complexes were detected by enhanced chemiluminescence (ECL) according to the manufacturer’s specifications (Amersham Biosciences UK Ltd, Buckinghamshire, England, UK).

Results

In a EURO-MRX panel of 66 patients, we detected in the DNA of one patient a novel variant in the SLC6A8 gene. This variant, c.1059_1061delICTT; p.Phe354del was not encountered in the DNA of 276 healthy control males [11].

In silico analysis with the ESE and ESS web-tools RescueESE (http://genes.mit.edu/burgelab/rescue-esc/), ESEFinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/
esfinder.cgi?process = home) and ExonScan (http://genes.mit.edu/exonscan/) did not reveal any negative or positive influence of the mutation on splicing. Pathogenicity of the c.1059_1061delICTT; p.Phe354del was confirmed by transient transfection of SLC6A8-deficient primary fibroblasts with the pEGFP-SLC6A8-Phe354del expression vector. In contrast to the pEGFP-SLC6A8 transfectants, which showed a significant increase of creatine uptake, the pEGFP-SLC6A8-Phe354del transfection did not restore the creatine uptake (Fig. 2a).

In all transfectants, except for the mock and pEGFP-N1, EGFP fusion proteins were detected by Western blotting, proving successful transfection of the SLC6A8-EGFP and SLC6A8-Phe354del-EGFP (Fig. 2b). The two proteins running with an apparent molecular mass of ~75 kDa and ~100 kDa visible in the pEGFP-SLC6A8 transfectants, probably represent the un-glycosylated and the N-linked glycosylated form of SLC6A8 [17]. The Western blot analysis using an antibody against EGFP (indicated by arrows). The two proteins running with an apparent molecular mass of ~75 and ~100 kDa visible in the pEGFP-SLC6A8 transfectants, probably represent the un-glycosylated and the N-linked glycosylated form of SLC6A8. The fusion proteins expressed by the pEGFP-SLC6A8-Phe354del transfectants, run with a different molecular weight, perhaps due to (partial) degradation of the mutated protein. The high molecular weight proteins indicated by an asterisk (*) could represent aggregates of creatine transporter fusion proteins. Please note that each creatine measurement and underlying Western blot lane originate from the same transfection.

Fig. 2 Creatine uptake and detection of EGFP fusion proteins in triplicate transient-transfected primary fibroblasts. a Creatine uptake of control fibroblasts (first column) and SLC6A8 deficient primary fibroblasts, transfected with pEGFP-SLC6A8 (n = 3), pEGFP-SLC6A8-Phe354del (n = 3), and pEGFP-N1 (n = 2) expression vectors (columns 2-11). After incubation with 25 μM creatine for 24 h, intracellular creatine content was measured in triplicate by stable-isotope dilution GC-MS. The hash (#) denotes an undetectable creatine level. b SLC6A8 deficient primary fibroblasts were transiently transfected with pEGFP-SLC6A8 (n = 3), pEGFP-SLC6A8-Phe354del (n = 3), and pEGFP-N1 (n = 2). The expression of the EGFPLSLC6A8 and EGFP-SLC6A8-Phe354del fusion proteins and EGFP was detected by
fusion proteins expressed by the pEGFP-SLC6A8-Phe354del transfectants, run with a different molecular weight, perhaps due to (partial) degradation of the mutated protein. The high molecular weight proteins could represent aggregates of creatine transporter fusion proteins. It is of note that the constructs have been transfected in triplicate, where different transfection efficiencies in all SLC6A8 transfectants cause variable levels of SLC6A8 fusion protein.

Magnetic resonance imaging of this 17-year-old patient showed incomplete myelination. In particular, the U-fibers and the baso-temporal regions still had a higher signal than the cortex on T2-weighted images. Quantitative localized single voxel MRS was performed as described previously [15]. It revealed a creatine concentration in the cerebral cortex of 1.1 mmol/l (normal 6.5 mmol/l, sd 0.5 mmol/l); in the cerebral white matter of 1.5 mmol/l (normal 4.8 mmol/l, sd 0.4 mmol/l); in the cerebellum of 2.0 mmol/l (normal 8.6 mmol/l, sd 0.7 mmol/l) and in the basal nuclei of 1.8 mmol/l (normal 7.7 mmol/l, sd 0.9 mmol/l). For quantification and normal-values, see Pouwels et al. [9] and Provencer [10], respectively. Investigation of the patient’s urine showed an increased creatine/creatinine ratio of 2.6 (normal 0.011–0.244) [1]. Formal psychological and psychiatric assessment revealed moderate mental retardation (TIQ 50) and autistic behavior. Neurological exam showed clumsy gait, pyramidal signs with high DTR and bilateral Babinski.

DNA samples of the mother and the brother of the proband were investigated by direct sequence analysis of the SLC6A8 gene. The mutation was detected in the DNA of the affected brother, but unexpectedly, not in that of the mother (Fig. 1b). Preferential amplification of the wild-type allele due to an SNP in any of the primer regions was ruled out by sequence analysis. To investigate if we could demonstrate somatic mosaicism, the presence of the mutation was further analyzed by heteroduplex formation analysis with the DHPLC system. We detected heteroduplex formation in DNA isolated from EDTA blood of the mother; the DHPLC chromatogram showed a small heteroduplex peak relative to the homoduplex peak with a 1:6.6 area ratio (13.1% vs 86.9%, respectively, of total peak area). Furthermore, the retention times of both peaks were similar to that of the 1:1 mix of PCR products of the proband and a control male, which indicates one and the same mutation (Fig. 1c). Using ARMS, with primers specifically designed for the c.1059_1061delCTT mutation, we could confirm that the mother was indeed a carrier of this mutation (Fig. 3).

On account of this finding, the detection limit of the DHPLC system for this mutation was investigated. The amplicon containing the mutation of the proband and a control male was amplified separately and mixed to obtain wild-type/mutant ratios ranging from 0–100% to 50–50%. Subsequent sampling of these mixtures on the DHPLC system showed that a 1% mutation frequency can be easily detected (Fig. 4). Furthermore, these results were used to obtain a conversion rate for area percentages of heteroduplex peaks relative to homoduplex peaks of females with somatic mosaicism. In our case, a heteroduplex peak area of 13.1% relative to the total peak area represents an in vivo somatic mosaic percentage of 6%.

**Discussion**

In 2004, we reported a prevalence of SLC6A8 deficiency of 2.1% (confidence interval [CI]: 0.43 to 3.73) in an XLMR panel of 288 males (6288) of the EURO-MRX consortium [11]. A different study performed by Lion-Francois et al. in 2006, revealed an SLC6A8 deficiency prevalence of 5.4% (CI: 0 to 12.7) in a panel of 37 families presumed to be affected with XLMR [5]. In the present study, 66 newly collected patients from the EURO-MRX consortium were investigated for mutations present in the SLC6A8 gene. This study resulted in the detection of an additional family with SLC6A8 deficiency, and showed a prevalence of 1.5% (CI: 0 to 4.46), which is in the range of the earlier reported prevalence of 2.1% (CI: 0.43 to 3.73) and the 5.4% (CI: 0 to 12.7) prevalence.

In this current study, we identified one family with a pathogenic mutation in the SLC6A8 gene. This mutation, c.1059_1061delCTT; p.Phe354del, has not been published before. The mutation is located in the highly conserved transmembrane domain VII. Multiple-sequence alignment
Fig. 4 Sensitivity of the detection of the c.1059_1061delCTT mutation by DHPLC. Wild-type and mutant PCR amplicons were pooled with ratios ranging from 0–100% to 50–50%. The elution profile of the 99%/1% wild-type/mut mix (second from top) indicates that with a mutant allelic percentage of 1%, heteroduplex formation is already present and detectable with the DHPLC system.

among SLC6A8 proteins and some of the other SLC6 family members (neurotransmitter transporters) of different species indicates conservation of the phenylalanine on the 354\textsuperscript{th} position of the human creatine transporter protein (Fig. 5). Brain MRS of the patient showed significantly decreased creatine levels in the cerebral cortex, white matter, cerebellum, and basal nuclei. Investigation of the patient’s urine showed an increased creatine/creatinine

\[\text{Transmembrane domain VII}\]

![Multiple-sequence alignment of SLC6A8 proteins of different species and the SLC6 superfamily (neurotransmitter transporters). The SLC6A8 protein sequence is shown for the following species: Homo sapiens (Hs), Mus musculus (Mm), Monodelphis domestica (Md), Danio rerio (Dr), Macaca mulatta (Mam), Pan troglodytes (Pt), Rattus norvegicus (Rn), Macaca fascicularis (Mf), Equus caballus (Ec), Bos Taurus (Bt), Canis lupus familiaris (Cf), Oryctolagus cuniculus (Oc), Torpedo marmorata (Tm), Tetradon nigroviridis (Tn), Drosophila melanogaster (Dm). The codes A01–A14 represent the SLC6A1–SLC6A14 protein sequences (A1–GABA\textsubscript{A}, A2–noradrenaline, A3–dopamine, A4–serotonin, A5–glycine\textsubscript{2}, A6–tau-\textsubscript{rine}, A7–proline, A9–glycine 1, A11–GABA\textsubscript{B}, A12–betaine/GABA, A13–GABA\textsubscript{B2}, and A14–ATB\textsuperscript{17}). Protein sequences were aligned using BioEdit software (Hall 1999). Identical amino acids are highlighted in black and functionally conserved amino acids are highlighted in gray. Functionally conserved amino acids are classified as follows: V, I, L and M; D, E, Q and N; F, Y and W; G, S, T, P and A; and K, R and H. Transmembrane domain VII is predicted by ExPASY (Swiss-Prot S6A8 Human P48029). Conservation of the phenylalanine amino acid at the 354\textsuperscript{th} position (*) of the human creatine transporter is indicated by the gray box.\]
ratio. Transfection of SLC6A8 deficient primary fibroblasts with the pEGFP-SLC6A8-Phe354del expression vector, resulting in the expression of the SLC6A8 protein lacking the phenylalanine amino acid on the 354th position, did not restore the creatine uptake, in contrast to the pEGFP-SLC6A8 expression vector, which restored the creatine uptake of SLC6A8 deficient primary fibroblasts. Implementation of all of the above findings in the flowchart of [12], used for the screening of SLC6A8 deficiency in patients with mental retardation of unknown etiology, indicates that this mutation is a proven pathogenic mutation.

The proband and his brother suffer from mental retardation, motor impairment, and autism. Both are diagnosed with SLC6A8 deficiency caused by the pathogenic c.1059_1061delCTT mutation. Subsequent DNA sequence analysis of the apparently unaffected mother did not reveal the presence of the mutation ascribed to the SLC6A8 deficiency. However, DHPLC analysis of the DNA of the mother revealed that the mutation was indeed present, yet only in about 6% of her blood cells, indicating that this mutation occurred spontaneously in her embryonic phase [16].

The fact that the mother has two affected sons indicates that the germline mosaicism of the mother is of a significantly high percentage [8], in contrast to the low-level somatic mosaicism found in the DNA of blood of the mother with an estimated occurrence of 6%. However, in spite of the fairly low chance of transmitting the mutated allele twice in the case of low-level germline mosaicism, this might have happened against the odds. This shows that low-level somatic mosaicism can still have substantial indicative importance and cannot be assumed to be trivial.

This is the first identified SLC6A8-deficient family with somatic mosaicism. Therefore, these findings may have consequences for the current counseling of not only families with SLC6A8 deficiency, but also for the diagnostics of families with mental retardation of unknown cause.

In the first place, counseling of families with a presumed de novo mutation in the SLC6A8 gene has to include information on the possibility and consequences of somatic and germline mosaicism as well as the option of prenatal diagnostics. Second, the DNA of mothers of affected males, or of both parents in the case of an affected daughter, should be analyzed for somatic mosaicism because the presence of somatic mosaicism may increase the risk of recurrence. Third, as somatic mosaicism may broaden the clinical spectrum of SLC6A8 deficiency, this disorder might also be considered in the differential diagnosis of females/males with milder symptoms than previously described (e.g. learning disabilities). Moreover, it should be noted that the X-linked pattern of inheritance will not be observed in the case of a de novo mutation. Therefore, diagnostic screening of males with sporadic mental retardation (prevalence of 1.4%; CI: 0 to 3.30 [5]) and females (prevalence data unknown) should include screening for SLC6A8 deficiency.

We show that low-level somatic mosaicism of 1% can be easily detected with DHPLC, which is similar to the 1% detection limit of [6] and more sensitive than the previously reported 5% [3, 18] and 10% [18] detection limit of other studies. It should be noted that the latter two detection limits were obtained with the screening of missense mutations, which are usually harder to detect with DHPLC, in contrast to the more easily detectable three base-pair deletion examined in this study.

We suggest DHPLC may be an ideal method of screening for SLC6A8 deficiency in females/males with no evident cause for their mental retardation or learning disabilities, as it is more sensitive than direct DNA sequencing. In conclusion, this study illustrates the importance of awareness of mosaicism in the counseling of families with a de novo mutation in the SLC6A8 gene.

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