BRIEF REPORT

Functional Analysis of Monocarboxylate Transporter 8 Mutations Identified in Patients with X-Linked Psychomotor Retardation and Elevated Serum Triiodothyronine


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Context: T₃ action in neurons is essential for brain development. Recent evidence indicates that monocarboxylate transporter 8 (MCT8) is important for neuronal T₃ uptake. Hemizygous mutations have been identified in the X-linked MCT8 gene in boys with severe psychomotor retardation and elevated serum T₃ levels.

Objective: The objective of this study was to determine the functional consequences of MCT8 mutations regarding transport of T₃.

Design: MCT8 function was studied in wild-type or mutant MCT8-transfected JEG3 cells by analyzing: 1) T₃ uptake, 2) T₃ metabolism in cells cotransfected with human type 3 deiodinase, 3) immunoblotting, and 4) immunocytochemistry.

Results: The mutations identified in MCT8 comprise four deletions (24.5 kb, 2.4 kb, 14 bp, and 3 bp), three missense mutations (Ala224Val, Arg271His, and Leu471Pro), a nonsense mutation (Arg245stop), and a splice site mutation (94 amino acid deletion). All tested mutants were inactive in uptake and metabolism assays, except MCT8 Arg271His, which showed approximately 20% activity vs. wild-type MCT8.

Conclusion: These findings support the hypothesis that the severe psychomotor retardation and elevated serum T₃ levels in these patients are caused by inactivation of the MCT8 transporter, preventing T₃ uptake and metabolism in central neurons.

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Abbreviations: D3, Type III deiodinase; ex, exon; IB, immunoblotting; ICC, immunocytochemistry; MCT8, monocarboxylate transporter 8; TMD, transmembrane domain.

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of six MCT8 mutations. The functional consequences of mutations in MCT8 on cellular uptake and metabolism of T3 were determined in JEG3 cells transfected with wild-type or mutant MCT8 alone or in cells cotransfected with MCT8 and D3.

Patients and Methods

Serum and DNA analyses

Approval for these studies was obtained from the medical ethical committee of the Erasmus Medical Center. Informed consent was obtained from the parents of all patients. Serum T4, free T4, T3, and TSH were measured by Vitros ECI technology (Immunodiagnostic System; Ortho-Clinical Diagnostics, Beerse, Belgium). The coding sequence of MCT8 was analyzed in patient DNA using intronic primers flanking the six exons.

Cloning and site-directed mutagenesis of human MCT8

Construction of a human MCT8 cDNA-containing pcDNA3 expression vector was described previously (14). The point mutations identified in patients (Table 1) were introduced in MCT8 cDNA using the QuickChange Site-Directed Mutagenesis protocol (Stratagene, Amsterdam, The Netherlands) and confirmed by sequencing.

Transfection of JEG3 cells

JEG3 cells were grown at 37 °C in DMEM-F12 medium containing 9% fetal bovine serum and 100 nm sodium selenite and transfected at 70–80% confluency using 0.3 μl FuGENE 6 transfection reagent (Roche Diagnostics, Almere, The Netherlands) per 100 ng DNA. For T3 uptake studies and immunoblotting (IB), cells were grown in six-well plates and transfected with 500 ng empty pcDNA3 or pcDNA3 containing wild-type or mutated MCT8 cDNA. Renilla luciferase vector (50 ng; pRL-SV40; Promega, Leiden, The Netherlands) was cotransfected to ensure correction for transfection efficiency. For intact-cell T3 metabolism assays, cells were cultured in 24-well plates and cotransfected with 100 ng pcDNA3 containing wild-type or mutant MCT8 and 100 ng pCI-Neo containing D3 cDNA. For analysis of D3 activity in cell lysates, cells were harvested in PE buffer and analyzed by HPLC as described previously (14). For analysis of D3 activity in cell lysates, cells were harvested in PE buffer containing 0.1 m phosphatase (pH 7.2), 2 mm EDTA containing 1 mm dithiothreitol and sonicated. Appropriate dilutions of the sonicates were incubated for 1 h at 37 °C with 1 nm (2 × 10−5 cpm) [125I]T3 in 0.1 ml PE buffer containing 10 mm dithiothreitol. Incubations were stopped, and samples were analyzed by HPLC. IB of transfected cell homogenates was performed as described previously (14). For ICC, cells were fixed and permeabilized with 4% paraformaldehyde and 0.2% Triton X-100 and stained with MCT8-specific polyclonal antibody 1306. The plasma membrane was stained using zona occludens 1 antibody (Invitrogen, Breda, The Netherlands). Alexa fluor 488 and 633 (Invitrogen) were used as detection antibodies; analyses were performed on a Zeiss Axiovert 100 confocal microscope (Zeiss, Sliedrecht, The Netherlands) using Zeiss LSM software.

Results

We report here on nine boys with severe psychomotor retardation, five of which have been presented previously (Table 1). The clinical phenotypes and serum thyroid parameters of the additional four patients are similar to those of patients with MCT8 mutations described by us and others. All of our patients have been identified with severe psychomotor retardation, characterized by axial hypotonia, spastic or flaccid quadriplegia, dystonic movements, and absence of speech. Mean serum T4 and free T4 are decreased, TSH is mildly increased, and serum T3 is markedly elevated. Patients are between 2.5 and 18 yr old and come from various ethnic backgrounds.

All nine patients were found to have different hemizygous mutations in MCT8 (Table 1). Patient 1 has a 24,527 bp deletion, stretching from 15 kb upstream to 9 kb downstream of exon 1. Patient 2 has a 671C→T missense mutation in exon 2, resulting in an Ala224Val substitution located in the second putative TMD (9, 15). Patient 3 has a 1412T→C mutation in exon 5, resulting in a Leu471Pro substitution located in the ninth TMD (9). Patient 4 has a 2374 bp deletion, which comprises a large part of exon 3, entire intron 3 and exon 4, and part of intron 4. Patient 5 has a nonsense 733C→T mutation in exon 2, resulting in premature translation termination (Arg245stop) (9). Patient 6 has a missense 812G→A mutation in exon 3, resulting in an Arg271His substitution located in the second extracellular loop. Patient 7 has a 14-bp deletion (nucleotides 631–644), resulting in a frame shift and truncation of the protein at amino acid residue 235. Patient 8 has a G→C mutation in the acceptor splice site of exon 3, i.e. ACCT instead of AGCT. RT-PCR analysis of mRNA isolated from fibroblasts of his affected brother indicated the loss of 282 nucleotides from exon 3 and, thus, of 94 amino acids, including TMDs 4–6. Patient 9 has a 3-bp (TCT) deletion in exon 2, leading to deletion of Phe230.

### Table 1. Nine patients with mutations in MCT8

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Year of birth</th>
<th>Mutation</th>
<th>Protein</th>
<th>T3 (N, Low:2.55 nmol/liter)</th>
<th>T4 (N, 54–127 nmol/liter)</th>
<th>Free T4 (N, 11–25 pmol/liter)</th>
<th>TSH (N, 0.10–4.0 mU/liter)</th>
<th>In vitro function (% of wild-type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1997</td>
<td>del ex1</td>
<td>Absent</td>
<td>6.10</td>
<td>58</td>
<td>9.0</td>
<td>8.79</td>
<td>0*</td>
</tr>
<tr>
<td>2</td>
<td>1999</td>
<td>671C→T</td>
<td>A224V</td>
<td>4.54</td>
<td>54</td>
<td>9.3</td>
<td>3.76</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2001</td>
<td>1412T→C</td>
<td>L471P</td>
<td>5.20</td>
<td>58</td>
<td>11.2</td>
<td>1.81</td>
<td>0*</td>
</tr>
<tr>
<td>4</td>
<td>1998</td>
<td>del ex3,4</td>
<td>Truncated</td>
<td>7.28</td>
<td>58</td>
<td>11.2</td>
<td>3.99</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>2002</td>
<td>733C→T</td>
<td>R245X</td>
<td>3.95</td>
<td>35</td>
<td>11.2</td>
<td>4.72</td>
<td>0.0</td>
</tr>
<tr>
<td>6</td>
<td>1987</td>
<td>812G→A</td>
<td>R271H</td>
<td>3.45</td>
<td>44</td>
<td>10.6</td>
<td>1.66</td>
<td>0*</td>
</tr>
<tr>
<td>7</td>
<td>1995</td>
<td>del 631–644</td>
<td>Truncated</td>
<td>3.45</td>
<td>36</td>
<td>9.6</td>
<td>1.73</td>
<td>0*</td>
</tr>
<tr>
<td>8</td>
<td>1999</td>
<td>ex3–1G→C</td>
<td>1412T→C</td>
<td>3.17</td>
<td>42</td>
<td>8.8</td>
<td>2.97</td>
<td>0*</td>
</tr>
<tr>
<td>9</td>
<td>2002</td>
<td>del 689–691</td>
<td>P290</td>
<td>5.46</td>
<td>88</td>
<td>15.1</td>
<td>0.20</td>
<td>0</td>
</tr>
</tbody>
</table>

N, Normal range.

*Synthesis of functional protein impossible.
Figure 1A shows the uptake of T₃ by JEG3 cells transfected with wild-type or mutant MCT8 cDNA after 5 min of incubation at 37°C. Significant T₃ uptake was observed in empty pcDNA3-transfected cells. JEG3 cells do not show endogenous expression of MCT8 (14). Therefore, this background uptake is likely facilitated by other, as yet unidentified, transporter(s). Transfection with wild-type MCT8 increased T₃ uptake 2.8-fold. Transfection of cells with MCT8 mutants Ala224Val, Leu471Pro, Arg245stop, splice site mutant ex3−1G→C, and delPhe230 did not increase T₃ uptake over control cells. However, transfection with the MCT8 Arg271His mutant induced a modest but significant 1.4-fold increase in T₃ uptake (P < 0.05). Uptake experiments using 100 nM T₃ produced similar results (data not shown).

JEG3 cells transfected with control plasmid or MCT8 cDNA alone did not show significant metabolism of T₃, indicating very low deiodination capacity of these cells (data not shown). Figure 1B shows that transfection with D3 cDNA resulted in 11% metabolism of T₃ after 4 h. Cotransfection of cells with wild-type MCT8 and D3 greatly increased T₃ metabolism to approximately 60%. Cotransfection of cells with the MCT8 Ala224Val, Leu471Pro, Arg245stop, or delPhe230 mutants and D3 did not increase T₃ metabolism compared with D3 transfection alone. However, cotransfection with the MCT8 Arg271His mutant and D3 showed 20% metabolism, again indicating that some T₃ transport is preserved in this mutant.

D3 activity in lysates of JEG3 cells cotransfected with the different MCT8 mutants amounted to 85–123% of that in cells cotransfected with wild-type MCT8 (Fig. 1D), indicating that the impaired T₃ metabolism in intact cells cotransfected with mutant vs. wild-type MCT8 is indeed attributable to inhibited T₃ uptake rather than decreased D3 expression. IB (Fig. 1C) showed marked expression of wild-type MCT8 and of mutants Ala224Val and Arg271His, little expression of Leu471Pro and delPhe230, and no expression of splice site mutant ex3−1G→C and Arg245stop. Control cells transfected with pcDNA3 also showed no expression of MCT8. ICC demonstrated marked plasma membrane expression of wild-type MCT8 and mutant Arg271His, whereas Ala224Val was mainly localized in the cytoplasm. Leu471Pro, ex3−1G→C, and delPhe230 showed very little expression of protein (data not shown).

**Discussion**

We present nine unrelated young males with severe X-linked psychomotor retardation and elevated serum T₃ levels...
with mutations in the MCT8 gene. We show that these mutations result in loss of function, demonstrated as reduced uptake and subsequent metabolism of T₃ in vitro. Several mechanisms may be involved in this loss of function, including reduced protein expression, impaired trafficking to the plasma membrane, or reduced substrate affinity. The mutant Ala224Val protein is clearly detectable by IB. However, ICC shows the protein to be mostly distributed in the cytoplasm, suggesting that this mutation inhibits trafficking to the plasma membrane. Much less Leu471Pro protein is detected by IB and ICC, suggesting that loss of function is correlated with reduced expression of the protein. The premature Arg245stop found in patient 5 results in a severely truncated MCT8 protein that cannot be detected with our polyclonal MCT8 antibody and does not have any functional activity. Mutant ex3 - 1G→C is not detected by IB, and only very limited protein is observed in ICC. This suggests that the expression of this splice variant, although clearly detectable at the RNA level, is very limited at the protein level. The lack of T₃ transport by mutant delPhe230 can also be explained by the low expression of this protein. Mutant Arg271His shows significant residual transport capacity. IB shows high expression of the protein, and ICC indicates expression at the plasma membrane. Possibly, the partial loss of function is caused by reduced affinity for T₃.

Our findings associate the psychomotor retardation observed in MCT8 patients with loss of T₃ transport capacity. This illustrates that MCT8 is crucial for normal thyroid hormone-dependent development of the central nervous system in humans. Thyroid hormone plays a crucial role in processes such as cell migration, dendritic outgrowth, the formation of synapses, and myelination (16). Neurons are the major target cells for thyroid hormone, expressing T₃ receptors (17) and D₃ and MCT8 (8). Loss-of-function mutations in MCT8 lead to reduced or absent supply of T₃ to neurons, resulting in impaired neurological development as well as a reduced clearance of T₃ by neuronal D₃. The role of MCT8 in neuronal T₃ uptake was recently studied in MCT8 knockout mice by Dumitrescu et al. (18) and Trajkovic et al. (19). They showed reduced T₃ concentrations, increased type II deiodinase activity, and reduced D₃ activity in brain, reflecting local hypothyroidism, despite elevated serum T₃. Trajkovic et al. (19) also showed reduced expression of the thyroid hormone-regulated genes TRH and RC3 in neurons, supporting hypothyroid state at the cellular level. It must be noted, however, that, although MCT8-deficient mice show reduced T₃ concentrations in the brain, they do not show an apparent neurological phenotype. This suggests differences in the role of MCT8 in the development of the central nervous system between the two species.

In conclusion, the experiments presented here support the hypothesis of reduced supply of T₃ to neurons in patients with mutations in MCT8. The severe psychomotor retardation observed in these patients clearly illustrates the important role of thyroid hormone in human neuronal development.

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References


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