Supplemental Appendix

Mutated thyroid hormone transporter OATP1C1 is associated with severe brain hypometabolism and juvenile neurodegeneration

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Measurements in the cerebrospinal fluid and serum

Total tau and the 42 amino acid form of amyloid-β (Aβ1–42) were measured using INNOTEST ELISAs (Fujirebio, Ghent, Belgium). Neurofilament light (NFL) protein was analysed using the NF-light ELISA kit (UmanDiagnostics AB, Umeå, Sweden). Glial fibrillary acidic protein (GFAP) was measured using an in house ELISA based on polyclonal antibodies as previously described in detail (1). Serum concentrations of TSH, free T4, and free T3 were measured according to standard laboratory procedures at our hospital.

Brain positron emission tomography–computed tomography (PET-CT) with 18F-FDG and 18F-flutemetamol

The examination was performed using a PET-CT scanner (Siemens Biograph, Siemens Healthcare, Oslo, Norway) with time of flight system and 46-slice computed tomography. PET-CT was essentially carried out following a well-defined protocol in paediatric patients (2,3). Briefly, the patient fasted over 6 hours before the examination (water was allowed). Blood glucose concentration was normal (4.9 mmol/L). The patient lay in a quiet room with eyes covered for 10 minutes before intravenous injection of 108 MBq 18F-FDG via a cannula. She was lightly sedated for the whole procedure with 12.5 mg midazolam (a benzodiazepine). The PET-CT scan was done 30 minutes after 18F-FDG injection, performing low dose CT first, then PET. During scanning, the head was placed in a head holder to prevent movement artefacts and to make the examination more comfortable. PET imaging from vertex to brain stem in one bed position lasted 6 minutes.

On a separate occasion, 135 MBq Vizamyl (18F-flutemetamol, GE Healthcare, Oslo Norway) was injected 94 minutes before scanning from vertex to the lower edge of the cerebellum, using
one bed position for 20 minutes. Low dose CT over the same area was done as a first step. No sedation was necessary. An experienced nuclear medicine physician assessed the 18F-FDG examination by viewing uptake intensity using PET rainbow colour scale. We used cerebellum as a reference region. The 18F-flutemetamol examination was also assessed visually and interpreted as amyloid- negative (no pathology), according to the EANM Practice Guideline for Amyloid PET Imaging of the Brain 1.0 (4).

Exome sequencing, data analysis, and Sanger sequencing

DNA from the patient and parents was sheared using a Covaris sonicator (Covaris, Woburn, MA) to produce fragments with an average size of 200 bp. Paired-end adapters were ligated to the fragments and exome capture was performed with the Illumina TruSeq Exome Enrichment kit (Illumina, San Diego, CA) according to the manufacturer’s recommendations. The final amplified exome captured library was quantified using a Qubit Fluorometer (Life Technologies, Carlsbad, CA) and qPCR using primers annealing to the adapter sequences and Power SYBR Green PCR Master Mix (Life Technologies). Illumina PhiX control kit v2.0 DNA was used for standard curve generation. Fragment size distribution of the input library was measured using a 2100 Bioanalyzer and Agilent High Sensitivity DNA Chip (Agilent Technologies, Santa Clara, CA). The exome captured library was sequenced on an Illumina HiSeq2000 with 100 bp paired-end reads. A total of more than 50 million reads were generated. Reads that did not pass Illumina’s chastity filter were removed prior to alignment. The remaining reads were aligned to the reference human genome (hg19) using Burrows-Wheeler Alignment tool (5). The alignment was refined by the Genome Analysis Toolkit (GATK) (6) and PCR duplicates were marked by Picard (picard.sourceforge.net). Variant calling was performed using GATK HaplotypeCaller. Variants were annotated by SnpEff v2.0.5 (7). The three variant calling files (VCFs) generated were simultaneously analyzed using the FILTUS program (8). We discarded...
variants with allelic frequency >0.01. We also discarded variants in silico predicted benign/tolerated for protein function according to the Combined Annotation Dependent Depletion (CADD) (CADD score <15) (9). We focused on missense, nonsense, frameshift, and small insertion/deletion variants. Exome data were analyzed with autosomal recessive (homozygous and compound heterozygous) and autosomal dominant mode of inheritance. Regions of homozygosity in the patient were estimated from the exome data using the autozygosity function in FILTUS. Only high quality variants were considered in the analysis (confidence of genotype assigned being accurate (GQ)>29; MQ= mapping quality of reads supporting variant call (MQ)>45; depth (number of reads) (DP) >50), and only homozygosity regions longer than 1 centi Morgan (cM) and containing more than 15 variants or regions of any size containing >100 variants were included in the output. The presence of the missense mutation in SLCO1C1 was verified using sanger sequencing on genomic DNA derived from the patient and her healthy parents and two younger sisters using the following primers: Fwd 5’-CTGACTGGATCACACATTTCTTG-3’ and Rev 5’-TTTGATAATTTCCACACTGTTCC-3’. PCR products were purified and Sanger sequenced using an ABI 3730xl DNA analyzer and ABI BigDye dye terminator cycle-sequencing kits v3.1 (Life Technologies). Sequences were aligned using EMBL-EBI Emboss Needle pairwise alignment tool for nucleotides (ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html).

**Cell culture and transfection**

For T4 uptake studies, cells were seeded in 24-well dishes and transiently co-transfected with 80 ng pSG5 empty vector, 20 ng wild-type or mutant OATP1C1-V5, and 100 ng CRYM. For immunoblot analyses on total lysates, cells were seeded in 6-well dishes and transiently transfected with 200 ng wild-type or mutant OATP1C1-V5. For cell surface biotinylation assays, cells were seeded on 10 cm dishes and transiently transfected with 2000 ng wild-type
or mutant OATP1C1-V5. For immunocytochemistry studies, cells were cultured on 20 mm
glass coverslips coated with poly-D-lysine (Sigma-Aldrich) and transfected with 100 ng wild-
type or mutant OATP1C1-V5. X-tremeGENE 9 (Roche Diagnostics, Almere, NL) was used as
a transfection reagent according to manufacturer’s protocol and all transfections were carried
at 70% cellular confluence. Optimal plasmid concentrations were obtained from plasmid dose
titration curves (data not shown). All experiments have been performed 48 h after transfection.

**T4 uptake studies**

Cells were washed with Dulbecco’s phosphate buffered saline with 0.9 mmol/L MgCl2 and 0.5
mmol/L CaCl2 (DPBS-CM) supplemented with 0.1% bovine serum albumin and 0.1% D-
glucose (Sigma-Aldrich), and subsequently incubated for 60 min at 37 °C with 1 nM (50,000
cpm) [125I]T4 in 0.5 ml DMEM/F12 with 0.1% BSA. After incubation, cells were briefly
washed with DPBS-CM containing 0.1% BSA and lysed with 0.1 M NaOH. Radioactivity in
the cell lysates was measured with a γ-counter.

**Cell surface biotinylation assays**

Cell surface biotinylation assays were performed as recently described (10). Briefly, surface
proteins were labeled with 1 mg/mL EZ-Link Sulfo-NHS-Biotin (Thermo Fisher, Bleiswijk,
NL) in DPBS-CM. After quenching of free biotin, cells were lysed with IP buffer (50 mM Tris-
HCl, 150 mM NaCl, 10 mM EDTA, 1% Triton-X-100), containing protease inhibitor cocktail
(Roche Diagnostics). An aliquot (5% of the total volume) of the clarified lysate was stored as
input control. Biotin-labeled surface proteins were isolated by incubating clarified lysates
overnight with 50 μL pre-washed NeutrAvidin agarose beads (Thermo Fisher). Extracted
proteins were eluted by incubating the beads for 10 min at 70°C with 20 μL 4× NuPAGE lithium
dodecyl sulfate loading buffer (Thermo Fisher). The complete eluates and input samples were used for immunoblot analyses as described below.

**Immunoblot analyses**

Cells were lysed in radioimmunoprecipitation (RIPA) buffer (50 mM Tris.HCl, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche Diagnostics), sonicated and clarified by centrifugation. Protein concentrations were measured using BCA protein quantification (Thermo Fisher) and 25 µg protein was used for immunoblots as previously described (11). OATP1C1 was visualized with rabbit anti-V5 (Cell Signaling Technology, Leiden, NL; mAB #13202; RRID:AB_2687461; 1:1,000). GAPDH was used as a loading control and detected with mouse anti-GAPDH (Millipore, Amsterdam, NL; mAB #374; RRID: AB_2107445; 1:10,000) antibody. For immunoblots on total lysates, membranes were incubated for 1 hour with goat anti-rabbit or goat anti-mouse IgG antibody labelled with horse-radish peroxidase (#172-1011; RRID: AB_11125936, Bio-Rad, Veenendaal, NL; 1:3,000) in TBS-T containing 5% milk, washed, and developed using enhanced chemiluminescence reagent (Bio-Rad) using the Alliance 4.0 Uvitec platform (Uvitec Limited, Cambridge, UK).

For cell surface biotinylation assays, OATP1C1-V5 and GAPDH were visualized by Odyssey detection systems as previously described (11). Expression levels were quantified by densitometry analyses using ImageJ software (12).

**Immunocytochemistry**

Immunocytochemistry (ICC) was carried out as previously described (10). Briefly, cells were fixed with 4% paraformaldehyde, and permeabilized with 0.2% triton X-100 in PBS. Samples were blocked for 1 hour at RT in PBS containing 2% BSA, and incubated overnight with rabbit anti-V5 antibody (1:1,000) and mouse monoclonal ZO-1 antibody (Invitrogen, Breda, NL, #61-
7300, RRID:AB_2533147; 1:500). After secondary staining with goat anti-rabbit Alexa Fluor
488 (Thermo Fisher, #A11008, RRID: AB_143165, 1:1,000) and goat anti-mouse Alexa 633
(Thermo Fisher, #A21050, RRID:AB_2535718, 1:1,000), cover slips were mounted on glass
slides with Prolong Gold containing DAPI (Invitrogen) and examined on a Zeiss Meta 510
microscope, using Zeiss LSM software (Zeiss NL, Sliedrecht, NL) as previously described (10).

OATP1C1 homology modelling

An OATP1C1 homology model was constructed similarly as we have recently described for
MCT8 using YASARA Structure Software (www.yasara.org) (10,13,14). Based on sequence
similarity, percentage coverage and model quality score, the crystal structure of the E.coli
multidrug transporter MdfA (PDB#4ZP0) in inward-open conformation was selected as the
most suitable template. The models based on the top ten putative structural alignments were
checked against the available in vitro data and compared to previously published homology
models of rat OATP1C1 (15). In line with these studies, we selected the model in which
previously identified mutation sensitive residues (Asp85, Glu89, Asn92, Arg597) were located
along the substrate pore. The KAZAL-like sequence motif present in extracellular loop (ECL)
5, spanning Arg470 to Cys523, was modelled separately based on the KAZAL-type inhibitor
Infestin 4 (PDB#2ERW) and integrated into the OATP1C1 model. The final alignment is
presented in Figure S1. The resulting model was subjected to energy minimization and validated
with WHAT_CHECK (16), yielding a protein structure quality (Z)-score of -1.29 (range: <-5,
terrible to >0, optimal). A T4 molecule was docked into the substrate pore using the substrate
docking algorithm of YASARA Structure and the OATP1C1 protein model was embedded into
a lipid bilayer as previously described (10). Despite the good resolution of the original template
crystals (4ZP0, 2.00 Å; 2ERW, 1.40 Å), the relatively low similarity score between target and
template (33% to MdfA and 51% to infestin 4 based on a BLOSUM62 score>0) limits the
prediction of the exact orientation of amino acid side-chains, especially in those regions that
contain differences in the (predicted) secondary structure of target and template(s) proteins. All
images were created using YASARA Structure and Pov-Ray v3.6 software (www.povray.org).

Supplemental Figures

Figure S1 Sequence alignment of hOATP1C1 and the E. coli multidrug transporter MdfA (4ZP0)
and Infestin 4 (2ERW).
Final alignment between OATP1C1 and the *E. coli* multidrug transporter MdfA (PDB# 4ZP0) using multiple sequence alignments and position-specific scoring matrices (PSSM) implemented in YASARA Structure as described previously. Here, the sequences of OATP1C1 (target in black) and MdfA (PDB# 4ZP0; template. In red) are shown and OATP1C1 residue numbers are indicated. Only template residues that keep their position in the model are displayed. All other residues (gaps in the template) are assumed to occupy a different position in the target model and are thus placed via loop modelling. The transmembrane domains of OATP1C1 predicted based on the membrane embedded OATP1C1 structural model are underlined. The KAZAL-like sequence motif present in extracellular loop (ECL) 5 spanning
from Arg470-Cys523 was modelled separately based on the highly homologues sequence of the KAZAL-type inhibitor infestin 4 (PDB#2ERW highlighted in blue). When only taking into account the aligned residues, the sequence similarity between target and template(s) was found to be 33% for MdfA and 51% for infestin 4 (similarity was defined by a BLOSUM62 score>0. calculated by YASARA Structure). The positions of the Lys248, Asp252 and Ser389 residues are highlighted in blue and the most evolutionary conserved domain in OATPs (“signature sequence” 267-279) is highlighted in grey.

Figure S2. Pedigree of the family, Sanger sequencing of the SLCO1C1 homozygous variant

Chr12(GRCh37):g.20870143G>A; NM_001145946.1:c.754G>A; p.(Asp252Asn) and its evolutionary conservation.
(a) Family pedigree and segregation of the *SLCO1C1* mutation. The patient (filled circle) was homozygous for the mutant allele (A/A) while her parents (I-1 and I-2) and one sister (II-2) were heterozygous (G/A). The youngest sister (II-3) was homozygous for the wild-type allele (G/G).

(b) Nucleotide profile obtained by Sanger sequencing of *SLCO1C1* with the missense mutation (indicated with an asterisk) in the family (R indicates G or A).

(c) Evolutionary conservation of the Asp residue (D), mutated in the patient, in a cross-species alignment of the OATP1C1 protein (only part of the protein is shown). The asterisk indicates the amino acid mutated in the patient.

References


