When interpreting serum creatinine, it is believed that LBM should be taken into account (1). However, as shown here and previously (3), the contribution of LBM to variations in serum creatinine is small. The group of subjects studied were all females, and this may account for the low contribution. However, their LBM covered a wide range (23.0–53.7 kg), and thus it is likely that the findings here would be widely applicable.

These results suggest that although creatinine production increases with increasing LBM, there is a concomitant increase in the volume of distribution of creatinine, thereby reducing the relationship between serum creatinine and LBM. Total body water is related to LBM, and Shutte et al. (9) have shown that LBM and total plasma creatinine (plasma volume \times creatinine concentration) are well correlated.

We conclude that the contribution of LBM to serum creatinine is small and that correction of serum creatinine according to LBM is unlikely to improve the usefulness of this measurement.

References

Rapid Detection of the Two Most Common CLN2 Mutations Causing Classical Late Infantile Neuronal Ceroid Lipofuscinosis, Marek Bodzioch,1,2 Charalampos Aslanidis,3 Marek Kacinski,1 Nanbert Zhong,3 Krystyna E. Wisniewski,4 and Gerd Schmitz2 (1 Department of Child Neurology, Polish-American Children’s Hospital, Collegium Medicum, Jagiellonian University, Wielicka 265, 30-663 Krakow, Poland; 2 Institute for Clinical Chemistry and Laboratory Medicine, University of Regensburg, Franz-Josef-Strauss-Allee 11, D-93042 Regensburg, Germany; 3 Molecular Neurogenetic Diagnostic Laboratory, Specialty Clinic Laboratories and 4 Department of Pathological Neuropathology, New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Rd., Staten Island, NY 10314; * author for correspondence: fax 49-941-944-6202, e-mail gerd.schmitz@klinik.uni-regensburg.de) Neuronal ceroid lipofuscinoses (NCLs) are a group of genetically transmitted neurodegenerative disorders characterized clinically by intellectual and motor decline, visual loss, and myoclonic seizures, in most cases preceded by a variable period of apparently normal development. A common pathological feature of all NCLs is the intracellular accumulation of an autofluorescent material resembling ceroid or lipofuscin. Five genes (CLN1, CLN2, CLN3, CLN5, and CLN8) have been identified that are mutated in different forms of NCL: respectively, infantile NCL (1); late infantile NCL (2, 3); classical juvenile NCL (4–6); Finnish variant late infantile NCL (7); and the progressive epilepsy with mental retardation (EPMR, also called Northern epilepsy) (8). Adult-onset NCL (CLN4) follows either an autosomal recessive (Kufs disease) or an autosomal dominant (Parry disease) pattern of inheritance and is likely to be linked to different, as yet unknown, gene loci.

The classical late infantile and juvenile forms, by far the commonest NCLs reported in different populations, are leading causes of neurodegeneration in childhood and adolescence. More than 30 mutations, scattered along the whole CLN2 gene, have been reported in association with the classical late infantile NCL (cLINCL) phenotype (9, 10). However, studies performed on large groups of cLINCL patients demonstrated that two mutations, 636C→T and T523G→C, are particularly common (9, 11). They occur in ~60% of cLINCL chromosomes, and at least one of these mutations can be identified in >75% of patients (12). We report here (a) successful development of a real-time multiplex fluorescence PCR with two dyes for the rapid detection of these two mutations and (b) genetic analysis of five new cLINCL families from South-Eastern Poland.

We obtained DNA samples for genotyping from five previously unreported cLINCL families with 5 cLINCL patients and 18 healthy relatives. Family members gave informed consent, and the study was approved by the appropriate ethics boards. The diagnosis of cLINCL was based on the typical clinical features and characteristic ultrastructural picture with curvilinear profiles. DNA was isolated from cultured skin fibroblasts using the QIAGEN Blood and Cell Culture DNA Mini reagent set (QIAGEN). Six DNA samples (C10516, C10557, C7153, C8878, C9542, and C11488) with known genotype (12) were obtained from Batten Disease Registry, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York. For mutation detection, we used the LightCycler (Roche Diagnostics) (13, 14). The method uses two hybridization probes, labeled with a pair of fluorescent dyes, that bind adjacent to each other on a single-stranded DNA template. After excitation by a laser beam, fluorescein, a donor dye attached to the 3’ end of one probe, transfers energy to an acceptor dye, located on the 5’ end of the other probe, which in turn emits light detected by the instrument. The use of thin glass capillaries allows rapid heat transfer and short cycling times. Each pair of hybridization probes consists of a longer anchor probe and a shorter detection probe that overlaps
Fig. 1. Binding sites for oligonucleotides used in the test (A) and analysis of the LightCycler fluorescence data (B).

(A), a pair of flanking PCR primers (dotted arrows) amplify a 464-nucleotide genomic fragment of CLN2, encompassing the 3′ part of exon 5, the entire intron 5 and exon 6, and the 5′ part of intron 6 (upper case letters indicate exonic sequences). Both anchor probes (dashed lines) contain fluorescein (F) on their 3′ ends. Detection probes (solid lines), which bind two nucleotides away from the respective anchor probes, have LC-Red 640 or LC-Red 705 covalently bound to their 5′ ends and phosphoryl (ph) residues, which prevent elongation, at their 3′ ends. The mutation sites are indicated by open arrowheads.

(B), panel I shows the overall change in fluorescence intensity with increasing temperature (mutation 636) in a melting curve analysis following PCR. Panel II shows the melting peaks for mutation 636, panel III shows the melting peaks for mutation T523-1, and panel IV summarizes the results. A single peak at a higher temperature (wild-type peak) indicates the wt/wt genotype. A single peak at a lower temperature (mutation peak) indicates the mut/mut genotype. A double peak pattern is consistent with the heterozygous mut/wt genotype. The letters A, B, and C refer to patients’ samples; D is a water control. mut, mutated allele; wt, wild-type allele.
the mutation site (Fig. 1A). The probe detecting the mutation at nucleotide position 636 is labeled with a LightCycler-Red 640 dye (LC-Red 640), whereas the probe for the T523-1 site is labeled with LightCycler-Red 705 (LC-Red 705). The processing of the fluorescence data involves color compensation to extract overlapping emission spectra of the two fluorescent dyes. Both detection probes have a wild-type sequence, and the presence of the predicted mutation produces a single nucleotide mismatch between the probe and template DNA (Table 1). The nucleotide in position T523-1 is localized very close to a run of six guanines, which causes technical difficulty because the probe "slips" on the template. We have overcome this problem by disrupting the run of identical base pairs in the probe's sequence by substituting one guanine with a thymidine.

Each reaction mixture for a single sample consisted of 50–100 ng of genomic DNA, a pair of flanking PCR primers (1 μL each from a dilution of 5 pmol/μL), two pairs of anchor and detection probes (1 μL of each probe from a dilution of 4 pmol/μL), 2 μL of the enzyme/buffer mixture [LightCycler-DNA Master Hybridization Probes (Roche Diagnostics)], 1.6 μL of 25 mmol/L MgCl₂, and PCR-grade water up to a final volume of 20 μL. Denaturation was at 95 °C for 2 min, followed by 40 cycles of denaturing at 95 °C for 0 s, annealing at 57 °C for 10 s, and elongation at 72 °C for 15 s. Melting curve analysis was done by increasing the temperature gradually from the starting point at 40 °C to the endpoint at 80 °C, at a rate of 0.8 °C/s. Acquisition of the fluorescence signal was performed in a stepwise manner at each increment of temperature. Incubation at 95 °C for 0 s means a rapid blast of hot air, which is sufficient to denature all of the DNA. Genotyping was confirmed by sequencing (ABI Prism Genetic Analyzer 310; PE Biosystems).

Panel I in Fig. 1B shows the relationship between fluorescence and temperature in melting curve analyses of three samples after PCR has been completed. The decrease in fluorescence intensity represents the melting point, when the detection probe detaches from the template. Because the probe is complementary to a wild-type sequence, the presence of a mutated nucleotide produces a mismatch. The binding of the detection probe to a mutated template is weaker than to a wild-type template. Consequently, melting occurs at a lower temperature (curve C) than for a wild-type sequence (curve A). Curve B has a two-step appearance, which is consistent with a heterozygous genotype, where the two reductions in fluorescence, at lower and higher temperatures, represent the mutated and wild-type alleles, respectively. The analysis of the results is easier when the variation of fluorescence is plotted as a change of fluorescence intensity (dF) vs a change of temperature (dT; Fig. 1B, panel II). The tops of the peaks mark the exact melting temperatures for the 636 mutation.

The analysis of the other mutation (T523-1) uses the fluorescence data emitted by the second detection probe at a different wavelength. Panel III in Fig. 1B shows the same samples as in panels I and II, but this time the peaks indicate the genotype status at position T523-1. Panel IV summarizes the results of genotyping at positions 636 and T523-1 for all three patients, A, B, and C. Patient A is homozygous wild-type for the 636 nucleotide but is homozygous for the mutation T523-1 (a single low temperature peak in panel III). The reverse is true for patient C (a homozygous 636C→T mutation with both alleles wild-type at position T523-1). Patient B has a double peak pattern for both mutations, which confirms compound heterozygosity with the 636C→T mutation on one allele and the T523-1 mutation on the other allele.

Using our assay for the genotyping of five patients suspected of cLINCL, we identified in just 1 h three individuals homozygous and one patient heterozygous for the 636C→T mutation. None of them had a mutation at position T523-1. Thus, with this simple test we established a definite genetic diagnosis in three of five patients. Moreover, the finding of a single mutated allele in another patient was strongly suggestive of the diagnosis of cLINCL despite missing information about a mutation on the second allele. A heterozygous carrier state was unlikely because the patient showed concordant clinical and ultrastructural features of cLINCL. In addition, some of his healthy first-degree relatives had the same heterozygous pattern, suggesting that the affected patient had a compounding mutation in addition to the 636C→T substitution. Sequencing of the whole CLN2 gene in this patient revealed the second mutation, a 2-bp deletion, 1691–1692delTC. The CLN2 sequencing in the last patient, who had no mutations at either of the two positions screened by the LightCycler test, revealed a homozygous C→T substitution at nucleotide 1438, causing a Ser475Leu amino acid exchange. It is worth noting that this muta-

<table>
<thead>
<tr>
<th>Type</th>
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<tbody>
<tr>
<td>PCR primers</td>
<td>FLA-f</td>
<td>TACGGAACCCATGTTGTAAG</td>
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<td>FLA-r</td>
<td>GTGGTAAAGGATTGAGCACT</td>
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<tr>
<td>Detection probes</td>
<td>DET 636</td>
<td>LC-Red 640-CGTAGGCTACACACTCTGAC-ph⁵</td>
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<tr>
<td></td>
<td>DET T523-1</td>
<td>LC-Red 705-CCTACGAGTCGGGTGACCTCA-ph⁶</td>
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<tr>
<td>Anchor probes</td>
<td>ANC 636</td>
<td>TGACTCCGGGATACCCCTCGTGGA-F</td>
</tr>
<tr>
<td></td>
<td>ANC T523-1</td>
<td>GCATTACGTCATAGATCTCTCATCAAGCCTGACTT-F</td>
</tr>
</tbody>
</table>

* Sequences given 5’ to 3’. The underlined nucleotides in the detection probes exactly match the mutation sites. The T in the DET T523-1 probe indicates a thymidine, which was substituted for a guanine to disrupt a long run of identical nucleotides.

⁵ ph, phosphoryl residue; F, fluorescein.
tion, reported for the first time in a homozygous state in a family without an apparent consanguinity, affects the very serine that forms a conserved Gly-Xaa-Ser motif, common for the catalytic serine in serine peptidases (15). This observation supports the notion that TTP1 is a serine peptidase.

The described assay detects the C636T and T523-1 mutations simultaneously in just 1 h in up to 32 samples processed in a single run. Because the sample tubes are not opened following amplification, the risk of cross-contamination is low. The simplicity of sample preparation and genotype detection makes the assay easy for technicians to perform.

The distinction between the wild-type and mutant alleles relies on the presence of a mutated nucleotide that does not match the probe’s sequence. Therefore, the probe can only differentiate between a wild-type and non-wild-type base pair. In the case of CLN2, it has practical implications because in addition to the common T523-1 G→C mutation, there is a rare G→A substitution in the same nucleotide position. This substitution produces a melting peak at a temperature slightly lower than the G→C variant, and the separation of the two peaks is not sufficient to make a safe distinction between these genotypes. However, this should not be considered a flaw. Indeed, in many commonly used PCR methods of mutational analysis (e.g., restriction fragment length polymorphism) such a variant mutation may be missed, or even worse, wrongly assigned a normal status. Moreover, the G→A change in position T523-1 has the same pathogenic consequences as the main G→C variant (16) and occurs rather infrequently (9).

Current estimations of allele frequency suggest that our assay will provide a definite genetic diagnosis in ~40% of cases, whereas in another 35–40% of patients it will detect one mutated allele, which is strongly supportive of the diagnosis in the presence of typical clinical and ultrastructural features of cLINCL. Although the test does not fully substitute for sequencing, it substantially reduces the need to use this much more complicated and expensive procedure and may serve as a model for an economical approach to genetic analysis in other hereditary disorders.

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References


Identification of Two Distinct Mutations at the Same Nucleotide Position, Concomitantly with a Novel Polymorphism in the Vasopressin-Neurophysin II Gene (AVP-NP II) in Two Dutch Families with Familial Neurohypophyseal Diabetes Insipidus, André P. Abbes,1 Ben nie Bruygenman,1 Erica L.T. van den Akker,2 Marco R. de Groot,3 Anton A.M. Franken,4 Valentijn R. Drexhage,5 and Henk Engler1 (Departments of 1 Clinical Chemistry and 2 Internal Medicine, Isala klinieken, Location Sophia, Dr. C.A. van Heesweg 60, 3015 GJ Rotterdam, The Netherlands; 3 Department of Clinical Chemistry, Isala klinieken, Location Sophia, Dr. C.A. van Heesweg 2, 8025 AB Zwolle, The Netherlands; 4 Department of Endocrinology, Sophia Children Hospital, Dr. Molewaterplein 60, 3015 GJ Rotterdam, The Netherlands; 5 Department of Internal Medicine, Medisch Spectrum Twente, Dr. Ariënsplein 1, 7500 KA Enschede, The Netherlands; * address correspondence to this author at: Department of Clinical Chemistry, Isala klinieken, Location Sophia, Dr. C.A. van Heesweg 2, 8025 AB Zwolle, The Netherlands; fax 31-38-424-7610, e-mail a.p.abbes@isala.nl)

Familial neurohypophyseal diabetes insipidus (FNDI) is a rare autosomal dominant inherited disease, characterized by serious polyuria and polydipsia, caused by deficient neurosecretion of the antidiuretic hormone, arginine vasopressin (AVP). Vasopressin is a hormone that affects