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Validity of a rapid and simple fluorometric tripeptidyl peptidase 1 (TPP1) assay using dried blood specimens to diagnose CLN2 disease

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ABSTRACT

Purpose: CLN2 disease is a genetic disorder caused by dysfunction of the lysosomal enzyme tripeptidyl peptidase 1 (TPP1) that belongs to the neuronal ceroid lipofuscinoses (NCL) and leads to epilepsy, dementia, and death in young persons. CLN2 disease has recently become treatable by enzyme replacement, which can only be effective when the disease is diagnosed early. We have investigated the reliability of a test for TPP1 deficiency in dried blood specimens (DBS) to detect CLN2 disease.

Results: During a 12-year period we have received 3882 samples for testing TPP1. Quality of samples was checked by measuring two additional lysosomal enzyme activities. For 50 samples with subnormal TPP1 activity and good sample quality, we obtained adequate clinical and molecular genetic data. All 50 patients had doubtless evidence of CLN2 disease (including seven atypical patients) as shown by clinical findings and the presence of known pathogenic CLN2 variants. Our institution is a major reference center for NCL, and we have never received information that a patient with a normal DBS test was later diagnosed with CLN2 disease.

Conclusions: We consider our TPP1 test on DBS to be a reliable, convenient and inexpensive tool for a first diagnostic step in suspected CLN2 disease.

1. Introduction

CLN2 disease is a genetic storage disorder caused by dysfunction of the lysosomal enzyme tripeptidyl peptidase 1 (TPP1) and primarily affects the brain and the retina. The disease belongs to the heterogenous group of neuronal ceroid lipofuscinoses (NCL) that lead to dementia, epilepsy, loss of vision and other skills in young persons [1]. CLN2 disease typically manifests itself at around 3 years of age with a stop of psychomotor development and seizures [2]. A rapid decay of all abilities ensues and children die at around 10 years of age.

CLN2 disease has recently gained much interest as enzyme replacement therapy with supply of biosynthetically produced TPP1 into a cerebral ventricle has become available and was shown to efficaciously stop the progression of the degenerative brain disease [3]. This therapeutic procedure is useless, however, when a significant destruction of brain tissue has already occurred, which is usually the case at the time when the diagnosis is made on clinical grounds. In order to benefit from the new therapy, the diagnosis must be made earlier [4], and the inclusion of CLN2 disease into neonatal screening programs is presently being discussed. We developed a dried blood spot (DBS) test for deficiencies of TPP1 and of palmitoyl protein thioesterase 1 (PPT1), the enzyme deficient in CLN1 disease, a different type of NCL [5]. We have now investigated the reliability of this DBS test to detect CLN2 disease in a large number of samples, taking advantage of our specialized NCL clinic for the verification of diagnoses.

2. Materials and methods

During a period of 12 years (2006–2017), we have received 3882 dB samples for the determination of TPP1 activity, which was performed as previously described [5]. The time interval between sampling and analysis was usually < 4 weeks. (We have verified that a decline of TPP1 activity was not detectable when samples were stored at room temperature for one year). To check the quality of the received DBS

Abbreviations: CLN2, gene coding for TPP1; NCL, neuronal ceroid lipofuscinoses; TPP1, tripeptidyl peptidase 1; DBS, dried blood specimen; PPT1, palmitoyl protein thioesterase 1; β-Gal, β-galactosidase

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samples, we determined the activities of two additional enzymes as controls (PPT1 and beta-galactosidase). PPT1 activity was determined according to [5], beta-galactosidase activity was determined as follows: dried blood spots (3 mm) are punched from filter paper cards and distributed in a 96-well microtiter plate. Each patient sample is run in duplicates. Subsequently, 100 µL of substrate buffer (3.4 mg 4-methylumbelliferyl-b-D-galactoside from Sigma, Taufkirchen, Germany are disolved in 5.0 mL 0.1 M citrate buffer to yield a 2 mM solution), and 100 µL of citrate buffer are added to each specimen. The plate is shaken for 45 min, sealed with tape, covered with aluminum wrapping, and incubated at 37 °C for 21 h. The reaction is terminated by the addition of 200 µL stop solution (12.8 g ammonia-free glycine from ICN Biomedicals, Eschwege, Germany and 18.0 g dry Na_2CO_3 from Merck, Darmstadt, Germany in 200 mL demineralized water) and the fluorescence is read with an excitation wavelength of 365 nm and an emission wavelength of 450 nm. For the interpretation of results, all enzyme activities were considered. If one or both activities of the control enzymes were below the respective reference ranges (0.25–2.50 nmol/spot*45 h for PPT1 and 0.50–3.20 nmol/spot*21 h for beta-galactosidase), the quality of the DBS was regarded as insufficient and a new sample was requested. The overall rate of samples considered poor because of subnormal activities of more than one enzyme was 1.7%.

Reliable samples with diminished TTP1 activity were carefully checked for clinical information compatible with the diagnosis of CLN2 disease, and respective patients were subject to molecular genetic testing and confirmation of CLN2 disease by detection of known variants within the *CLN2* gene (www.ucl.ac.uk/ncl/mutation.shtml). No novel variants were found. Samples where no such information was available were excluded from the study. Sufficient information was eventually available from 50 patients who could all be evaluated at our own institution. Of these, 43 patients had a classical phenotype with onset of symptoms during the late infantile age (< 4 years) and seven had a variant clinical course, characterized by a later onset and less rapid neurological deterioration [6] (Table 1).

Table 1
Classical (late-infantile) and variant (late-onset) phenotype in 50 patients with CLN2 disease detected by DBS testing, with results of molecular genetic testing.

Clinical phenotype	Number of patients	Molecular genetic results	
Classical phenotype	N = 43		
	15[a]	c.622C > T	c.622C > T
	6	c.622C > T	c.509-1G > C
	1	c.622C > T	c.230-13T > A
	1	c.622C > T	c.1087delinsTT
	1	c.622C > T	c.1448G > A
	1	c.622C > T	c.1678_1679del
	6[b]	c.509-1G > C	c.509-1G > C
	2[c]	c.509-1G > C	c.833A > C
	1	c.509-1G > C	c.2T > A
	1	c.509-1G > C	c.311T > A
	2[c]	c.1094G > A	c.1094G > A
	1	c.1568A > G	c.1568A > G
	1	c.380+5G > A	c.380+5G > A
	1	c.229G > C	c.229G > C
	1	c.617G > A	c.617G > A
	1	c.380G > A	c.1417G > A
	1	c.225A > G	c.225A > G
Variant phenotype	n = 7		
	1	c.509-1G > C	c.380G > A
	1	c.509-1G > C	c.1049G > A
	1	c.509-1G > C	c.1439T > G
	1	c.509-1G > C	c.1333_1344dup
	1	c.3G > C	c.1261T > A
	1	c.1261T > A	c.622C > T
	1	c.1015C > T	c.731T > C

[a] 2 sets of 2 siblings.
[b] 1 set of 2 siblings, 1 set of 3 siblings.
[c] 1 set of 2 siblings.

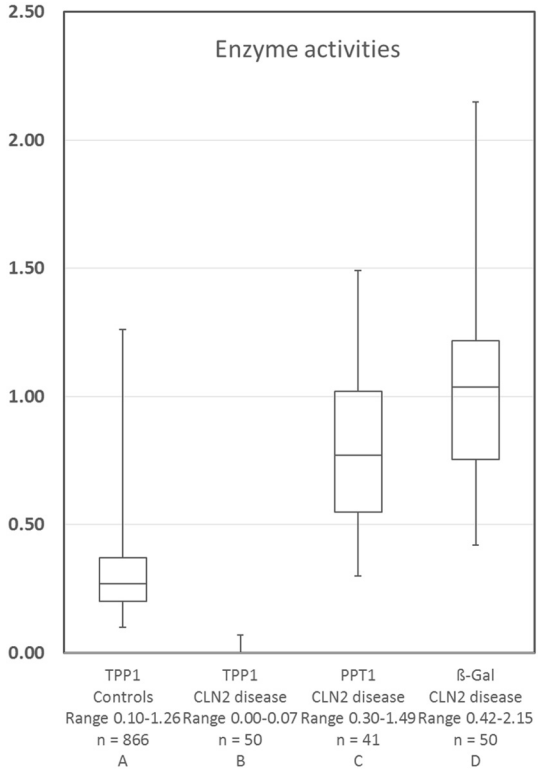

Fig. 1. Activities of lysosomal enzymes in dry blood spot samples. TPP1: tripeptidyl peptidase 1; PPT1: palmitoyl peptidase 1; β-Gal: β-galactosidase. Activities shown as nmol/spot*45 h for TPP1 and PPT1, nmol/spot*21 h for β-galactosidase. Boxplot presentation of results indicating minimum, lower quartile, median, upper quartile and maximum. A: TPP1 controls; B,C,D: TPP1, PPT1 and β-Gal activities in 50 patients with proven CLN2 disease. Normal activities of PPT1 and β-Gal in the blood spots of these patients indicated sufficient sample quality.

Diagnosis of CLN2 disease was regarded as confirmed when doubtless specific symptoms of a progressive degenerative brain disease and biallelic pathogenic variants of the *CLN2* gene were present. Second samples of previously diagnosed patients have not been counted for this study. However, no discrepant TPP1 activities were observed in any such repeat test.

3. Results

Activities of enzymes TPP1, PPT1 and beta-galactosidase in DBS samples of sufficient quality from 50 patients of whom adequate clinical information was available (male 22, female 28; median age 58 months, range 7–114 months) are shown in Fig. 1. All 50 patients with a low TPP1 activity in a DBS sample had a doubtless diagnosis of CLN2 disease, confirmed by disease-specific symptoms and molecular genetic demonstration of known pathogenic variants on both patient's alleles of the *CLN2* gene (Table 1).

4. Discussion

In this series of 50 consecutive DBS samples with low TPP1 activity, which were selected on the basis of sufficient sample quality and availability of adequate clinical information, the diagnosis of CLN2 disease was unequivocally confirmed in all cases. This speaks for a high specificity of the test when performed in the way described. A precaution of great practical value is concomitant testing of other enzyme activities in the same DBS specimen (we used PPT1 and beta-galactosidase), as low activity of more than one enzyme may indicate poor quality of the specimen, due to detrimental pre-analytical conditions.

The rejection rate for poor sample quality on the grounds of low enzyme activity of more than one enzyme was very low (1.7%). High sample quality results from good blood sampling routine, in particular from immediate spotting after the skin prick as well as proper drying and storage of the sample.

The question of the sensitivity of the test cannot be answered directly on the basis of our data, but there is strong circumstantial evidence that the sensitivity is high. Our NCL clinic has been a recognized national and international reference center for the diagnosis and care of patients with degenerative diseases of childhood for > 20 years and is in close contact with NCL research centers and NCL patient organizations in Germany and worldwide (https://cordis.europa.eu/result/rcn/161066_en.html). During the 12-year period of this study, our laboratory has communicated normal results of TPP1 activity in > 3800 dB samples to senders. We are frequently being contacted for diagnostic problems in suspected NCL disorders and would expect to have heard of discrepancies between the result of a TPP1 test on DBS and a confirmed diagnosis. However, we have not received any feedback information that a patient with normal TPP1 activity in our DBS test was later diagnosed with CLN2 disease. In addition, no discrepancy between results of the DBS test for CLN2 disease and results of enzymatic studies in fibroblasts or leukocytes or by genetic testing was brought to our attention.

It is noteworthy that seven patients with atypical clinical variants of CLN2 disease (with later onset than patients with the classical late-infantile type) also showed a diminished enzyme activity.

The fluorometric assay used in this study for the detection of suspicious cases can be modified to accommodate higher throughput for neonatal screening. Early detection of CLN2 disease in infants at an elevated risk (such as a retarded language development, which typically precedes overt symptoms [7]) or in symptom-free infants allows timely start of enzyme replacement therapy with a potentially better outcome. More recently, a new TPP1 substrate has become available for the application of mass spectrometry with modifications for mass throughput [8]. Its reliability should be properly evaluated against the fluorometric assay as used in this study. Eventually, mass spectrometry will offer the advantage of multiplexing further lysosomal diseases and thereby, expediting diagnosis of such disorders.

In conclusion, the TPP1 test on DBS as used in this study, had a high sensitivity and specificity for detecting CLN2 disease, including late-onset clinical variants. It is a convenient, inexpensive and reliable tool for a first diagnostic step in a suspected case of CLN2 disease that allows sending dry blood specimens from any region of the world to a specialized laboratory by regular mail. The test has gained particular importance as an effective therapy for CLN2 disease has become available [3].

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