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Characteristics of PPT1 and TPP1 enzymes in neuronal ceroid lipofuscinosis (NCL) 1 and 2 by dried blood spots (DBS) and leukocytes and their application to newborn screening

Rina Itagaki[a], Masahiro Endo[a], Hiroko Yanagisawa[a], Mohammad Arif Hossain[a], Keiko Akiyama[a], Keiko Yaginuma[a], Takashi Miyajima[a,b], Chen Wu[a,b], Takeo Iwamoto[a,c], Junko Igarashi[b], Yu Kobayashi[d], Jun Tohyama[d], Kazuhiro Iwama[e], Naomichi Matsumoto[e], Haruo Shintaku[f], Yoshikatsu Eto[a,g,*]

[a] Advanced Clinical Research Center, Institute of Neurological Disorder, Kanagawa, Japan
[b] Institute of Rare disease, AnGes Co., Tokyo, Japan
[c] Core Laboratory, Institute of Medical Science, Tokyo Jikei University School of Medicine, Tokyo, Japan
[d] Department of Child Neurology, Epilepsy Center, Nishi-Niigata, Chuo National Hospital, Niigata, Japan
[e] Department of Human Genetics, Yokohama City University Graduate School of Medicine, Kanagawa, Japan
[f] Department of Pediatrics, Osaka City University Graduate School of Medicine, Osaka, Japan
[g] Jikei University School of Medicine, Tokyo, Japan

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ABSTRACT

We first characterized PPT1 and TPP1 enzymes in dried blood spots (DBS), plasma/serum, and leukocytes/lymphocytes using neuronal ceroid lipofuscinosis (NCL) 1 and 2 patients and control subjects. PPT1 enzyme had only one acid form in control DBS, plasma/serum, and leukocytes/lymphocytes and showed deficient activities in these samples from NCL 1 patients. Conversely, TPP1 enzymes in control DBS and leukocytes/lymphocytes consisted of two forms, an acidic form and a neutral form, whereas serum TPP1 enzyme had only a neutral form. In control subjects, the optimal pH of PPT1 enzyme in DBS, plasma/serum, and leukocytes/lymphocytes was 4.5 to 5.0 in the acidic form, whereas TPP1 enzyme in control DBS and leukocytes/lymphocytes was pH 4.5 and 6.5, respectively. In NCL 1 and 2, both PPT1 and TPP1 enzyme activities in DBS, plasma, and leukocytes/lymphocytes were markedly reduced in acidic pH, whereas heterozygotes of NCL 1 and 2 in the acidic form showed intermediate activities between patients and control subjects. In neutral conditions, pH 6.0, the PPT1 enzyme activities in NCL 1 patients showed rather higher residual activities and intermediate activities in heterozygotes in NCL 1, which was probably caused by mutated proteins in three cases with NCL 1 patients. TPP1 enzyme activities at neutral pH 6.5 to 7.0 in DBS and leukocytes/lymphocytes showed higher enzyme activities in NCL 2 patients and heterozygotes. The reason for the increases of neutral TPP1 enzyme activities at pH 6.5 to 7.0 in NCL 2 DBS and leukocytes/lymphocytes, is obscure, but possibly caused by secondary activation of neutral TPP1 enzyme due to the absence of the acidic form. Interestingly, TPP1 activity in serum only consisted of a neutral form, no acidic form, and was not deficient in any NCL 2 patient. Therefore, we can diagnose NCL 1 patients by plasma/serum enzyme assay of PPT1, but not diagnose NCL 2 by serum TPP1 enzyme assay.

A pilot study of newborn screening of NCL 1 and 2 has been established by more than 1000 newborn DBS assays. Using this assay system, we will be able to perform newborn screening of NCL 1 and 2 by DBS.

1. Introduction

Neuronal ceroid lipofuscinosis (NCL) is a lysosomal storage disease with progressive neurodegenerative brain disorders caused by an accumulation of ceroid/lipofuscinoscine in various tissues, especially in the brain [1–3]. The clinical symptoms vary according to disease type, and NCLs were genetically classified into 14 types [1,2]. Type 1 and type 2 are the two most prevalent types. NCL 1 is caused by a deficiency of palmitoyl protein thioesterase I (PPT1), whereas NCL 2 is caused by a deficiency of tripeptidylpeptidase I (TPP1) [4,5]. Clinically, NCL 1 and

* Corresponding author at: Advanced Clinical Research Center, Institute of Neurological Disorder (in Shiyurigaoka General Hospital), Furusawa Tsuko 255, Asao-Ku, Kawasaki city, Kanagawa, Japan.
E-mail address: yosh@sepia.ocn.ne.jp (Y. Eto).

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NCL 2 are further classified according to the age of onset as follows: the infantile type (INCL), late infantile type (LINCL), juvenile type (JNCL), and adult type (ANCL) [5–7]. Especially in INCL, LINCL, and JNCL, the patient usually shows progressive rapid clinical courses and becomes bedridden within a few years associated with intractable seizure, myoclonus, and ataxia.

Currently, clinical therapeutic trials for NCL are in development, such as intraventricular enzyme replacement therapy [8,9] and gene therapy [10,11]. Recently, intrathecal administration of cerliponase alfa (Brineura™) was approved to slow the loss of ability to walk or crawl (ambulation) in children with symptoms of NCL 2 disease who are 3 years of age and older. Furthermore, gene therapy using adeno-associated virus (AAV) or lentivirus gene therapy has been used in various type of NCLs [3, 12]. Therefore, early diagnosis of NCL is essential for early treatment. For early diagnosis of NCL 1 and 2, the simplest screening method is to use dried blood spot (DBS) for high risk and newborn screening. The enzyme activities of PPT1 and TPP1 were measured by artificial fluorescent substrates [4]. This fluorometric measurement method is simple and useful to diagnose NCL 1 and 2.

Recently, a tandem mass spectrum method was used for PPT1 and TPP1 enzyme assay [13]. There were two distinct pH optima for TPP1 activity in normal fibroblasts at pH 4.5 and 7, and the acidic TPP1 activity was totally absent from NCL fibroblasts [14]. However, until now, there have been no reports examining the detailed characteristics of PPT1 and TPP1 enzymes in DBS, plasma, and lymphocytes.

In this study, we identified one acid form of PPT1 and two forms of TPP1 enzymes, acidic and neutral forms, in DBS, plasma/serum, and leukocytes/lymphocytes; in serum, the TPP1 enzyme consists of only a neutral form, pH 6.

We also investigated the measurement conditions and characteristics of NCL 1 and 2 enzymes by changing pH on PPT1 and TPP1 enzymes in NCL 1 and 2 patients, using DBS, plasma/serum, and leukocytes/lymphocytes. Furthermore, we examined these two enzyme activities in newborn DBS to investigate the possibility of newborn mass screening and established the usefulness of newborn screening using 1000 or more DBS.

2. Materials and methods

2.1. Samples

DBS were taken from 89 control individuals, one NCL 1 patient (case 1, a 5-year-old female, infantile type NCL 1 with paternal unilateral isodisomy, c20_47 del 128) [15], and two juvenile NCL 1 sibling patients (case 2, 26-year-old male and 31-year-old female, gene mutation; c550G > A/c646A > G). In addition, samples included the first patient with NCL 2 (case 1, a 6-year-old female with gene mutation; c731T > C/c.1106dupC) and second patient with NCL 2 (case 2, a 6-year-old female with gene mutation c1015C > T/ c731C > G). Eighty-five DBS from control subjects were used for TPP1 enzyme assay. All control DBS samples, plasma/serum, and leukocytes/lymphocytes were obtained by consent. Leukocytes and lymphocytes were obtained from 10 control individuals, two NCL 1 patients, and one NCL 2 patient. To determine a normal range of PPT1 and TPP1 enzyme activity of newborns, 1191 newborn DBS were used. All blood sampling was approved by the ethics committee and also permitted by patients and their parents.

2.2. Measurement of PPT1 and TPP1 activities

We measured PPT1 and TPP1 activity using DBS according to the protocol of Lukacs et al. [4]. DBS was punched into a 3 mm diameter disk. PPT1 enzyme assay in DBS was performed in a mixture of 0.64 mM 4-methylumbelliferyl-6-thiopalmitoyl-β-glucoside (MUTG; Carbosynth, EM06650) and 400 mM citrate-phosphate buffer (pH = 4.0). TPP1 enzyme measurement in DBS was performed in a mixture of 300 μM Ala-Ala-Phe-7-amido-4-methylcoumarin (SIGMA, A3401), 1 μM Pepstatin A, 10 μM trans-epoxysuccinyl-l-leucylamido-butane (E-64; SIGMA, E3132), acetate buffer (pH = 4.0), and 0.9% NaCl buffer. DBS eluate was incubated at 37 °C for 45 h. After incubation, PPT1 enzyme activity was stopped by the addition of 150 mM ethylenediamine tetraacetic acid (EDTA, pH = 11.5), and TPP1 enzyme activity was stopped by glycine-carbonate buffer (pH = 9.7). Each enzyme activity was measured as fluorescence by a microplate reader (Ex355nm/Em460nm). Other samples (plasma, serum, leukocytes, and lymphocytes) were measured by the same procedure except using 10-μL samples, and the incubation time was changed to 3 h. All assays were performed in duplicate.

2.3. Enzyme activities at different pH in controls, carriers, and patients with NCL 1 and NCL 2

PPT1 and TPP1 enzyme activities in DBS, plasma, serum, leukocytes, and lymphocytes were measured at different pH. The pH was adjusted by citrate-phosphate buffer and phosphate buffer changing from pH 3.0 to 8.0.

With this condition, PPT1 and TPP1 enzyme activities were measured changing pH in the aforementioned samples from controls, carriers, and patients.

3. Results

3.1. Measurement of PPT1 and TPP1 enzyme activities using DBS

In PPT1 enzyme activities, the mean value was 197.0 (± 115.5) nmol/L/h in 89 normal control subjects, whereas the mean values of TPP1 enzyme activities in 85 normal controls were 181.7 (± 93.7) nmol/L/h (Fig. 1A and Table 1A). Three NCL 1 patients showed markedly reduced activity, 2.0 nmol/L/h in case 1, and 6.2 nmol/L/h and 8.7 nmol/L/h in case 2 siblings. The infantile form of NCL 1 (case 1) showed more reduced residual enzyme activity compared with those of the juvenile type of NCL 1 (case 2 siblings). TPP1 enzyme activity in two NCL 2 patients were deficient, 4.4 nmol/L/h and 7.1 nmol/L/h, respectively (Fig. 1B and Table 1B). Both enzyme activities of PPT1 and TPP1 in heterozygous parents showed intermediate values as shown in Table 1.

3.2. Enzyme characteristics of PPT1 and TPP1 in NCL 1 and 2

The PPT1 enzyme in control subjects is optimal at pH 4.5 to 5.0 in DBS, plasma, serum, and leukocytes/lymphocytes (Fig. 2A–F). In NCL 1, two patients showed deficient activities at acidic condition (pH 4.0–5.0) in DBS, plasma, serum, leukocytes, and lymphocytes. PPT1 enzyme activities at approximately pH 6.0 have significantly higher residual activity at pH 6.0 in DBS and plasma of NCL 1 patients.

TPP1 activities in control DBS and leukocytes/lymphocytes have two distinct optimal activities at pH 4.0 and 6.5, respectively, but in serum there is only a neutral form (Fig. 3A-E). In patients with NCL 2, these enzyme activities were absent in acidic condition (~pH 4.0), whereas neutral TPP1 enzyme activities showed significantly higher residual activities at pH 6.0 to 7.0. Serum TPP1 enzyme activity at neutral pH showed normal values in a NCL 2 patient (Fig. 3C).

Heterozygous carriers of both NCL 1 and 2 showed intermediate PPT1 and TPP1 enzyme activities in DBS, plasma, leukocytes, and lymphocytes at acidic pH, compared with those of controls (Fig. 2–3).

3.3. Newborn screening of NCL 1 and 2 by PPT1 and TPP1 enzyme assay using DBS

In newborns (n = 1191), the mean value of PPT1 enzyme activity is 370.8 (± 174.1) nmol/L/h, whereas the mean value of TPP1 enzyme activity is 272.3 (± 125.4) nmol/L/h. Results are shown in Table 2,