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A two-dimensional protein fragmentation-proteomic study of neuronal ceroid lipofuscinoses: Identification and characterization of differentially expressed proteins

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ABSTRACT

The neuronal ceroid lipofuscinoses (NCLs) are a group of neuronal degenerative diseases that primarily affect children. Previously we hypothesized that the similarity of the phenotypes among the variant subtypes of NCL suggests that the NCLs share a common metabolic functional pathway. To test our hypothesis, we have studied several candidate proteins identified using a proteomic approach. We analyzed their differential expression and cataloged their functions and involved pathways. Forty protein peaks, differentially expressed in NCLs, were selected from two-dimensional protein fragmentation (PF2D) maps and twenty-four proteins were identified by MALDI-TOF-MS or LC–ESI-MS/MS. Six proteins were verified by further Western blotting. Our results showed that annexin A1, annexin A2, and vimentin were significantly down-regulated in NCL1, NCL2, NCL3, and NCL8 cells; galectin-1 was down-regulated in NCL1, NCL3, and NCL8 but up-regulated in NCL2 cells; and isoform 5 of caldesmon was up-regulated in all NCL cell types. The histone 2B was down-regulated in NCL3. Functional analysis showed that the differentially expressed proteins identified by PF2D could be grouped into categories of intermediate filaments, cell motility, apoptosis, cytoskeleton, membrane trafficking, calcium binding, nucleosome assembly, pigment granule and cell development. Immunocytochemistry revealed nuclear translocalization of annexin A1 in CLN2-deficient fibroblasts and abnormal distribution of L-caldesmon in cultured *CLN1*, *CLN2*, *CLN3* and *CLN8*-deficient fibroblasts. Finding differentially expressed proteins in variant NCLs, which showed disturbances of cytoskeleton, RAGE-dependent cellular pathways and decreased glycolysis provides evidence supporting our hypothesis. These findings may contribute to the discovery of molecular biomarkers and may help further elucidate the pathogenic mechanisms underlying the NCLs.

1. Introduction

The neuronal ceroid lipofuscinoses (NCLs) are a group of neuronal degenerative diseases affecting primarily children and commonly referred to as the "Batten disease", comprise one dominant and nine recessive variants as well as several rare and atypical unclassified subtypes [1]. Clinically, the NCLs are characterized by seizures, rapid deterioration of vision, progressive development of mental retardation, movement disorders, behavioral changes and early death. Pathologically, NCLs are characterized by intralysosomal accumulations of mitochondrial ATPase subunit C and ceroid lipopigments, considered to be biomarkers for the NCLs, except for NCL1 for which the sphingolipid activator proteins (SAPs) or saposins A and D [2,3] are the main protein components of the stored lysosomal lipopigment. Genetically, eight genes have been identified for the NCLs. They are *CLN1*, *CLN2*, *CLN3*, *CLN5*, *CLN6*, *CLN7* [4,5], *CLN8* and *CLN10* [6]. *CLN1*, *CLN2*, and *CLN10* encode lysosomal enzyme palmitoyl-protein thioesterase 1 (PPT1), tripeptidyl peptidase I (TPP1) and cathepsin D, respectively. *CLN7* encodes a putative lysosomal transporter, major facilitator superfamily domain-containing protein-8 [4]. However, the primary functions of the proteins encoded by *CLN3*, *CLN5*, *CLN6*, or *CLN8* are still unclear. Similar phenotypes including deregulated cell growth, apoptosis, and abnormal sphingolipid/phospholipids levels, exist

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in the variant NCLs, have been observed [7], and there are multiple points at which the NCL proteins may associate [8,9]. However, the *CLN1*, *CLN2*, and *CLN3* encoded proteins may not interact with each other [10]. Despite extensive investigation, the precise biological mechanism underlying the development of the NCLs has remained elusive [11].

Earlier studies on transcriptional and lipidomic profiling have provided information on the role of lipid metabolism, synaptic membrane trafficking and inflammation, which mediated neuronal apoptosis [12–14]. Data on transcriptional profiling obtained from homozygous *Cln1*-knockout mice [13], *Cln3*-knockout mice [13,15], *Cln5*-knockout mice [16], and *CLN6*-deficient patient fibroblasts have highlighted some metabolic pathways. This has also demonstrated by metabolic profiling of *Cln3*-knockout mice [15]. However, neither transcriptional nor metabolic profiling has provided information at the protein level that is directly relevant to the pathogenesis of the disease. Investigation of differentially expressed protein profiles of NCLs may lead to increased understanding of the mechanism of NCLs.

Although genetic heterogeneity has been demonstrated in the NCLs in which various subtypes are due to differing genes, it is unclear why the individual variant subtypes of the NCLs share similar clinical, neuropathological and biochemical features, including lipofuscin storage and accumulation of mitochondrial ATPase subunit C in all NCLs (except NCL1) and of SAPs in three variants of NCLs. To explain this, we earlier proposed a hypothesis that the clinical similarity of variant NCLs may result from a common pathogenic pathway that is shared by the individual subtypes of NCLs [17]. In this hypothesis, all *CLN* gene-encoded proteins may be, directly or indirectly, involved or linked within this pathway. An alternative possibility is that they may associate with an unidentified protein that plays a key role in the development of NCLs [17]. Proteins are the functional readout of genetic information and there can be a substantial discordance between mRNA abundance and protein expression levels [18,19]. Systematic proteomic studies are necessary for the analysis of protein activity, which can be influenced by many factors, including post-translational modifications, interactions with biomolecules or sub-cellular localization. Such information is not reflected in the genomic or transcriptomic assays. In this study, we have employed a proteomic approach, the two-dimensional liquid chromatography performed by a two-dimensional protein fragmentation (PF2D) platform, to test our hypothesis.

2. Materials and methods

2.1. Cell lines and antibody

Fibroblasts were derived from skin biopsy of controls or patients who had been identified and characterized with mutations in *CLN1*, *CLN2*, *CLN3* or *CLN8* gene that encodes PPT1, TPP1, CLN3 or CLN8 protein, respectively. These cell lines are C10 and c14768 that derived from two unrelated normal controls, c11029 and c10316 from NCL1 patients who carry compound heterozygous mutations of Y109D/R151X or R151X/c.749G>T, respectively, in the *CLN1* gene [20], c8419 with a homozygous R280X mutation from one NCL2 patient in the *CLN2* gene [21], c9282 and c8614 from two NCL3 patients with an identical mutation of homozygous1.02-kbdeletion in the *CLN3* gene [21], and c12628 from a NCL8 patient with homozygous c70C>G mutation in *CLN8* gene [22]. Antibodies used were: annexin I and annexin II monoclonal antibodies (mAb) (BD pharmingen), caldesmon mAb (Abcam), β-actin mAb or polyclonal (pAb) (Abcam), vimentin mAb (Chemicon International), and galectin-1 mAb and H2B mAb (Santa Cruz).

2.2. Cell culture and sample preparation

Fibroblasts mentioned above were grown in RPMI 1640 medium (GIBCO) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C with 5% of CO_2. All fibroblasts cell strains used in this study were at three to four passages. When monolayer cultures were 85–95% confluent, the cells were harvested and the total proteins were extracted according to the ProteomeLabTM PF2D procedure (http://www.beckmancoulter.com). Briefly, about 1×10^6 cells were rinsed with PBS, pelleted, and lysed with 2 ml of lysis buffer (6 M urea, 2 M thiourea, 10% glycerol, 50 mM Tris–HCl (pH 7.8–8.2, 10–25 °C), 2% (w/v) n-octylglucoside (octyl α-D-glucopyranoside), 5 mM TCEP ((Tris-[carboxyethyl)phosphine hydrochloride]), and 1 mM protease inhibitor). The insoluble material was removed by ultracentrifugation at 20,000 g at 4 °C for 60 min, and the supernatant was stored at −80 °C for further use.

2.3. Proteomic study with ProteomeLab™ PF2D system and mass spectrometry (MS)

Procedures of PF2D analysis were conducted following the manufacture's protocol (http://www.beckmancoulter.com), as we described earlier [10] with slight modification.

Before the first dimensional chromatofocusing separation, the cell lysate was desalted and the buffer was exchanged into start buffer (pH 8.6) using a PD 10 column (Amersham Bioscience). The protein concentration was determined using a BCA protein assay kit (Pierce) and adjusted to 1.5 mg/ml. 3 mg protein were loaded on HPCF-1D column, and fractions were collected at 0.3-pH intervals.

200 μL of each first dimensional p*I* fraction from pH 3.9 to 8.6 was further separated by reversed-phase HPLC using UV detection (214 nm) with a gradient of solution B from 0% to 100% in 30 min with solution A:0.1% TFA in water and B:0.08% TFA in acetonitrile. The fractions of 0.2 min were collected and stored at −80 °C for further analysis.

The repeatability of the chromatofocusing was assured running all the samples in parallel and identical conditions. The reproducibility of the reversed phase separations was confirmed by injecting the same first-dimensional pH-fraction (pH 4.76–5.06) twice independently. The protein peaks with UV absorption at 214 nm larger than 0.05 and the peaks with height ratio of NCL fibroblasts compared to normal fibroblasts larger than 1.2 or less than 0.8 fold were subjected to protein identification using MALDI-TOF-MS or LC–ESI-MS–MS. For MALDI-TOF-MS analysis (ABI4800, Applied Biosystems, Framingham, MA, USA), the samples were digested with trypsin for 16 h at 37 °C. The tryptic peptides were dried and extracted into 5% ACN and 0.1% formic acid for MS analysis. HCCA was the MALDI matrix. MS spectra were acquired between 600 and 3500 *m*/*z*. The five most intensive peptides with S/N exceeding 20 of each spot were selected for MS/MS analysis. Peptide identification was done with a software of MASCOT 2.1. For the LC–ESI-MS/MS analysis, the samples were dried and digested with 5 μL of trypsin (15 ng/μL) at 37 °C for 16 h. The tryptic peptides were dried and redissolved with 0.1% formic acid previous to LC–ESI-MS/MS analysis. A ThermoFinniganT linear IT mass spectrometer (LTQ) equipped with an ESI source was used. Chromatography was carried out on a LCPackings Ultimate nanoLC. About 19 μL digested peptide mixture was injected into one C18 trap column for desalting and afterwards, eluted through a PicoFritT tip column (BioBasic®18, 5 mm, 75 mm id × 10 cm, 15-mm id spray tip, New Objective, Woburn, MA, USA) using a gradient of 0.1% HA in water and 80% ACN. The LTQ-MS was operated in data-dependent mode (one full MS scan followed by five MS/MS scans on the five most intense ions) using normalized collision energy of 35%. The temperature of the ion transfer tube was set at 200 °C and the spray