CASE REPORT

Atypical CLN2 with later onset and prolonged course: a neuropathologic study showing different sensitivity of neuronal subpopulations to TPP1 deficiency

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Abstract This is the first neuropathology report of a male patient (born 1960–died 1975) with an extremely rare, atypical variant of CLN2 that has been diagnosed only in five families so far. The clinical history started during his preschool years with relatively mild motor and psychological difficulties, but with normal intellect and vision. Since age six there were progressive cerebellar and extrapyramidal symptomatology, amaurosis, and mental deterioration. Epileptic seizures were absent. The child died aged 15 years in extreme cachexy. Neuropathology revealed neurolysosomal storage of autofluorescent, curvilinear and subunit c of mitochondrial ATP synthase (SCMAS) rich material. The neuronal storage led to laminar neuronal depopulation in the cerebral cortex and to a practically total eradication of the cerebellar cortical neurons. The other areas of the central nervous system including hippocampus, which are usually heavily affected in classical forms of CLN2, displayed either a lesser degree or absence of neuronal storage, or storage without significant neuronal loss. Transformation of the stored material to the spheroid like perikaryal inclusions was rudimentary. The follow-up, after 30 years, showed heterozygous values of TPP1 (tripeptidylpeptidase 1)

activity in the white blood cells of both parents and the sister. DNA analysis of *CLN2* gene identified a paternal frequent null mutation c.622C > T (p.Arg208 X) in the 6th exon and a maternal novel mutation c.1439 T > G in exon 12 (p.Val480Gly). TPP1 immunohistochemistry using a specific antibody gave negative results in the brain and other organs. Our report supports the notion that the spectrum of CLN2 phenotypes may be surprisingly broad. The study revealed variable sensitivities in neuronal subpopulations to the metabolic defect which may be responsible for the variant's serious course.

Keywords CLN 2 · Atypical course · Neuropathology · Storage pattern

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Introduction

The family of neuronal ceroid lipofuscinoses (NCL) consists, to date, of ten entities [13], two of which still resist exact definition at the molecular level, while the others have been well defined. Three are caused by mutations of genes coding for enzymes: cathepsin D [17, 20], palmitoyl-protein thioesterase [23] and tripeptidylpeptidase 1 (TPP1) [18]; in five the mutated gene codes for transmembrane proteins or for soluble lysosomal proteins with unknown functions. All the relevant information concerning the responsible genes, mutated proteins, their structural and functional relationship to the lysosomal system, and the nature of the stored compounds, which are mainly proteins, has been reviewed [9, 13, 15]. A molecular classification has replaced the former phenotype based one and unified the diverse phenotypes under a single precisely defined disorder [16, 22, 26]. The CLN2 phenotype is generally considered to be repetitive and uniform, well defined by age of

onset (late infantile) and having specific neurological symptoms and electrophysiological findings [24]. There are only three reports referring to variants with protracted courses [11, 19, 25]. This is only the fourth report of atypical CLN2 having one novel mutation in the *CLN2/TPP1* gene and differing clinically from previously published cases with respect to the absence of epilepsy. It indicates that the phenotypic spectrum of this type of NCL may be unexpectedly broad. It is also the first neuropathology report showing different sensitivities of various neuronal systems to TPP1 deficiency.

Clinical history

A boy with an unremarkable family history (mother healthy, father treated for diabetes, one healthy sister) was born in 1960. The first problem reported by his parents was at the age of 3 years when the child experienced difficulty in trying to run. According to the parents, the child was quite clumsy and awkward. Nevertheless, at this age, he was able to feed himself using cutlery. He spoke in sentences, but with a clear lisp; speech therapy started at age 4. The boy's intellect was above average. During kindergarten he exhibited behavioral problems and had difficulty in communicating with other children. He detested noise (vacuum cleaners, washing machines) and was afraid of them. He was unable to learn to ride a bicycle during his preschool years. An investigation for regression of motor functions and mental difficulties was undertaken by neurologists and psychologists during the first class of primary school; a slight cerebral palsy with normal intellect was diagnosed. The child's ability to draw corresponded to the age of 3 years. At the age of 7 years, following a measles vaccination, his condition worsened. He was sent to hospital at the age of 8 years for thorough investigations. Routine biochemical tests of the blood, urine, and cerebrospinal fluid revealed nothing abnormal. The EEG revealed diffuse abnormalities with a parieto-occipital maximum. Episodic high-voltage, slow waves were induced by hyperventilation. A neurologist found paleocerebellar, neocerebellar, and extrapyramidal syndromes and dementia. Vision was 7/18, eye grounds were normal and skull X-rays were normal. Mental deterioration, speech, and motor difficulties progressed gradually and over time, and eventually total blindness and spasticity developed. Therapy was limited to Chlorpromazine (2×10 mg) and to vitamin B12. After several years, he became wheelchair bound and shortly thereafter, he became bedridden. The patient never experienced myoclonic or any other types of convulsions. He died at the age of 15 years (1975) in extreme cachexia from aspiration bronchopneumonia while residing in a social care institution.

The autopsy revealed pronounced cerebral and cerebellar atrophy (850 g). The substantia nigra was unpigmented.

Materials and methods

Samples of central nervous system and visceral organs (heart, kidney, liver, spleen, and lungs) were fixed in 10% formalin and processed for histology and after osmification for electron microscopy. For histology, the sections were stained using a battery of techniques used for analysis of tissues in neuronal ceroid lipofuscinoses [4, 7, 8]. In our review of the archived tissue samples, cathepsin D was detected with rabbit polyclonal antibodies (DAKO, Copenhagen, Denmark; dilution 1:2,000 or 4,000). Subunit c of mitochondrial ATP synthase (SCMAS) and tripeptidylpeptidase 1 (TPP1) enzyme protein were also detected using antibodies kindly supplied by Dr. Ezaki (Juntedo University, Tokyo, Japan). After dewaxing, the sections were incubated with the primary antibodies (diluted 1:100–200) overnight in the refrigerator, washed and exposed to the appropriate Envision™+kit (DAKO, Copenhagen, Denmark).

DNA extraction from the archived paraffin blocks of proband's spleen, brain and heart was unsuccessful. Total genomic DNA from proband's parents was isolated from Ficoll separated lymphocytes using the QIAamp Blood Mini Kit. Total RNA was isolated from the maternal blood according to the method described [2] with Trizol lysis (Invitrogen). cDNA was prepared by reverse transcription using SuperScript II Reverse Transcriptase (Invitrogen) and oligo dT_{18} primer (Invitrogen). The entire gene-coding region (13 exons) as well as 5′ and 3′ untranslated regions and exon/intron boundaries of the CLN *2* gene were examined by direct sequencing of PCR and RT/PCR products. Primers for PCR were designed according to GenBank reference sequence (NC_000011.8; NM_000391.2). PCR and RT/PCR products were prepared using thermostable DNA polymerase PPP Master Mix (Top Bio) and purified on 1% agarose gel by Wizard SV Gel and PCR clean-up System (Promega). PCR products were used as a template for cycle sequencing on capillary sequencer (ABI 3100-Avant Genetic Analyzer).

TPP1 activity was measured in leukocytes fluorimetrically using the synthetic substrate Ala-Ala-Phe-7-amido-4-methylcoumarin (Sigma A3401) accordingly [28].

Results

Storage process

Regardless of cell types, storage was characterized by accumulation of autofluorescent granules, and for the most part, was maximally expressed in central neurons which

led to variable distension of the perikarya and axon hillocks which, on occasion, were remarkably distended. The storage frequently led to neuronal regression and loss (see below). There were strong signals for SCMAS and for cathepsin D. The deposits displayed uniform curvilinear ultrastructure with variable admixture of dense homogenous droplet like deposits; no fingerprints were observed. Infrequently, the neuronal storage lysosomes displayed fusion with transformation of the stored material, a process which has been shown to exist, to variable degrees in other NCL types and is characterized by altered staining, absence of detectable SCMAS and electron densification [4, 8]. It was accompanied by either reduced or absent cathepsin D signals. The process was expressed in some of the nigral neurons but to a low degree and was rarely seen in locations typical of the classical late infantile CLN2 variants [4].

Storage distribution

Neuronal storage in the brain neocortex was uniform and was expressed in layers II, III, IV and VI, layer I being unaffected while layer V was severely depleted of neurons, severely astrogliosed and infiltrated by CD68 positive microglial phagocytes containing autofluorescent storage granules (Fig. 1a). Layer V was also somewhat spongy in appearance. Other cortical layers displayed a lesser degree of astrogliosis. Neuronal storage in the entorhinal cortex had a similar distribution with moderate neuronal depletion in layers II and IV, and was expressed in all transitional fields of the hippocampal formation (*parasubiculum, praesubiculum*), as well as in the subiculum, although, without significant neuronal reduction. In the hippocampal fields CA1–CA4, neuronal storage was restricted to the *stratum pyramidale* with decreasing order from CA1 to CA4. In both CA3 and CA4 fields it was restricted to

isolated neurons. There were discrete storage granules in the dentate gyrus neurons. The cerebellar cortex was depleted of the majority of its neurons in all layers and massively astrogliosed (Fig. 1b). There were only exceptional persisting Purkinje cells, which were loaded with storage granules. The dentate nucleus exhibited neuronal storage and only mild neuronal loss.

Neurons in the thalamus and in the basal ganglia displayed variable degrees of storage with questionable signs of regression and loss. Large striatal neurons displayed borderline storage (borderline autofluorescence and borderline SCMAS signal) and differed from the surrounding small and medium-sized neurons (Fig. 2a, b). In other parts of the CNS, storage was present without significant regression. There were neurons in the oblongata and brain stem that were either without detectable or with only borderline focal perikaryal storage especially on the floor of the 4th ventricle (Fig. 2c, d). In the spinal medulla, maximal storage was found in the neurons of the posterior columns, while being virtually absent from the bulk of the anterior horn neurons (Fig. 2e, f). Myelin staining was not significantly reduced. The ependyma displayed discrete storage. No autofluorescent granules were detectable in the choroid plexus epithelium. Signs of active demyelinization were absent. CD68 positive microglial phagocytes containing autofluorescent SCMAS positive granules were exceptional and concentrated in areas of neuronal degeneration. Occasional perivascular macrophages harbored lipofuscin with a nonspecific ultrastructure.

Extraneuronal storage was characterized by autofluorescent and SCMAS positive granules. It was observed, in decreasing order, in cardiocytes, kidney tubules, smooth muscle cells of visceral arteries, adipocytes, hepatocytes, spleen (macrophages and sinusoidal endothelium), and in vascular endothelium. Comparisons with classical CLN2 showed a major difference in hepatocytes, which displayed
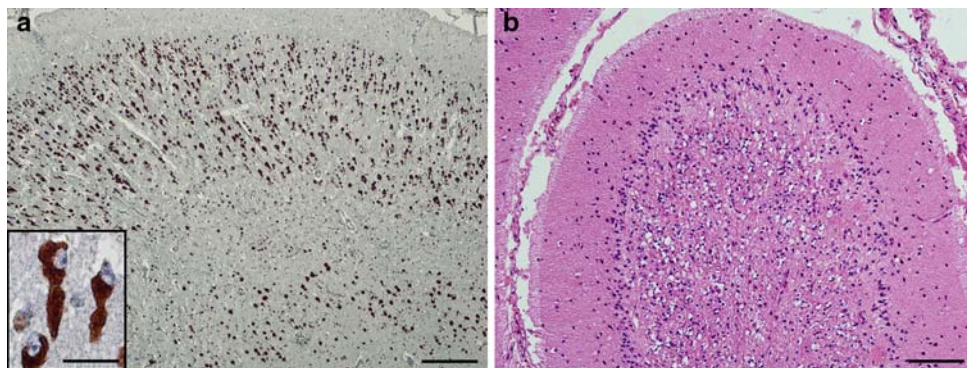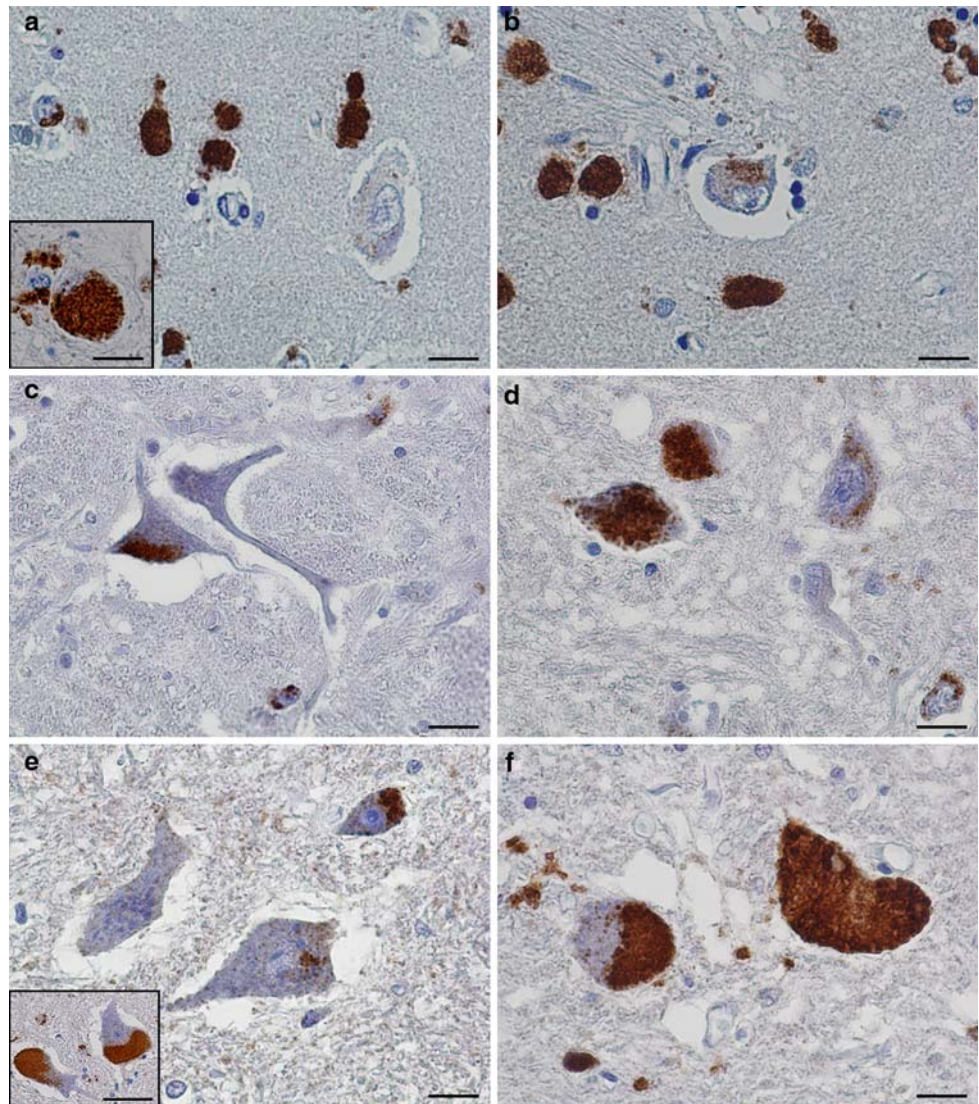

Fig. 1 **a** Paraffin section of cerebral cortex showing storage confined to layers II, III, IV, and VI. Layer V is almost totally depleted of storage neurons. SCMAS staining (bar 200 μm). *Insert* shows detail of perikaryal and axon hillock distension (bar 50 μm). **b** Cerebellar cortex with extreme depopulation of the cortical neuronal layers and pronounced gliosis in the Bergmann astrocytic layer. H&E (bar 200 μm)

much less storage in the presented case. The storage lysosomes contained curvilinear profiles. Occasionally, there was typical lipofuscin in separate lysosomal populations (hepatocytes, renal tubules).

TPP1 activities in both parents and sister showed typical heterozygote values: mother 129, father 138, sister 171 nmol mg prot^{-1} h^{-1} (controls: 202–482 (mean $\pm$ SD 297 $\pm$ 70, $n = 43$)).

Direct sequencing revealed that the proband's father and sister are carriers of frequent null mutation c.622C > T (p.Arg208X) in the 6th exon of the *CLN2* gene. In the maternal gene a novel variation c.1439T > G in exon 12 was found. Its predicted effect on protein structure is an amino acid exchange of a valine for a glycine residue at position 480 (p.Val480Gly). Sequencing of the transcript (cDNA) showed the heterozygous substitution c.1439T > G but did not reveal any effect of this transversion on mRNA splicing. Restriction analysis, using endonucleases *BsrI or ScrFI* (NEB, Ipswich, MA, USA) confirmed the substitu-

tion c.1439T > G in the mother's gene and was negative in 300 control alleles. This result together with high phylogenetic conservation of valine residue in *CLN2* gene, in 10 species, supports our consideration, that this missense nucleotide change is causative. Thus the sequence variations c.622C > T (p.Arg208X) and c.1439T > G (p.Val480Gly) confirm the diagnosis of cLINCL.

Discussion

Cases of CLN2 with atypical clinical manifestation are extremely rare. So far only seven patients (in five families) have been published [11, 19, 25]. Their clinical course consisted of progressive loss of motor functions (extrapyramidal and cerebellar signs), dementia, and in some of them uncontrollable seizures; visual impairment was variable. Formally the course corresponded to the juvenile phenotype. Dominant ultrastructure was curvilinear, but an

admixture of other profiles was also seen. No autopsy reports have been made available.

Our case shared both basic features of the NCL2 storage process, i.e. the ultrastructural appearance with the uniform curvilinear profile pattern of the stored material [6] and the extracerebral SCMAS storage pattern [7]. Therefore, the original pathology report, in the late 1970s, concluded ceroid lipofuscinosis, supposedly of the late infantile type. The case was mentioned as atypical CLN2 in the national report [5] due to the notably atypical clinical manifestation, relative to classical CLN2, which is characterized by an early, full blown onset, rapidly progressive course with fits, frequently of the myoclonic type, early amaurosis and ERG extinction, EEG with a spike response to photic stimulation, and death within the first decade. The crucial contemporary electrophysiological tests were not available at that time. The final confirmation had to wait as only recently it has been possible to contact the family and offer diagnosis verification using diagnostic criteria at the molecular level [18].

The neuropathology of this variant case, published for the first time, showed significant sparing and survival of neurons in regions extensively affected in classical CLN2 such as large striatal neurons, neurons in the medulla oblongata and spinal alpha motor neurons [1, 3, 10] as well as our unpublished observations (see inserts to Fig. 2a, e). Sparing of the hippocampal neuronal population contrasted with its complete eradication in full blown CLN2 [21]. This indicates that brain cortical neuronal damage is more severe in the six-layered neocortex than in the developmentally older cortical formations which have reduced lamination (hippocampal formation, entorhinal cortex).

The described case differed from the typical phenotype by the complete absence of epileptic paroxysms of any type. Our report strengthens the evidence that the spectrum of CLN2 phenotypes may be surprisingly broad, not only with regard to the rate of progression but also the variability of neurological manifestations. This in turn makes an intravital diagnosis and effective genetic counseling, based on established clinical criteria only, extremely difficult if even possible.

The genotype phenotype correlation is difficult. In our case, the frequent null mutation (p.Arg208X) was combined with the novel one (p.Val480Gly) which might be responsible for the variant phenotype. However, the position of the valine residue near the active center and its high phylogenetic conservation [27] does not suggest a mutation of a mild type. Mutation in the vicinity (p.Phe481Cys) was described [12] without any details of the clinical phenotype. The suggestion that the variant course described in two patients is caused by the p.Arg447His exchange at the highly conserved and protein stabilizing arginine residue [27], could not be confirmed experimentally [14]. The

genotype in the other published variant cases was also characterized by mutations with heavy impact on the enzyme at the structural level [11, 23]. These data suggest that the explanation of the variant course, at the genotype level, is hardly possible. We would like to emphasize that only when this case was studied at the cellular and tissue levels, it was possible to unravel the significantly different sensitivity of various neuronal populations to TPP1 deficiency in the described genotype. We, therefore, suggest that *different degrees of affection of brain neuronal populations* may be responsible for the variant's serious course. We would like to stress that neuropathology should be performed in other variant cases as well as this approach seems to be effective in evaluating the given genotype/phenotype correlation in individual neuronal populations.

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