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Neuronal ceroid lipofuscinosis type CLN2: A new rationale for the construction of phenotypic subgroups based on a survey of 25 cases in South America

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Supplementary Fig. 1.

ClustalW2 analysis demonstrates evolutionary conservation. (A) Arg350; (B) Ala453; and (C) Gly535.

Supplementary Fig. 2.

The location of missense mutations in the TPPI three-dimensional structure, obtained with PyMol 1.1. (A) Arg350 (light blue); (B) Ala453 (yellow); and (C) Gly535 (yellow). Amino acids depicted in pink correspond to the pro-segment (residues 20 to 195), while those in green correspond to the catalytic domain (residues 196–563). The active site according to Pal et al. 2009 (Ser475-Glu272-Asp360) is depicted in red. According to Guhaniyogi et al. 2009, the active site would be formed by Ser475-Glu272-Asp276.

Supplementary Fig.3.

Global TPPI/CLN2 mutations. Red circles highlight those found in South American families. Countries of origin (red): AR, Argentina; BE, Belgium; BR, Brazil; CA, Canada; CH, Switzerland; CL, Chile; CN, China; CZ, Czech Republic; DE, Germany; DK, Denmark; ES, Spain; FI, Finland; FR, France; GB, England; HU, Hungary; IE, Ireland; IL, Israel; IN, India; IQ, Iraq; IT, Italy; LB, Lebanon; MX, Mexico; NED, Netherlands; NO, Norway; OMA, Oman; PL, Poland; PT, Portugal; RO, Romania; RU, Russia; SE, Sweden; SK, Slovakia; TR, Turkey; US, United States; ZA, Africa. Phenotypes (blue): LINCL, late infantile, JNCL, juvenile; v, variant; prot, protracted; unk, unknown. The number of affected families is depicted in green. Global information: <http://www.ucl.ac.uk/ncl/cln2.shtm>; Country codes: <http://inf.ufrgs.br/~cabral/Paises.html>.

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Abstract

Tripeptidyl-peptidase 1 (TPP1) null or residual activity occurs in neuronal ceroid lipofuscinosis (NCL) with underlying *TPP1/CLN2* mutations. A survey of 25 South American CLN2 affected individuals enabled the differentiation of two phenotypes: classical late-infantile and variant juvenile, each in approximately 50% of patients, with residual TPP1 activity occurring in approximately 32%. Each individual was assigned to one of three subgroups: (I) n=11, null TPP1 activity in leukocytes; (II) n=8, residual TPP1 activity of 0.60–15.85 nmol/h/mg (nr 110–476); (III) n=6, activity not measured in leukocytes. Curvilinear bodies (CB) appeared in almost all studied CLN2 subjects; the only exceptions occurred in cases of subgroup II: two individuals had combined CBs/fingerprints (FPs), and one case had pure FPs. There were 15 mutations (4 first published in this paper, 3 previously observed in South America by our group, and 8 previously observed by others). In subgroup I, mutations were either missense or nonsense; in subgroups II and III, mutations prevailed at the non-conserved intronic site, c.887-10A>G (intron 7), and to a lesser extent at c.89+5G>C (intron 2), in heterozygous combinations. Grouping phenotypically and genetically known individuals on the basis of TPP1 activity supported the concept that residual enzyme activity underlies a protracted disease course. The prevalence of intronic mutations at nonconserved sites in subgroup II individuals indicates that some alternative splicing might allow some residual TPP1 activity.

Keywords

Neuronal ceroid lipofuscinoses; Tripeptidyl-peptidase 1; *TPP1/CLN2* gene; phenotype; South American population; mutational spectrum

1. Introduction

The neuronal ceroid lipofuscinoses (NCLs) are the most common group of inherited progressive neurodegenerative diseases of childhood (Goebel, 1995), and are classified as lysosomal disorders (LDs) (Cox and Cachon-Gonzalez, 2012). The childhood forms are characterized clinically by progressive motor and mental deterioration, vision loss, behavioral changes, and severe seizures, resulting in a persistent vegetative state and ending in premature death (Rider and Rider, 1999). Dementia is the main clinical symptom in the adult forms (Siintola et al., 2006a; Noskova et al., 2011; Velinov et al., 2012), and is often confused with other neurodegenerative diseases of adulthood. The storage of autofluorescent ceroid-lipofuscin-like bodies in the brain and in peripheral tissues is characteristic of these diseases (Goebel, 1995; Kida et al., 2001), which can exhibit various fine structural

phenotypes, such as granular osmiophilic deposits (GRODs), curvilinear bodies (CBs), fingerprint profiles (FPs), rectilinear bodies (RBs), or mixed. NCLs are inherited in an autosomal recessive manner, except for one autosomal dominant adult form (Noskova et al., 2011; Velinov et al., 2012). According to the newly proposed classification scheme recently published by Williams and Mole (2012), nine NCL-causative genes have been identified and can be functionally grouped as “soluble lysosomal enzyme deficiencies” *PPT1/CLN1* (Vesa et al., 1995), *TPP1/CLN2* (Sleat et al., 1997; Liu et al., 1998), and *CTSD/CLN10* (Siintola et al., 2006b; Steinfeld et al., 2006), or “nonenzyme deficiencies” *CLN3* (The International Batten Disease Consortium, 1995), *DNAJC5/CLN4* (Noskova et al., 2011; Velinov et al., 2012), *CLN5* (Savukoski et al., 1998), *CLN6* (Gao et al., 2002; Wheeler et al., 2002; Arsov et al., 2011), *MFSD8/CLN7* (Siintola et al., 2007), and *CLN8* (Ranta et al., 1999). Remaining genes whose classification is still uncertain are *CLCN6* (Poet et al., 2006), *SGSH* (Sleat et al., 2009), *CLN9* (Schulz et al., 2004), *GRN/CLN11* (Smith et al., 2012), *ATP13A2/CLN12* (Bras et al., 2012), *CTSF/CLN13* (Tang et al., 2006), and *KCTD7/CLN14* (van Bogaert et al., 2007; Krabichler et al., 2012; Staropoli et al., 2012).

Mutations in the *TPP1/CLN2* gene located on chromosome 11p15.4 (Gene ID: 1200) result in a deficiency of the lysosomal enzyme Tripeptidyl-peptidase 1 (TPP1; EC 3.4.14.9) (CLN2; OMIM ID: 204500) (Zhong, 2005). Pathogenic *TPP1/CLN2* mutations were assumed to typically result in the classical late infantile phenotype (LI-NCL), with onset of symptoms between 2 and 4 years of age, including seizures, progressive encephalopathy, visual failure, and motor abnormalities (Jalanko and Braulke, 2009). Pathologic material has been characterized in the literature by lysosomal deposits with mainly CB morphology (Wisniewski et al., 1999). Variant forms with a later onset or protracted disease course have been reported worldwide in a small number of patients (Hartikainen et al., 1999; Wisniewski et al., 1999; Zhong et al., 2000; Steinfeld et al., 2002; Bessa et al., 2008; Elleder et al., 2008). In most cases, the phenotype of these individuals resembled juvenile forms (J-NCL) and the patients were usually compound heterozygotes (Mole et al., 2005). In addition to the typical CB morphology observed in the “classical” LI-NCL phenotype, electron microscopy revealed FPs (Kohan et al., 2009) and occasional GRODs (Williams et al., 1999). A variant infantile phenotype described in a few CLN2 patients, with onset before the first year of age, was also reported (Simonati et al., 2000; Ju et al., 2002).

CLN2 is the prevalent genotype in South America, and is recognized in individuals with the classical LI and milder vJ presentations (Kohan et al., 2009; Kohan, 2011). Null or residual TPP1 activity occurs in NCLs with underlying *TPP1/CLN2* mutations. The aim of this study was to determine whether the two subtypes, null or residual TPP1 activity, correlate with the mutational spectrum, clinical phenotypes, and morphotypes, to support consistent differences among them.

2. Material and methods

2.1. Study participants

We profiled the phenotypes and genotypes of 25 CLN2 individuals (n=9 retrospective (Kohan et al., 2009) and n=16 prospective) belonging to Argentinean and Chilean families. Controls for enzyme and molecular studies were obtained from volunteer donors from a similar ethnic population. Each individual was assigned to one of three subgroups: (I) null TPP1 activity in leukocytes; (II) residual TPP1 activity; and (III) activity not measured in leukocytes. Subgroup III comprised three Chilean patients for whom dried blood spots (DBSs) were the only available sample, and three additional retrospective individuals related to other NCL patients, who died before the implementation of the NCL Program in Argentina. All procedures were performed in accordance with the Universal Declaration on Bioethics and Human Rights, UNESCO-2005. The investigation was approved by the CIEIS

- Polo Hospitalario of the Province of Córdoba, Argentina (registration no. 132). All study participants provided written informed consent for participation in the research project and authorization to release medical information.

2.2. Determining TPP1 enzyme activity

TPP1 activity was evaluated in samples of leukocytes (the gold standard) (Sohar et al., 1999; Sohar et al., 2000), saliva (Kohan et al., 2005), and/or DBSs (Lukacs et al., 2003), according to previously published methods. Palmitoyl protein thioesterase 1 (PPT1; EC 3.1.2.22) activity was measured as a control of the sample's integrity (van Diggelen et al., 1999). Briefly, the substrate for TPP1, 0.25 mM Ala-Ala-Phe-7-amido-4-methylcoumarin (Sigma-Aldrich, Buenos Aires, Argentina) diluted fresh from a 25 mM stock solution in dimethyl sulfoxide and kept at -20°C was prepared in 0.15 M sodium chloride, 1 g/L Triton X-100, 0.1 M sodium acetate, at pH 4.0 at 20°C (Sohar et al., 1999; Sohar et al., 2000). The leukocyte pellets were prepared as published (Kohan, 2011). The saliva (+/-0.3 ml) was collected with a disposable Pasteur pipette from the mouth under the tongue after at least 3 h of fasting, without any mouth cleansing. It was cleared by centrifugation at 15,000 × g for 45 min. The samples were stored at -20°C until assayed (Kohan et al., 2005). Protein measurements were performed according to the method of Lowry et al. (1951). The assay fractions contained 3 µg/10µL protein in a final volume of 0.5 mL. DBSs (3 mm) were punched from filter paper (S&S903) into Eppendorf tubes (Lukacs et al., 2003). Each spot was eluted with 20 µL substrate buffer and 40 µL NaCl (9 g/L). The tubes were shaken for 45 min at room temperature. Incubation times were 1 h for leukocyte pellets, 24 h for saliva, and 45 h for DBSs at 37°C. The reaction was stopped by the addition of 0.1 M monochloroacetic acid/0.13 M NaOH/0.1 M acetic acid, pH 4.3. Measurements were performed using an LS 50 B fluorometer (Perkin Elmer, Waltham, MA, USA) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Product formation was converted from fluorescent units to nanomoles using 7-amino-4- methylcoumarin calibrators.

2.3. Transmission electron microscopy

Transmission electron microscopy (TEM) was performed on skin biopsies. These were fixed in 1.5% Karnovsky/1% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.2), and embedded in Durcupan (Sigma-Aldrich, Buenos Aires, Argentina). Thin sections were cut with a diamond knife on a JEOL JUM-7 ultramicrotome (Nikon, Tokyo, Japan), stained with uranyl acetate and lead citrate, and examined with a Zeiss LEO 906-E electron microscope equipped with a Megaview III digital camera (Oberkochen, Germany).

2.4. PCR amplification

Blood samples were collected in tubes containing ethylenediamine tetraacetic acid (EDTA), and genomic DNA was extracted using the Wizard® DNA Purification Kit (Promega, Fitchburg, WI, USA), according to the manufacturer's instructions. Each of the 13 exons and the adjacent intronic sequences in the *TPP1/CLN2* gene (**GenBank ID: AF017456.1**, with nucleotide 1 counted as the first nucleotide of the translation initiation codon) was amplified using previously published intron-based primers (Liu et al., 1998). PCR amplification was performed using *AmpliTaq* Gold (Roche, NJ, USA) and an amplification protocol comprising an initial hold of 5 min at 95°C; 30 cycles of 30 sec at 95°C, 30 sec at 62°C, and 1 min at 72°C; and an extension step of 5 min at 72°C. PCR products were purified using the QIAquick Multiwell PCR Purification System (Qiagen, Valencia, CA, USA), and the purified products were sequenced bi-directionally on an ABI 3730XL capillary gel electrophoresis system (Applied Biosystems, Inc., Foster City, CA, USA) using the same primer sets mentioned above (Liu et al., 1998). Positive mutations and

polymorphisms were identified by comparing bi-directional sequence data against normal control sequence, and were confirmed by independent re-amplification and bi-directional sequencing from the patients' original DNA. Polymorphisms were re-named according to the SNP database (<http://www.ncbi.nlm.nih.gov/snp?term=tp1%20human>).

2.5. Experimental validation of DNA changes

A series of complementary methods were used to differentiate pathogenic mutations from common polymorphisms in the population (den Dunnen and Antonarakis, 2000). New missense changes were presumed pathogenic through analysis of co-segregation of the change in the parents. Direct sequencing was used to verify their absence in 200 chromosomes from healthy individuals of the same ethnic group.

2.6. Bioinformatic analysis

A variety of web tools were used for bioinformatic analysis of the new missense mutations. We used the ClustalW2 program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) to set the alignment of TPP1 sequences from different species in the protein sequence region of the new missense changes with UniproKW (<http://www.uniprot.org>). Polymorphism Phenotyping-2 (PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2>) (Adzhubei et al., 2010) predicted the possible impact of an amino acid substitution on the structure and function of a human protein through direct physical and comparative considerations. The program Sorting Intolerant from Tolerant (SIFT, <http://sift.jcvi.org>) (Ng and Henikoff, 2003; Kumar et al., 2009) sorted missense substitutions and classified them as tolerated or deleterious. The program PopMusic 2.1 (<http://babylone.ulb.ac.be/popmusic>) (Dehouck et al., 2011) predicted changes in the thermodynamic stability of proteins caused by mutations, using a statistical potential linear combination whose coefficients depend on the solvent accessibility of the mutated residue. Finally, the Protein Data Bank (PDB, <http://www.rcsb.org/pdb/home/home.do>) and PyMOL version 1.1 (<http://www.pymol.org>) were used to analyze the three-dimensional structure of TPP1 protein (**PDB ID: 3EDY**) and the location of the new mutations.

3. Results

3.1. Patient classification

The phenotypes and genotypes of the 25 CLN2 affected individuals were profiled through clinical data collection, enzymology, electron microscopy, and genotyping. The individuals were separated into three subgroups: I, null TPP1 activity; II, residual TPP1 activity of any magnitude in leukocytes (exceptionally, one result in saliva); and III, enzyme activity obtained in DBSs or not measured (Table 1). Because the DBS technique often gives false negative results owing to its low sensitivity, DBS results were not considered for the classification of individuals in subgroups I or II. The residual TPP1 activity in leukocytes ranged from 0.60 to 15.85 nmol/h/mg protein, for a control range of 110–476 nmol/h/mg protein. Among subgroup II individuals, the percentage of residual TPP1 activity in leukocytes ranged from 0.35% to 9.30% of the mean for the controls, occurring in approximately 32% of the studied CLN2 subjects (n=8 of 25).

Table 1 shows the relevant phenotypic data and the mutational spectrum of each individual in the three subgroups. The presented data are age at onset, present age or age at death, TPP1 activity, morphotypes with CBs or combined with FPs, designation of mutations, and polymorphisms. The genotypes of all CLN2 patients rendered a total of n=15 mutations; of these, eight were known worldwide, three had been observed previously in South America by our group (Kohan et al., 2008; Kohan et al., 2009), and four were novel (Table 2). The novel mutations comprised three missense mutations (E8 p.Arg350Trp, E11 p. Ala453Asp,

and E13 p.Gly535Arg) and one deletion (E9 c.1107-1108delTG). Pedigrees of some interesting families are shown in Fig. 1.

3.2. Experimental validation of novel missense mutations in the TPP1/CLN2 gene

To rule out the possibility of common South American sequence polymorphisms, direct sequence analysis of 200 samples from healthy individuals in Argentina's general population was performed for each of the three missense mutations identified for the first time in the prospective subjects of this study. No mutation change was identified in any of the 200 control samples. Co-segregation studies among direct family members and bioinformatics programs were also applied to support their validation as disease-causing mutations.

3.3. Bioinformatic predictions for new missense pathogenic mutations

Three novel missense mutations were analyzed using bioinformatics tools: p.Arg350Trp (exon 8), p.Ala453Asp (exon 11), and p.Gly535Arg (exon 13). The scores obtained for each of the analyzed missense mutations applying Polymorphism Phenotyping-2 indicated the likelihood of harmful effects, as they were all within a value that was likely damaging (data not shown). SIFT results showed that no substitutions were tolerated by the TPP1 protein structure (data not shown). Popmusic 2.1 predicted that E8 p.Arg350Trp, E11 p.Ala453Asp, and E13 p.Gly535Arg would be destabilizing mutations (data not shown). ClustalW2 alignment of the TPP1 sequence with the mutations showed the selected residues to be highly conserved when compared among nine different species (Supplementary Fig. 1). The three-dimensional structure of TPP1 (**PDB ID: 3EDY**) obtained through the application of PDB Protein Workshop 3.3 and PyMOL 1.1 programs is shown in Supplementary Fig. 2 (the positions of the different South American new missense mutations are depicted by arrowheads). The proximity between the sites of the new missense mutations and the TPP1 active site is notable.

4. Discussion

The occurrence of 30 cases of NCL disorders was first published in Argentina by Taratuto et al. almost 20 years ago (Taratuto et al., 1995). It was later observed that the disease, caused by mutations in the *TPP1/CLN2* gene, is prevalent in the region, and genetic findings were published for CLN1 and CLN2 by our group as part of a systematic detection program implemented in Córdoba, Argentina (Kohan et al., 2005; Noher de Halac et al., 2005; Kohan et al., 2009; Kohan, 2011).

In the literature, TPP1-deficient individuals were usually classified as one of three phenotypes according to their age at onset (Wisniewski et al., 2001; Williams et al., 2006): late infantile (LI), variant juvenile (vJ), or variant infantile (vI). The LI-NCL phenotype is the most abundant CLN2 form globally, characterized by early-onset seizures (2–4.5 years onset age), progressive encephalopathy, and visual failure; the vJ-NCL phenotype is characterized by a protracted course and later onset age (2.5–10 years of age), and the rare infantile form features onset at approximately 1 year of age (Simonati et al., 2000; Ju et al., 2002). We propose a new rationale for grouping the CLN2 phenotypes under the indicator trait of a quantifiable feature, such as the amount of TPP1 enzyme activity (Table 1): subgroup I “null TPP1 activity”, subgroup II “residual TPP1 activity”, and subgroup III “TPP1 activity not measured in leukocytes”. Notably, the residual TPP1 activity in leukocytes corresponded to a milder phenotype in five of eight individuals classified in subgroup II; although the remaining three subjects experienced an earlier age of onset than the other individuals in subgroup II, it is still too early to know whether the later course of the disease will exhibit a milder phenotype in these patients. Although significant variation

in the ability of different observers to detect the onset of signs is highly likely, it is remarkable that the clinical data of the South American patients show a clear tendency: a more severe phenotype appeared within subgroup I, with ages of onset ranging from 3 to 5 years (mean age of onset 3.60 years; SD=0.65 years) and a lifespan of 15 years or fewer. In subgroup II, the ages of onset ranged from 2 to 9 years (mean age of onset 5.56 years; SD=3.04 years) and the lifespan was up to 36 years. In the six individuals in subgroup III, with TPP1 enzyme assays not performed or performed only using DBSs, molecular studies were performed because the clinical picture and morphotypes were compatible with a TPP1 deficiency. Although DBSs are useful for a first diagnostic approach, the results must be confirmed in leukocytes and/or saliva (Lukacs et al., 2003; Kohan et al., 2005). However, the clinical data from patients of subgroup III reinforce the high frequency of the CLN2 vJ phenotype in our region.

Morphotypes different from the typical CBs were observed in three individuals from subgroup II (Table 1), who exhibited combined or pure FPs. The significance of these findings is still unclear, and more detailed studies are needed to determine correlation traits for this variability.

A total of 15 pathogenic mutations were recognized (seven apparently unique to the South American population, and eight identified throughout the world). The eight pan-ethnic mutations include two nonsense (E3 p.Gln66* and E6 p.Arg208*), three missense (E8 p.Arg339Gln, E11 p.Arg447His, and E11 p.Ser475Leu), and three intronic (I2 c.89+5G>C, I5 c.509-1G>C, and I7 c.887-10A>G) mutations, all of which were described earlier in Europe, while the seven South American mutations include one nonsense (E4 p.Leu104*), one deletion (E9 c.1107-1108delTG), and five missense (E7 p.Asp276Val, E8 p.Arg350Trp, E11 p.Ala453Val/p.Ala453Asp, and E13 p.Gly535Arg) mutations (Tables 1 and 2). Of these, the E8 p.Arg350Trp, E11 p. Ala453Asp, E13 p.Gly535Arg, and E9 c.1107-1108delTG mutations are first being documented in this publication.

The most frequent mutations were E7 c.827A>T/p.Asp276Val, in 14 alleles (28%), followed by I7 c.887-10A>G in seven alleles (14%), E6 c.622C>T/p.Arg208* in six alleles (12%), and E4 c.311T>A/p.Leu104* and E3 c.196C>T/p.Gln66* in three alleles each (6% each). The allele frequency of each globally known or South American mutation with regard to enzyme phenotypes is shown in Table 2. The three new missense mutations were proved to be disease-causing mutations, following the recommendations provided by the Human Genome Organization (HUGO) (<http://www.hgvs.org/entry.html>): 1) the entire *TPP1/CLN2* gene was sequenced, and no other DNA changes were observed (with exception of polymorphisms); 2) every mutation was validated by co-segregation studies (parents and other family members were evaluated for carrier status); 3) they were excluded as common regional polymorphisms by direct sequencing performed in 200 control chromosomes from individuals of the same ethnic background. The pathogenicity of mutations was also deduced by the use of a combination of bioinformatics experiments to predict the theoretical degree of pathogenic potential. The consistency and coherence of the results achieved by the different resources is remarkable (Supplementary Figs. 1 and 2).

The only two intronic mutations located in non-consensus sites (I7 c.887-10A>G and I2 c.89+5G>C, both found in heterozygous combinations) correlate well with the presence of residual TPP1 activity within subgroup II, independent of the age of onset and the second allele (Table 1). Aberrant splicing may be caused by intronic or exonic DNA changes that exclude an entire exon, activate cryptic splice sites within the intron that led to inclusion of intronic sequences, exclude exonic sequences, promote inclusion of pseudo-exons in the mature transcript, generate multiple abnormal transcripts, or result in a combination of several of these events (Wilton and Fletcher, 2011). It is generally accepted that mutations at

the conserved splice dinucleotide consensus sites GU (donor) and AG (acceptor) disrupt pre-mRNA splicing, and those creating alternative novel acceptor and donor sites may interfere with splicing efficiency at wild-type sites. The presence of novel splice sites that compete with the wild-type sites would allow the alternative existence of normal and mutated mRNAs, which may in turn be related to some residual enzyme activity that ameliorates the pathological phenotype. In fact, the I7 c.887-10A>G mutation was extensively studied by Bessa et al. (2008). They found this intronic mutation in homozygous condition in one Portuguese patient exhibiting a protracted phenotype and residual TPP1 activity. After conducting *in vivo* and *in vitro* TPP1 mRNA and protein assays and *in silico* studies, the authors concluded that the translation of the mutant transcript produced a polypeptide with three extra amino acids after Proline 295 (p.Pro295_Gly296insGluAsnPro). Therefore, the residual enzyme activity might be directly related to the mild phenotypes observed in homozygous (in the case of the Portuguese patient) or heterozygous (in the Latin American patients) conditions because of a defectively folded protein that retains some enzyme activity, or to the possibility of alternative splicing among the wild type and the novel splice sites that generate a small percentage of TPP1 with normal activity.

There is a growing realization that splicing efficiency is a significant contributor to phenotypic variability, and the contribution of splicing to phenotype has become particularly apparent through its impact on modifying the severity of human disease and its contribution to disease susceptibility (Wang and Cooper, 2007). The identification of abnormal splicing as the primary mechanism of disease raises the possibility of therapeutic approaches that specifically target splicing. For example, antisense oligomer-mediated splice manipulation promises potential therapies for a wide range of inherited or acquired disorders (Wilton and Fletcher, 2011), and successful experiments have been described regarding *in vitro* studies of β -thalassemia (Sierakowska et al., 1996) and cystic fibrosis (Friedman et al., 1999), among other genetic entities.

Eight polymorphisms were found in the population. Of these, five were previously recognized in the SNP database (<http://www.ncbi.nlm.nih.gov/snp?term=tppl%20human>) and three were newly identified (Table 1). It is now clear that genetic variants that are linked with a phenotype, whether these are disease-causing mutations or common SNPs, must be evaluated for disruption of the correct splicing pattern. Normal genetic variation in the splicing code creates differences in splicing efficiency that may modify disease severity (Wang and Cooper, 2007).

The E7 p.Asp276Val mutation is the most common of these mutations in South America, as it is present in 14 (28%) of the *TPP1/CLN2* alleles (Table 2). To date, it has only been reported in families from Argentina and Chile (<https://www.ucl.ac.uk/ncl/cln2mutationtable.htm>). It was shared among all patient subgroups, although its homozygous state was only observed in patients without residual TPP1 activity. Only one subgroup II individual exhibited this mutation in heterozygous combination with a novel deletion (081-1004-CL, Table 1). The p.Asp276Val mutation is assumed to severely affect enzyme activity, as the Asp276 amino acid has been proposed as part of the TPP1 active site (Guhaniyogi et al., 2009; Pal et al., 2009). The pathogenic nature of p.Asp276Val is supported by studies revealing that the catalytic activity of TPP1 is reduced by 79% when Asp276 is replaced with alanine (Walus et al., 2005).

A comparative summary of the clinical, enzymatic, morphological, and genetic data from the two CLN2 subgroups in South American patients is shown in Table 3.

Table 1 lists the mutations found in this South American cohort, including those published in earlier works by our group (Kohan et al., 2008; Kohan et al., 2009) or other authors (Sleat

et al., 1999; Bessa et al., 2008; Kousi et al., 2012). We have added to the mutational spectrum those of 18 additional subjects, identifying four novel mutations. The global *CLN2* mutational spectrum is shown in Supplementary Fig. 3. The mutations found in our cohort (15.3% of all globally recognized *TPP1/CLN2* mutations) are highlighted. It is worth emphasizing the heterogeneity of mutations, which is in keeping with the multiethnic population of South America: five missense mutations and 1 nonsense mutation, one deletion, and three polymorphisms not previously described in patients from other parts of the world. The mutations were observed in both homozygous (28%, n=7) and heterozygous (72%, n=18) combinations (Table 1).

5. Conclusions

CLN2 is the most frequently observed NCL form in South America. *TPP1*-deficient individuals exhibit either null enzyme activity (in relation to a generally more severe clinical phenotype) or residual enzyme activity (associated with a mostly protracted phenotype). Clustering the data into three groups, “null *TPP1* activity”, “residual *TPP1* activity” and “activity not measured in leukocytes”, suggested that slight residual *TPP1* activity of any magnitude might have an effect on the attenuation of the phenotype. On the basis of the concept that residual enzyme activity is expected to underlie a protracted disease course, this paper creates subgroups of clinically and genetically defined *CLN2* individuals for the first time. Residual *TPP1* enzyme activity may allow a therapeutic approach based on modifier molecules (Pierret et al., 2008; Kohan et al., 2011).

The protracted *CLN2* phenotype was previously considered globally rare. In South America, the frequency is approximately 50% of affected individuals, with phenotypic characteristics not published before in a relevant number of subjects from other parts of the world.

The heterozygous combination of intronic mutations in non-consensus sites with missense/nonsense mutations correlated with residual *TPP1* activity, demonstrating that splice site mutations may allow some enzyme activity either by generating a variable percentage of alternative splicing using the wild type or mutated splice site, or by producing an incorrectly folded enzyme that still retains some activity. Some mild missense mutations may also cause incorrect protein folding that would enable a small percentage of enzyme activity in correlation with a more protracted phenotype.

The molecular studies rendered a total of 15 mutations in South America, including one deletion mutation and three novel missense mutations (Tables 1 and 2). To simplify the future genetic analysis of *TPP1*-deficient patients in our region, one might consider ruling out E7 p.Asp276Val, I7 c.887-10A>G, E6 p.Arg208X, E3 p.Gln66* and E4 p.Leu104* before starting costly complete *TPP1/CLN2* screening. At present, these five most common South American mutations comprise 66% of the detected pathological alleles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CB	curvilinear body
DBS	dried blood spot
FP	fingerprint profile
GROD	granular osmiophilic deposit
J-NCL	juvenile NCL
LD	lysosomal disorder
LI-NCL	late-infantile NCL
NCLs	neuronal ceroid lipofuscinoses
PDB	protein data bank
PolyPhen-2	polymorphism phenotyping-2
PPT1	palmitoyl protein thioesterase 1
RB	rectilinear body
SIFT	sorting intolerant from tolerant
SNP	single nucleotide polymorphism
TEM	transmission electron microscopy
TPP1	tripeptidyl-peptidase 1
vI-NCL	variant infantile NCL
vJ-NCL	variant juvenile NCL

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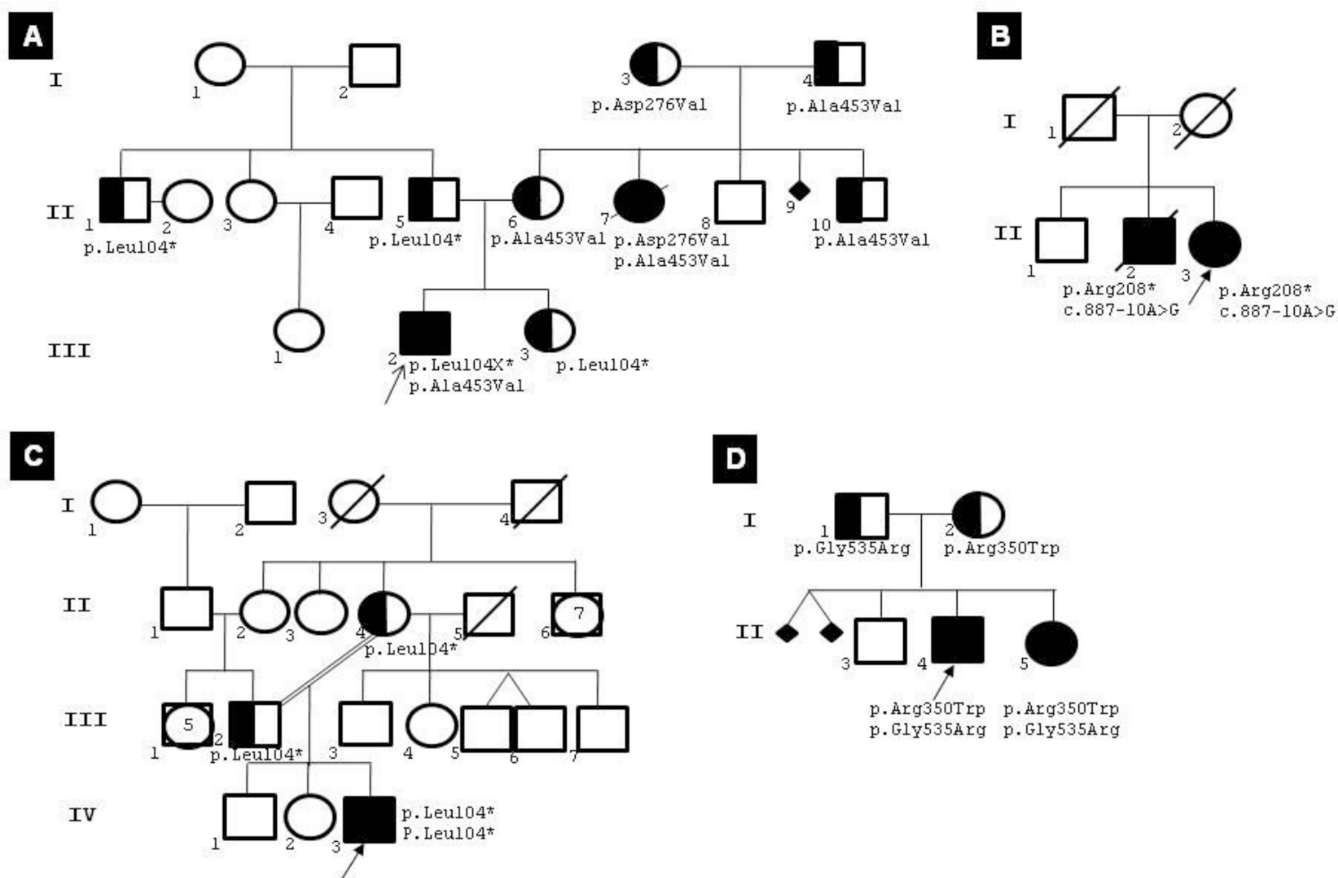


Fig. 1. Genealogies. (A) 82-2219 (III-2) and 82-1806 (II-7); (B) 173-1307 (II-3) and 173-1308 (II-2); (C) 117-1312 (IV-3); and (D) 147-1805 (II-4) and 147-1807 (II-5). Although II-7 and II-2 were not screened, their genotypes were deduced from those of their relatives.

Table 1

Subgroups I, II, and III: phenotypes and genotypes of CLN2 individuals from Argentina and Chile.

ID Number- COUNTRY	ONSET AGE	CURRENT AGE/ † AGE AT DEATH	TPPI ACTIVITY ^a	MORPHO TYPE	ALLELE 1	ALLELE 2	POLYMORPHISMS (SNPs)
SUBGROUP I: NO RESIDUAL TPPI ACTIVITY							
016-0303-AR	3 y	† 9 y	0.00	CB	E6 p.Arg208* ^e	E7 p.Asp276Val ^e	-
070-0701-AR	3 y	15 y	0.00 ^b	CB	E7 p.Asp276Val ^e	E7 p.Asp276Val ^e	-
073-0312-AR	3 y	12 y	0.00	CB	E7 p.Asp276Val ^e	E7 p.Asp276Val ^e	-
082-2219-AR	4 y	11 y	0.00	CB	E4 p.Leu104* ^e	E11 p.Ala453Val ^e	I6 c.687+32T>C, (rs1800710) ^e
117-1312-AR	3 y	10 y	0.00	CB	E4 p.Leu104* ^e	E4 p.Leu104* ^e	I6 c.687+32T>C, (rs1800710) ^e
127-0414-AR	3.5 y	8 y	0.00	CB	E6 p.Arg208*	E7 p.Asp276Val	-
134-1107-AR	4 y	11 y	0.00	CB	E7 p.Asp276Val	E7 p.Asp276Val	-
147-1807-AR	5 y	7 y	0.00	nd	E8 p.Arg350Trp	E13 p.Gly535Arg	-
153-0601-AR	?	?	0.00	CB	E6 p.Arg208*	E6 p.Arg208*	-
154-2010-AR	3.5 y	10 y	0.00	CB	I5 c.509-1G>C	E7 p.Asp276Val	I6 c.687+32T>C, (rs1800710)
162-0411-AR	4 y	7 y	0.00	CB	E8 p.Arg339Gln	E8 p.Arg339Gln	I5 c.508+26A>T, (rs1800738)
SUBGROUP II: RESIDUAL TPPI ACTIVITY							
005-1507-AR	9 y	36 y	4.80	nd	E3 p.Gln66* ^e	I7 c.887-10A>G ^e	-
005-1503-AR	9 y	† 27 y	0.60	CB+FP	E3 p.Gln66* ^e	I7 c.887-10A>G ^e	I2 c.89+4A>G ^e
081-1004-CL	2 y	9 y	2.90	CB	E7 p.Asp276Val	E9 c.1107- 1108delTTG	I5 c.508+26A>T, (rs1800738)
147-1805-AR	5 y	17 y	9.70	FP	E8 p.Arg350Trp	E13 p.Gly535Arg	I5 c.508+26A>T, (rs1800738) E12 p.Gly514Gly, (rs1128396)
149-0110-AR	3 y	8 y	8.76	nd	E11 p.Ala453Asp	I2 c.89+5G>C	I5 c.508+26A>T, (rs1800738)
149-0112-AR	2.5 y	6 y	15.85	nd	E11 p.Ala453Asp	I2 c.89+5G>C	-

ID Number-COUNTRY	ONSET AGE	CURRENT AGE / † AGE AT DEATH	TPPI ACTIVITY ^a	MORPHO TYPE	ALLELE 1	ALLELE 2	POLYMORPHISMS (SNPs)
173-1307-AR	9 y	19 y	6.45	CB+FP+ GROD	E6 p.Arg208*	I7 c.887-10A>G	-
185-1903-AR	5 y	13 y	4.16	CB	E11 p.Arg447His	E11 p.Ser475Leu	-
SUBGROUP III: TPPI ACTIVITY DETERMINED IN DBS^c / NOT DETERMINED							
005-1501-AR	9 y	† 27 y	nd	nd	E3 p.Gln6* ^d	I7 c.887-10A>G^d	-
082-1806-AR	4.5 y	† 13 y	nd	nd	E7 p.Asp276Val ^d	E11 p.Ala453Val ^d	-
099-1303-CL	3 y	16 y	0.07	CB	E11 p.Ser475Leu	I7 c.887-10A>G	-
123-0713-CL	4 y	10 y	(0.11) [#]	CB	E7 p.Asp276Val	E7 p.Asp276Val	-
126-0113-CL	10y	16 y	(0.15) [#]	CB	E7 p.Asp276Val	I7 c.887-10A>G	-
173-1308-AR	5 y †	21 y	nd	nd	E6 p.Arg208* ^d	I7 c.887-10A>G^d	-

Reference sequence: **GenBank ID: AF017456.1**; AR, Argentina; CL, Chile; y, years; nd, not determined;

^aTPPI measured in leukocytes: χ 170.5 nmol/h/mg protein; normal range 110–476;

^bTPPI measured in saliva: χ 218 nmol/24 h/mg protein; normal range 92–476;

^cTPPI measured in DBS: χ 0.26 nmol/spot; normal range 0.1–0.81;

[#]These results correspond to activities in DBSs in the control range (false negatives);

^dMolecular results deducted from studies performed on other family members or using banked DNA samples;

^eResults published previously by the authors. Note: DNA pathogenic mutations in intronic splice sites and other DNA changes in non-coding positions are designated in bold type in the table. Siblings or other family members share the first three ciphers of the notation.

Table 2

Frequency of mutated *TPPI/CLN2* alleles in the three subgroups of TPP1-deficient patients.

Mutations	No. of alleles	Subgroup I	Subgroup II	Subgroup III	Overall %
Worldwide mutations	7	n=0	n=3	n=4	14
I7 c.887-10A>G (Bessa et al., 2008)					
E6 p.Arg208* (Sleat et al., 1999)	6	n=4	n=1	n=1	12
E3 p.Gln66* (Sleat et al., 1999)	3	n=0	n=2	n=1	6
E8 p.Arg339Gln (Kousi et al., 2012)	2	n=2	n=0	n=0	4
E11 p.Ser475Leu (Sleat et al., 1999)	2	n=0	n=1	n=1	4
I2 c.89+5G>C (Kousi et al., 2012)	2	n=0	n=2	n=0	4
I5 c.509-1G>C (Sleat et al., 1999)	1	n=1	n=0	n=0	2
E11 p.Arg447His (Sleat et al., 1999)	1	n=0	n=1	n=0	2
South American mutations	14	n=9	n=1	n=4	28
E7 p.Asp276Val (Kohan et al., 2009)					
E4 p.Leu104* (Kohan et al., 2008; Kohan et al., 2009)	3	n=3	n=0	n=0	6
E8 p.Arg350Trp	2	n=1	n=1	n=0	4
E11 p.Ala453Val (Kohan et al., 2009)	2	n=1	n=0	n=1	4
E11 p.Ala453Asp	2	n=0	n=2	n=0	4
E13 p.Gly535Arg	2	n=1	n=1	n=0	4
E9 c.1107-1108delTTG	1	n=0	n=1	n=0	2

Mutations	No. of alleles	Subgroup I	Subgroup II	Subgroup III	Overall %
	50	n=22	n=16	n=12	100%

Reference sequence: **GenBank ID: AF017456.1**

Table 3

Comparative summary of CLN2 South American patients grouped according to their null/residual TPP1 activity.

CLN2	Subgroup I	Subgroup II
Onset age	3–5 y (average: 3.6 y)	2–9 y (average: 5.5 y)
Evolution time	2–12 y (average: 6.4 y)	3.5–27 y (average: 11.3 y)
Presenting symptoms	Seizures (85%)	Seizures (75%)
Epileptic syndrome	100%	83%
Altered MRI	3.5–5 y	7–11 y
Altered EEG	3 y -	11–13 y
Visual failure	5 y (77%); no (23%)	10 y (66.7%); no (33.3%)
Ataxia	3.5 y (85.7%); no (14.3%)	10 y - (100%)
Motor regression	2.5 y (100%)	7 y - (100%)
Language difficulties	2.5 y (100%)	11 y - (88.9%); no (11.1%)
Mental regression	3 y (100%)	5–11 y (100%)
TPP1 residual activity	No (100%)	Yes (100%)
Vacuolated lymphocytes	Negative (100%)	Negative (87.5%); positive (12.5%)
TEM	Pure CB (100%)	Pure CB (40%); atypical deposits (60%); CB+FP/CB+FP+GROD/FP
Mutations	<i>Novel:</i> Missense: E8 p.Arg350Trp, E13 p.Gly535Arg <i>Known:</i> Missense: E7 p.Asp276Val ^a , E8 p.Arg339Gln ^b , E11 p.Ala453Val ^a Nonsense: E4 p.Leu104* ^a , E6 p.Arg208* ^b Intronic: 15 c.509-1G>C ^b	<i>Novel:</i> Missense: E8 p.Arg350Trp, E11 p.Ala453Asp, E13 p.Gly535Arg Deletion: E9 c.1107-1108delTG <i>Known:</i> Missense: E7 p.Asp276Val ^a , E11 p.Ser475Leu ^b , E11 p.Arg447His ^b Nonsense: E6 p.Arg208* ^b , E3 p.Gln66* ^b Intronic: 17 c.887-10A>G ^b ; 12 c.89+5G>C ^b
Allele combination	Homozygous (54.5%); heterozygous (45.5%)	Heterozygous 100%

MRI, Magnetic resonance images; EEG, electroencephalogram; TEM, transmission electron microscopy; y, years;

^a mutations previously published by our group in South American patients;

^b globally recognized mutations.