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A new large animal model of CLN5 neuronal ceroid lipofuscinosis in Borderdale sheep is caused by a nucleotide substitution at a consensus splice site (c.571+1G>A) leading to excision of exon 3

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Batten disease (neuronal ceroid lipofuscinoses, NCLs) are a group of inherited childhood diseases that result in severe brain atrophy, blindness and seizures, leading to premature death. To date, eight different genes have been identified, each associated with a different form. Linkage analysis indicated a CLN5 form in a colony of affected New Zealand Borderdale sheep. Sequencing studies established the disease-causing mutation to be a substitution at a consensus splice site (c.571+1G>A), leading to the excision of exon 3 and a truncated putative protein. A molecular diagnostic test has been developed based on the excision of exon 3. Sequence alignments support the gene product being a soluble lysosomal protein. Western blotting of isolated storage bodies indicates the specific storage of subunit c of mitochondrial ATP synthase. This flock is being expanded as a large animal model for mechanistic studies and trial therapies.

Keywords: CLN5; NCL; Batten disease; mRNA splicing; Animal model; Borderdale sheep; Lysosomal storage disease

Introduction

The neuronal ceroid lipofuscinoses (NCLs, Batten disease) are a group of fatal inherited neurodegenerative diseases affecting an estimated 1 in 12,500 live births worldwide (Rider and Rider, 1999). They are characterized by severe brain atrophy and the accumulation of fluorescent lysosome-derived organelles (storage bodies) in neurons and most other cells throughout the body. Retinal degeneration is also a common feature. Affected children start life normally but then develop progressive visual failure, as well as mental and motor deterioration. They sleep poorly, suffer nightmares, hallucinations, fits and seizures which are difficult to control, and die between infancy and early adulthood. Adult onset cases have also been reported. Presently, there are no effective therapies.

A number of different mutations in at least eight genes underlie the group (see www.ucl.ac.uk/ncl), and more have been suggested. The clinical features, characteristic pathologies and ultrastructure of each form have been well described along with the genetics and biochemical characteristics (Goebel et al., 1999; Haltia, 2003, 2006). Better understanding of the genetics has led to improved diagnosis and an increase in the number of cases reported.

The NCLs are lysosomal storage diseases in which protein is stored in lysosome-derived organelles. Mutations in lysosomal enzymes are responsible for some forms. Mutations (i) in palmitoyl protein thioesterase 1 (PPT1) cause the infantile CLN1 form (Vesa et al., 1995), (ii) in cathepsin D cause a congenital form (Siintola et al., 2006; Tyynelä et al., 2000), (iii) in tripeptidyl peptidase 1 (TPP1) cause the classical late infantile CLN2 form (Sleat et al., 1997) and (iv) in a soluble lysosomal protein of unknown function cause the variant late infantile CLN5 form (Bessa et al., 2006; Holmberg et al., 2004, 2000; Pineda-Trujillo et al., 2005; Savukoski et al., 1998). Another group of proteins, all of unknown function, reside in the lysosomal membrane or membranes of pre-lysosomal compartments, and are associated with (i) the juvenile CLN3 form (Cao et al., 2006; Ezaki et al., 2003; Fossale et al., 2004), (ii) the Northern epilepsy CLN8 form (Lonka et al., 2004), (iii) the CLN6 variant late infantile form (Heine et al., 2004; Mole et al., 2004) and (iv) the CLN7 Turkish variant (Siintola et al., 2007).

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The specific storage of subunit c of mitochondrial ATP synthase in most NCLs was unequivocally established by direct protein sequencing (Chen et al., 2004; Fearnley et al., 1990; Palmer et al., 1989, 1992; Tyynelä et al., 1997) and inferred from immunohistochemical studies. Sphingolipid activator proteins A and D are stored in the CLN1 infantile form (Tyynelä et al., 1993). Traditionally, it was thought that neuropathology and gliosis were consequences of this storage but a recent study in the CLN6 form in sheep revealed that activation began prenatally, long before significant storage or neuron loss (Kay et al., 2006; Oswald et al., 2005). Indications from mouse models suggest that this may be general to the NCLs (Cooper, 2003; Pontikis et al., 2004) and other lysosomal storage diseases (Jeyakumar et al., 2003; Ohmi et al., 2003; Wada et al., 2000). Glial activation has also been reported in other neurodegenerative conditions (Hunot and Hirsch, 2003; Minagar et al., 2002; Neumann, 2001; Stoll and Jander, 1999).

Despite these advances in genetics and biochemistry, understanding of the pathobiology of the NCLs is still limited, yet understanding of the interconnections between the gene lesions, subunit c storage and neurodegeneration is central to determining the options for therapy. Studies in humans are very restricted and progress depends on studying genetically defined animal models. Several mouse models are very useful but, in general, do not show the severe cortical atrophy, profound neuronal loss and retinal degeneration characteristic of the sheep and human diseases. Sheep have gyrencephalic human-like brains and are a convenient size for experimentation. Being production domestic animals, they are used to human handling, and are straightforward and economic to maintain.

Recently, an NCL was reported in New Zealand Borderdale sheep (Jolly et al., 2002). We report here that this disease is caused by a nucleotide substitution at a consensus splice site in the *CLN5* gene (c.571+1G>A) and excision of exon 3, and the establishment of a flock for research particularly relevant to the human CLN5 late infantile form and to soluble enzyme forms of NCLs in general.

Materials and methods

Animals

Animals were mated in single pairs or by artificial insemination and maintained under standard New Zealand pastoral conditions on university research farms with adjacent animal hospital facilities. All procedures accorded to NIH guidelines and the New Zealand Animal Welfare Act (1999). Affected lambs were diagnosed at 2 to 3 months of age by histopathology of needle brain biopsies (Dickson et al., 1989) and/or analysis of mRNA extracted from blood (see below).

Leukocyte isolation and enzyme assays

Blood was collected from the jugular vein of affected and normal animals into heparinized tubes and mixed by inversion with 2.5 volumes of ice-cold lysis solution (150 mM NH_4Cl, 10 mM KCl, 0.1 mM Na_2EDTA). Leukocytes were pelleted by centrifugation, 3000 rpm, 10 min, 4 °C and washed twice in 10 ml of TKME buffer, pH 7.4 (50 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA), 4 °C, by resuspension and repelleting. The pellets were freeze dried and sent to Rotterdam, The Netherlands, and enzyme assays for PPT1, TPP1 and cathepsin D were performed as previously described (van Diggelen et al., 2001).

Genomic DNA and BAC (bacterial artificial chromosome) clones

Sheep genomic DNA was extracted from heparinized venous blood by NaCl fractionation (Montgomery and Sise, 1990), from Whatman FTA cards (Whatman, Brentford, Middlesex, UK), or from EDTA blood samples using the QIAamp DNA mini extraction kit (Qiagen, Hilden, Germany). Four BAC clones (126L2, 16J4, 223D24 and 433I11) were kindly provided by Dr. Sue Galloway (AgResearch Limited, Dunedin, New Zealand) after screening of the ovine BAC library CHORI-243 (BACPAC Resources, Children's Hospital Oakland Research Institute, Oakland, CA, USA) with the bovine CLN5 mRNA sequence (GenBank accession no. NM_001046299) (Houweling et al., 2006). DNA was extracted from the BAC clones using a Qiagen plasmid midi prep kit.

Linkage analysis

Microsatellite markers were genotyped on 6% polyacrylamide gels in a LICOR 4200 analyzer (LICOR Biosciences, NE, USA) after PCR amplification using M13 tailed primers and a modified "touchdown" PCR cycle protocol (Oetting et al., 1995). This consisted of 10 μl PCRs with a template of 50 ng of genomic DNA in 1× QBuffer (Qiagen), 2.5 mM $MgCl_2$, 200 mM dNTP, 0.08 pmol of each primer, 0.4 pmol of IRD700 (Licor) and 1 unit of Hotstar *Taq* polymerase (Qiagen). The cycles used were: 95 °C for 15 min; 5 cycles of 95 °C for 45 s, 68 °C annealing for 1 min 30 s decreasing by 2 °C/cycle and 72 °C for 1 min; 4 cycles of 95 °C for 45 s, 58 °C for 1 min decreasing by 2 °C/cycle and 72 °C for 1 min and finally 25 cycles of 95 °C for 45 s, 50 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 5 min.

Digital gel images of each microsatellite were transferred to RFLP scan software (BD Biosciences Bioimaging/Scanalytics, Rockville, MD, USA) and individual genotypes were determined. The program CRI-MAP (Green, 1992) was used to determine linkage between the NCL phenotype and selected markers. The *CLN6* SNP genotype was determined as previously described (Tammen et al., 2006).

Sequencing

PCR products were separated on agarose gels, excised and purified using the Perfectprep Gel Cleanup kit (Eppendorf, Hamburg, Germany) before sequencing using the PCR primers. Direct BAC sequencing was performed on 1.5 μg of purified BAC DNA per reaction and using only one primer.

Sequence reactions were performed using BigDye terminator v3.1 Cycle sequencing kits and the 5× BigDye v1.1/3.1 sequencing buffer (Applied Biosystems, Foster City, CA, USA) after pre-sequencing clean-up of excess dye terminator with CleanSEQ dye-terminator removal (Agencout Bioscience, Beverly, MA, USA). Samples were run on an ABI PRISM 3100-Avant Genetic Analyser (Applied Biosystems).

Sequence compilation and analysis

The ovine sequence produced (GenBank accession no. NM_001082595) was analyzed and aligned to CLN5 mRNA and protein sequences published on NCBI using Genedoc (Nicholas et al., 1997). Alignments were made to cattle (NM_001046299), human (NM_006493), mouse (NM_001033242) and dog (NM_001011556) sequences (Fig. 1).