Molecular screening of Batten disease: identification of a missense mutation (E295K) in the CLN3 gene

Received: 12 May 1997 / Accepted: 21 August 1997

Abstract  Batten disease, the juvenile form of neuronal ceroid lipofuscinosis, is a prevalent neuron degenerative disorder of childhood. A 1.02-kb genomic deletion in the Batten disease gene CLN3 has been determined to be a common mutation. We developed a PCR method to screen for this deletion and tested 43 Batten disease probands. We found 36% (31/86) of Batten disease chromosomes did not carry the 1.02-kb deletion. Of the three heterozygotes for the 1.02-kb deletion, a novel G-to-A missense mutation at nucleotide 1020 of the \textit{CLN3} cDNA sequence was found on two of the non-1.02-kb deletion chromosomes. The missense mutation resulted in a substitution of glutamic acid (E) by lysine (K) at position 295 (E295 K). The E295 K mutation causes a change in predicted local protein conformation. This glutamic acid is a highly conserved acidic amino acid, being present in human, mouse, dog and yeast, which suggests it may play an important role in the function of the Batten disease protein.

Introduction

Batten disease (MIM 204200), the juvenile form of neuronal ceroid lipofuscinosis (JNCL), is a progressive neurodegenerative disease characterized by rapid deterioration of vision, cognitive and motor dysfunction, and seizures in childhood (Zeman and Donahue 1963) with an autosomal recessive inheritance so that the neuropathological changes can be detected only in affected individuals (Burrig et al. 1982). Clinical onset of Batten disease is usually from 5 to 10 years of age. Batten disease is diagnosed by finding characteristic “fingerprint” profile inclusions in different cells, including lymphocytes, fibroblasts and neurons; “granular,” “curvilinear” or “mixed” inclusions are also found in clinically variant cases that present atypical JNCL forms (Wisniewski et al. 1992). A biochemical marker, elevated subunit C of mitochondrial ATP synthase complex, is nonspecific for Batten disease, as it is also seen in other forms of NCL (Kida et al. 1993; Tanner and Dice 1996). No treatment is available at present, and Batten disease is usually fatal within a decade after the onset of clinical symptoms. The incidence of Batten disease is estimated at 1/20,000 to ~1/100,000 live births, making it one of the more common neurodegenerative diseases of childhood (Mitchison et al. 1995; Zeman 1974).

The gene underlying Batten disease, designated as \textit{CLN3}, has been mapped to chromosome 16p12.1 (Callen et al. 1991; Gardiner et al. 1990; Lerner et al. 1994; Mitchison et al. 1995). Linkage disequilibrium studies provide evidence that \textit{CLN3} lies between D16S299 and D16S298 (Lerner et al. 1994; Mitchison et al. 1993, 1994, 1995; Taschner et al. 1995), and 73% of Batten disease chromosomes share a haplotype, designated 5–6, defined by microsatellite markers D16S299 and D16S298 (Mitchison et al. 1994). Exon amplification was used to isolate a \textit{CLN3} clone, cDNA2-3, which detects a 1.7-kb transcript from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas tissue (International Batten Disease Consortium 1995). The gene encodes a predicted protein.
of 438 amino acids of unknown function. The gene appears to be highly conserved as clone cDNA2-3 generates strong signals on Southern hybridization when tested against a wide variety of mammals. Genomic structure of CLN3 consists of 15 exons, which range in size from 47 bp to 356 bp (Mitchison et al. 1997).

A 1.02-kb deletion in genomic DNA, including 217 bp of the cDNA sequence, was found to be a common mutation being present in 81% of tested Batten disease chromosomes (International Batten Disease Consortium 1995). The 1.02-kb genomic deletion can be detected by Southern hybridization after restriction enzyme PsiI digestion, where the normal chromosome is seen as a 3.8-kb band but the Batten disease chromosome is seen as a 2.8-kb band. The 1.02-kb deletion can also be detected by PCR using cDNA primers F2 and P3 that flank the deleted 217-bp fragment coding sequence (International Batten Disease Consortium 1995). This “common mutation” was found in all affected Batten disease individuals with haplotype 5–6 chromosomes. Deletion of this 217-bp fragment produces a coding sequence frameshift and generates a stop codon 84 bp downstream from the deletion junction. The truncated CLN3 protein (CLN3P) is composed of 153 N-terminal residues, followed by 28 novel amino acids before the stop codon. Two other mutations have been identified in Finnish patients including a 3-kb genomic deletion of the entire 1.3-kb PsiI fragment, which results in a termination codon 84 bp downstream from the deletion junction and an RNA alternative splicing error (International Batten Disease Consortium 1995).

We have analyzed a series of 43 presumed Batten disease probands and found that 36% (31/86) of their chromosomes did not have the common 1.02-kb deletion. Analysis of one of these cases revealed a novel missense mutation of E295 K.

Materials and methods

PCR amplification of the common deletion region

PCR amplification with primers F2 and P3 (International Batten Disease Consortium, 1995) and an annealing temperature of 55°C resulted in a high level of non-specific background bands. One of these was a 2.5-kb band, which was not detected in normal controls but only in Batten disease samples (Zhong et al. 1996). To develop a reliable method for detecting this band in the absence of other background bands, two new primers near the original F2 and P3 sequences were designed. These new primers, BDNZ1F (5′-ATT CTG TGG GGA CCA GCC TGT GTG) and BDNZ2R (5′-GCC TCA GGA GAT GTG AGC AAC AAG), allowed combination of the annealing and extension PCR cycles and use of a minimal PCR reaction volume. The protocol employed was: A 0.5-µl sample of genomic DNA (approximately 2.5–5 ng) was extracted with a Puregene kit (Genta, Ill.) by a 1.7-kb CLN3 cDNA fragment released from clone CLN2-3 (kindly supplied by Dr. Lerner) that was labeled with the RadPrime DNA Labeling System (Gibco-BRL). The positive clones were sequenced by a dCycle sequencing kit (Gibco-BRL) from both directions with kit primers T7 and Sp6.

Direct genomic sequencing analyses of the nt 1020G→A missense mutation

Since the nt 1020G→A mutation did not change any restriction pattern, a direct sequencing analysis of the genomic fragment containing this mutation was conducted. The genomic fragment was PCR-amplified with primers BDNZ9F (5′-CCT CAT AAG AAC CGA GGC) and BDNZ12R (5′-GAC TCA GGG AAG TCT GCC AG) in a 50-µl reaction containing 25–50 ng genomic DNA, 1 × Taq buffer II, 1.5 mM MgCl2, 0.2 mM dNTPs, 25 pmol of primers, and 0.25 units AmpliTaq DNA polymerase (Perkin-Elmer). The cycling condition was optimized to denature double-stranded genomic DNA at 95°C for 4 min, followed by 30 cycles of denaturing at 95°C, annealing at 58°C, and extension at 72°C with 30 s at each temperature. The PCR product was gel purified with a QIAEX gel purification kit (Chatsworth, Calif.) and the final elution was in 75 µl of dH2O. Five microliters of the eluate was used for a thermocycle sequencing analysis by the same primers in both directions.

Single-strand conformational polymorphism (SSCP) analyses of the nt 1020G→A missense mutation in non-1.02-kb deletion alleles

Genomic DNA was subjected to SSCP analysis (Bardeesy and Pelletier 1995) to detect the nt 1020G→A missense mutation. SSCP analysis was carried out in a 10-µl reaction volume that contained 5–10 ng genomic DNA, 5 pmol of primers (BDNZ8F: 5′-CAT CTC CAG CCT CTC CCT TC and BDNZ12R: see sequence above), 1 × PE Taq buffer II, 1.5 mM MgCl2, 0.2 mM dNTPs, 10 µCi α-32P-dCTP, and 0.5 units of AmpliTaq polymerase. The thermal cycling conditions were the same as for the genomic DNA amplification for direct sequence analysis. SSCP were analyzed on a 6% polyacrylamide nondenaturing mini-gel with 1 × TBE buffer.
at room temperature and a constant 200 V for 4 h. The gel was dried and exposed for 15 min.

**Results**

Normal control polymorphism of nt 968A→G and CLN3 RNA alternative isoforms

Employing RT-PCR and DNA sequence analysis, we identified a new polymorphism, a substitution of A (33.3%) by G (66.7%) at nucleotide 968 (nt 968 A→G), with a heterozygosity of 44.4%. This polymorphism does not result in an amino acid substitution. When cloning of a specific allele is necessary, we have used this polymorphism to distinguish one allele from the other after DNA sequencing analyses. In addition, two normal RNA splicing alternative isoforms were observed – one spliced-out exons 7, 8, and 9, and a spliced-out exon 11.

PCR screening of Batten disease chromosomes for the 1.02-kb common deletion

A total of 43 clinically and pathologically diagnosed Batten disease probands, including 27 clinically typical cases and 16 atypical cases, were analyzed. With our PCR protocol, we were able to detect the common 1.02-kb deletion without background from Batten disease chromosomes. As illustrated in Fig. 1, PCR analyses showed only a single 2.3-kb band amplified from affected patients homozygous for the 1.02-kb deletion (Fig. 1, lanes 1 and 5). Two bands of 2.3 kb and 3.3 kb were detected from heterozygous parents of affected individuals (Fig. 1, lanes 3 and 6), and a single 3.3-kb band was present in normal siblings (Fig. 1, lanes 2, 4 and 7) as well as 26 unrelated normal controls.

Among the Batten disease subjects studied, 53.5% (23/43) probands with typical Batten disease phenotypes carried the common 1.02 kb deletion on both alleles, 20.9% (9/43) including 4 typical and 5 atypical probands carried the 1.02 kb deletion on one allele but no deletion on the other allele, and 25.6% (11/43) “atypical” probands did not have 1.02 kb deletion on either allele. In total, 36% (31/86) of Batten disease chromosomes did not carry the common 1.02 kb deletion. The frequency of the common deletion in this study, 64%, is lower compared to the 81% figure as found by The International Batten Disease Consortium (1995).

E295 K missense mutation

A Batten disease proband (C11189MH), a heterozygote for the 1.02-kb deletion, was found to carry a novel point mutation of nt 1020G→A on the nondeletion allele (Fig. 2). The point mutation in the CLN3 cDNA sequence was identified by sequencing analyses carried out in both directions on 13 RT-PCR clones containing a 3.3-kb insert. The mutation resulted in an amino acid substitution within exon 11 from glutamic acid (E) to lysine (K) at residue 295 (E295 K).
Because the peptide between residues 281 and 303 is within the fourth transmembrane fragment, based on CLN3P model analysis (Janes et al. 1996), the E295 K missense mutation would be predicted to affect local structure within the membrane and may result in a dysfunctional protein.

Detection of the nt 1020G→A mutation by SSCP in non-1.02-kb deletion chromosomes

The nt 1020G→A missense mutation did not alter any identifiable restriction site in the cDNA sequence of CLN3. To investigate whether the E295 K mutation occurred in the remaining 31 chromosomes, including the 9 heterozygotes of the 1.02-kb deletion and the 22 homozygous “atypical” cases that did not carry the 1.02-kb deletion, we undertook SSCP analysis to detect the nt 1020G→A
missense mutation (Bardeesy and Pelletier 1995). In addition to C11189MH, one other non-1.02-kb deletion chromosome from a heterozygote showed the nt 1020G→A pattern on SSCP gels (Fig. 4). Thus, the E295 K missense mutation accounted for about 2% (2/86) of the Batten disease chromosomes in our study.

Discussion

We have confirmed that the 1.02-kb deletion is a very common mutation in Batten disease (International Batten Disease Consortium 1995). Of the 86 Batten disease chromosomes tested, 55 (64%) were found to carry the 1.02-kb common deletion. An explanation for the finding of a lower frequency of the common deletion than in a previous report (International Batten Disease Consortium 1995) may be that some of the 11 clinically “atypical” variants of Batten disease are not the true JNCL form. Since 23 homozygotes and 9 heterozygotes for the 1.02-kb deletion were identified, and assuming Hardy-Weinberg equilibrium, we calculate that the expected number of homozygotes for non-1.02-kb deletions would be small, approximately 0.7. This would suggest that most of the 11 non-1.02-kb deletion homozygotes (~10/11) do not have mutations allelic to CLN3. Correcting for this, the number of true Batten disease probands in our study can be calculated to be ~33. In this case, the frequency of the 1.02-kb deletion would be 83% (55/66), which is close to the previously reported figure of 81% (International Batten Disease Consortium 1995). Based on these considerations, we conclude that the majority of these “atypical” cases are probably the late infantile form of NCL, having an overlap of clinical symptomatology. A more detailed genotype and phenotype study on these “atypical” cases will be presented elsewhere (Wisniewski et al. 1997).

Except for the common deletion, only a few other mutations underlying Batten disease have previously been characterized (International Batten Disease Consortium 1995), and the remaining mutations are still unknown. We have identified a novel missense mutation in this study. A transversion of G to A at nt 1020 of the CLN3 cDNA sequence resulted in a substitution of a highly conserved acidic glutamic acid at residual position 295 (E295 K). This E295 K missense mutation was also found in the patient’s mother and affected brother, but not in the normal sister. Since the nt 1020G→A resulting in E295 K was detected from a Batten disease patient who carried a 1.02-kb deletion on the second allele and there was no other abnormality observed from the allele that contains nt 1020G→A, we conclude the E295 K is a novel mutation underlying Batten disease.

The high conservation of glutamic acid (E) at residue 295 (Fig. 3) among species suggests it may have a fundamental function in the CLN3 protein. The localization and function of the CLN3 protein are unknown as of yet, as is the pathogenesis of Batten disease. The major component of the accumulated material in the Batten disease “fingerprint” is subunit C, a peptide that contributes to form the proton channel of mitochondrial ATP synthase in the inner mitochondrial membrane (Hall et al. 1991; Komiami et al. 1992; Pederson and Amzel 1993). The accumulated subunit C results from delayed degradation within mitochondria before being taken up by lysosomes (Ezaki et al. 1995). One possible explanation is that the CLN3 mutation may result in dysfunction of the mitochondrial hydrolysis process. In addition, Janes et al. (1996) recently proposed that the CLN3 protein functions as a chaperone involved in the folding and unfolding of subunit C in the mitochondrial ATP synthase complex in the mitochondrial membrane. If the predicted model (Janes et al. 1996) proves to be true, the novel missense mutation of E295 K identified in this study could involve the fourth transmembrane domain (TM4) of the CLN3 protein, and may affect normal mitochondrial membrane structure and function. However, the relation between the mutation and the accumulation of subunit C of ATP synthase remains unclear.

We hypothesize that the mutant transmembrane CLN3P is involved in a macro-molecular complex that consists of at least three components: subunit C, CLN3P, and subunit C hydrolytic enzyme. The normal structure and function of CLN3P may mediate hydrolysis of subunit C. Possibly, it interacts with subunit C through transmembrane domains and with hydrolytic enzymes through extramembranous sequences. Not only is subunit C the major accumulating substance in Batten disease cells, it also accumulates in the late infantile form of the neuronal ceroid lipofuscinoses and in Kuf’s disease, the adult form of NCL. We suggest that CLN3 may function as a regulator of a series of downstream functions, such as hydrolytic enzyme degradation of subunit C of the ATP synthase complex. The accumulation of subunit C from the mitochondrial ATP synthase complex may represent a final common pathogenetic pathway in NCL neurons and lead to neuronal degeneration.

Acknowledgements This study was supported in part by grants from the Children’s Brain Research Foundation (N. Z.) and from

---

Fig. 4 SSCP analysis of nt 1020G→A transversion showed that lane 2 (DNA of C11189MH) and lane 13, which had been determined to be 1.02-kb deletion heterozygotes, had the same pattern. Arrow points to the mutant band; lane 9, in a normal control, and the remaining lanes were from clinically “atypical” Batten disease cases without a 1.02-kb deletion on either allele. M was a pBR322/ MspI marker.
the New York State Mental Hygiene Foundation (N. Z.). We thank Mrs. Maureen Marlow for her assistance and Dr. Lerner for supplying the CLN3 cDNA clone. We thank Drs. S. Mole and H. Mitchison for personal communications.

References


