Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation: clinical and genetic characterization and target for therapy

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Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation is a disorder caused by recessive mutations in the gene DARS2, which encodes mitochondrial aspartyl-tRNA synthetase. Recent observations indicate that the phenotypic range of the disease is much wider than initially thought. Currently, no treatment is available. The aims of our study...
were (i) to explore a possible genotype-phenotype correlation; and (ii) to identify potential therapeutic agents that modulate the splice site mutations in intron 2 of DARS2, present in almost all patients. A cross-sectional observational study was performed in 78 patients with two DARS2 mutations in the Amsterdam and Helsinki databases up to December 2012. Clinical information was collected via questionnaires. An inventory was made of the DARS2 mutations in these patients and those previously published. An assay was developed to assess mitochondrial aspartyl-tRNA synthetase enzyme activity in cells. Using a fluorescence reporter system we screened for drugs that modulate DARS2 splicing. Clinical information of 66 patients was obtained. The clinical severity varied from infantile onset, rapidly fatal disease to adult onset, slow and mild disease. The most common phenotype was characterized by childhood onset and slow neurological deterioration. Full wheelchair dependency was rare and usually began in adulthood. In total, 60 different DARS2 mutations were identified, 13 of which have not been reported before. Except for 4 of 42 cases published by others, all patients were compound heterozygous. Ninety-four per cent of the patients had a splice site mutation in intron 2. The groups of patients sharing the same two mutations were too small for formal assessment of genotype-phenotype correlation. However, some combinations of mutations were consistently associated with a mild phenotype. The mitochondrial aspartyl-tRNA synthetase activity was strongly reduced in patient cells. Among the compounds screened, cantharidin was identified as the most potent modulator of DARS2 splicing. In conclusion, the phenotypic spectrum of leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation is wide, but most often the disease has a relatively slow and mild course. The available evidence suggests that the genotype influences the phenotype, but because of the high number of private mutations, larger numbers of patients are necessary to confirm this. The activity of mitochondrial aspartyl-tRNA synthetase is significantly reduced in patient cells. A compound screen established a ‘proof of principle’ that the splice site mutation can be influenced. This finding is promising for future therapeutic strategies.

Keywords: white matter disorder; DARS2; genotype-phenotype correlation; enzyme activity; compound screen

Abbreviations: EYFP = enhanced yellow fluorescent protein; GMFCS = gross motor function classification system; LBSL = leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation; MACS = manual ability classification system

Introduction

Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL, MIM 611105) is a rare autosomal recessive disease that was initially described as a relatively mild disorder, characterized by juvenile onset of slowly progressive ataxia, spasticity and dorsal column dysfunction (Van der Knaap et al., 2003; Linnankivi et al., 2004; Serkov et al., 2004; Tavora et al., 2007; Uluc et al., 2008). LBSL is associated with a highly distinctive MRI pattern, consisting of signal abnormalities in the periventricular cerebral white matter and specific brainstem and spinal cord tracts (Van der Knaap et al., 2003; Schepers et al., 2007). In most patients, proton magnetic resonance spectroscopy of the abnormal white matter reveals increased lactate (Van der Knaap et al., 2003; Petzold et al., 2006; Labauge et al., 2007). The definitive diagnosis of LBSL is established by the demonstration of mutations in the gene DARS2 (MIM 610956) (Scherper et al., 2007). No treatment is available for LBSL.

DARS2 encodes mitochondrial aspartyl-tRNA synthetase, the enzyme that attaches the amino acid aspartate to the correct mitochondrial transfer RNA. Aspartyl-tRNA is necessary in the translation of mitochondrial messenger RNA into protein. It is striking that almost all patients are compound heterozygous for two DARS2 mutations and that one of the mutations is almost invariably a splice site mutation in intron 2, upstream of exon 3 (Scherper et al., 2007). As a consequence of such a mutation, exon 3 is not included in the messenger RNA, leading to a frameshift, premature stop and absence of functional protein. These splice site mutations are, however, ‘leaky’. This means that for part of the mutated messenger RNAs, exon 3 is included, from which normal full-length protein is formed (Van Berge et al., 2012).

Recent observations indicate that the phenotypic spectrum in LBSL is much wider than originally assumed. Adult-onset oligosymptomatic cases were described (Petzold et al., 2006; Labauge et al., 2007; Synofzik et al., 2011; Moore et al., 2012), as well as patients with infantile onset, rapid neurological deterioration and early demise (Galluzzi et al., 2011; Miyake et al., 2011; Steenweg et al., 2012). The explanation for this wide clinical variation is unclear.

The first aim of the present study was to explore a possible genotype-phenotype correlation in LBSL. We made an inventory of the clinical characteristics and DARS2 mutations in a cohort of 78 LBSL patients and evaluated a possible genotype-phenotype relationship. The second aim of the study was to investigate the potential application of agents modulating the common intron 2 splice site mutations, with the aim to increase the amount of normal enzyme produced. A compound screen was performed to identify modulators of the splicing event.

Materials and methods

The study was performed with approval of the Institutional Review Board of the ‘VU University Medical Centre’, Amsterdam. Written informed consent for research on patients’ cells was obtained from all patients or guardians of patients participating in the study, in agreement with the Declaration of Helsinki.
**Phenotypic inventory**

A cross-sectional observational study was performed including all 68 patients with two DARS2 mutations present in the Amsterdam LBSL patient database up to December 2012. The database contains patients referred to the VUMC Centre for Childhood White Matter Disorders in Amsterdam for genetic testing. Additionally, 10 Finnish patients, investigated in the Helsinki University Central Hospital, were included.

Clinical questionnaires were completed primarily by the patient’s physician (77% of the patients). If this source was not available, the information was derived from medical records, supplemented by information provided by the family. To avoid differences in rating of the clinical phenotype, robust outcome measures were chosen: age of onset, age at achieving unsupported walking, age at loss of walking without support, full wheelchair dependency, Gross Motor Function Classification System (GMFCS), Expanded and Revised version) and Manual Ability Classification System (MACS) scores, cognitive ability and age at death. The GMFCS and the MACS are international standards for the classification of locomotion and hand function in patients with cerebral palsy (Supplementary Table 1) (Eliasson et al., 2006; Palisano et al., 2008). For cognitive ability we used a 3-point score: normal level of function, learning disabilities and severe cognitive impairment, as assessed by developmental milestones, school performance and occupation.

Magnetic resonance images of all patients were available for evaluation. The studies were evaluated according to a standard protocol (Van der Knaap et al., 1999).

**Genotypic inventory**

Mutation analysis of DARS2 was performed as described previously (Scheper et al., 2007). Several new primers were used, which are listed in Supplementary Table 2. The presence of the c.228-20T>C polymorphism on the same allele as another intron 2 mutation was determined by sequence analysis of the equivalent PCR fragment obtained from the parental DNA. If both parents carried the c.228-20T>C polymorphism, the patient's PCR fragment was cloned into a pGEM-T vector (Promega), which was then transformed into E. coli and individual clones were sequenced.

Genotype data of all 68 patients of the Amsterdam database up to December 2012 and the 10 patients of the Helsinki database were documented. Additionally, the mutations of 42 published patients with two DARS2 mutations were reviewed (Mikhaylova et al., 2009; Galluzzi et al., 2011; Labauge et al., 2011; Miyake et al., 2011; Synofzik et al., 2011; Huang et al., 2012; Moore et al., 2012; Tsouilis et al., 2012).

**Statistical analysis**

Descriptive statistics were used to review the phenotypes and genotypes. To test whether there is an association between age of onset and disease progression in the first 10 years after disease onset, we performed a non-parametric resampling procedure with 100 000 permutations to study the differences between the empirical cumulative distribution functions over the ordinal severity scale of consecutive age-of-onset groups. Cox regression analysis was performed to study the relation between the continuous covariate ‘age of onset’ and disease duration at the time of loss of ambulation. The recursive partitioning technique of Hothorn et al. (2006) was used to analyse whether categories of patients with different ages of onset could be formed. Kaplan-Meier curves were constructed to visualize the probabilities for the loss of the ability to walk without support and full wheelchair dependency relative to the duration of the disease, with a distinction for age of onset. For patients in whom the event of loss of walking without support or full wheelchair dependency had not yet occurred at the last clinical evaluation, the event was indicated as censored. The log rank test was used to compare subgroups. Student's t-test was used to assess the difference in the levels of DARS2 intron 2 inclusion. Analyses were performed using the statistical software program R and SPSS for Windows version 20.

**Cell culture**

Control and patient-derived lymphoblasts were obtained and cultured as described previously (van Kollenburg et al., 2006). HEK293T cells were grown in Opti-MEM® (Invitrogen) and 10% foetal bovine serum. Cells were cultured at 37°C and 5% CO2.

**Enzyme assay**

Mitochondria were isolated from lymphoblasts using the Mitochondrial Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol. Isolated mitochondria were resuspended in reaction buffer (50 mM HEPES—KOH pH 7.6, 25 mM KCl, 12 mM MgCl2 with protease inhibitors (Roche)). Mitochondrial extracts were obtained after incubation in 0.5% NP40 for 10 min and centrifugation at 13 000 rpm for 10 min. Mitochondrial extracts were run on a SDS-PAGE gel with 0.5% trichloroethanol and protein loading was analysed with the Gel Doc™ EZ system (Bio-Rad). Aminoacylation assays were carried out in 50 mM HEPES—KOH pH 7.6, 25 mM KCl, 12 mM MgCl2, 2.5 mM ATP, 0.2 mg/ml bovine serum albumin, 1 mM spermine, 32 mM stable-isotope labelled aspartate, 40 μM total E. coli tRNA (Roche) and mitochondrial extract at 37°C. NaAc (0.1 mM) pH 5.2 was added to the samples at indicated time points to stop the reaction. Transfer RNAs were isolated immediately, using acidic phenol/CHCl3/IAA and ethanol precipitation. Aspartate was released from the transfer RNA by incubation in 62.5 mM borate buffer at pH 10.0 for 1 h at 42°C. Stable-isotope labelled aspartate was measured by liquid chromatography–tandem mass spectrometry.

**Compound screen**

For the compound screen, a splicing reporter construct was used, containing the most common mutation in patients with LBSL (c.228-21_20delTTinsC), transfected in HEK293T cells together with a pmCherry-N1 plasmid (Clontech) as control. Splicing reporter constructs contained exon 2, intron 2, exon 3, intron 3 and part of exon 4 of DARS2 fused to enhanced yellow fluorescent protein (EYFP), as previously described (van Berge et al., 2012). This reporter construct leads to expression of EYFP when exon 3 is skipped. Compounds from the Spectrum Collection (Microsource), a library of 2000 FDA approved and natural compounds, were screened for their effect on expression of the splicing reporter construct.

HEK293T cells were transfected with polyethyleneimine (Polysciences) at 50% confluency. Cells were co-transfected with a pmCherry-N1 plasmid to control for the number of cells, transfection efficiency and possible other effects of the compounds on, for example, protein stability and cell viability. At an earlier stage, we had found that sodium orthovanadate increased correct splicing of the third exon of DARS2, and this compound was therefore used as a positive control. The stock solution of each compound was 100 μM dissolved in 1% dimethyl sulphoxide. Compounds were added 24 h after transfections at a final concentration of 1 μM. After incubation for 24 h, cells
were fixed in 4% paraformaldehyde and EYFP and mCherry expression was measured on a Cellomics ArrayScan VTI HCS Reader. The screen was done in duplicate. The 2000 screened compounds were ranked based on the EYFP/mCherry ratio with the rank product method (Breitling et al., 2004).

The top five hits from this analysis were used for further validation on transfected HEK293 cells in different concentrations. The compounds that were used for validation were obtained from Sigma. These selected compounds were also used in combination with two additional constructs, one with intron 2 mutation c.228-20_−11delinsCCCCCCCCCG and the other with c.228-20_−15delinsCCCCCA, to demonstrate that the effects were not restricted to one single mutation, c.228-21_−20delTTinsC.

RNA isolation and quantitative polymerase chain reaction

Transfected cells were harvested 48 h after transfection and 24 h after addition of the compounds. Total RNA was extracted using TRIzol® (Invitrogen). First-strand complementary DNA synthesis was carried out with SuperScript® III RT (Invitrogen). PCR was done on complementary DNA with Platinum® Taq according to the manufacturer’s protocol (Invitrogen). Quantitative PCR was performed using SYBR® Green (Roche) on a LightCycler 480 (Roche). The primers are described in Supplementary Table 2.

Results

Phenotypic spectrum

The databases contained 78 patients with two DARS2 mutations. Patients for whom no clinical information could be collected (n = 11) were excluded from the study. One patient was excluded because of co-morbidity (serious hypoplasia of the right cerebellar hemisphere; Isohanni et al., 2010). For some patients, the available clinical information was incomplete; these patients were only excluded from the analysis for the subject of the missing information.

Sixty-six patients with LBSL (36 female and 30 male patients, 58 families, see Supplementary Table 3) were available for the clinical inventory. The average age of the patients at the latest clinical evaluation was 24 years [standard deviation (SD) 14 years, range 1–59 years].

Age of onset

The mean age at which patients first showed neurological signs, was 8 years (SD 8 years, median 5 years, range 0.4–40 years). In 53% of patients, the disease began before the age of 6 and in 88% before the age of 18 years (Fig. 1). Onset in late teens was rare. Eight patients had adult onset, seven of whom were female. The most common neurological sign was cerebellar ataxia, especially gait ataxia. One patient (Patient LBSL236) has thus far been asymptomatic. He had been referred for a neurological examination at the age of 3 months because of abnormal muscle tone. Cranial ultrasound examination revealed hyperechogenecity of both cerebral hemispheres and MRI showed abnormalities typical of LBSL, after which the diagnosis was confirmed by DNA analysis. He is presently a normal boy of 2 years.

Early motor development

The majority of patients achieved unsupported walking at a normal age. Nine of 63 patients showed a delayed development and three patients never achieved unsupported walking.

Loss of ambulation

Only eight patients required an aid for walking before the age of 12 years and only four became fully wheelchair dependent before 12 years. Patients in their teens were more likely to have lost walking without support, but not to be fully wheelchair dependent. In patients of 18 years and older, 50% required support for walking and 13% were fully wheelchair dependent.

GMFCS and MACS

The majority of patients was able to walk without (GMFCS I-II) or with (GMFCS III) support. Most patients had no (MACS I) or limited (MACS II) problems with handling objects. Manual ability was generally less severely affected than ambulation. It was exceptional for patients to score on the highest level V of both systems, corresponding to complete wheelchair dependency and a severely limited manual ability. This was only the case in a small number of patients with disease onset before 2 years and rapid deterioration. Two middle-aged patients are currently also severely handicapped (GMFCS IV or V and MACS IV).
Cognitive ability

Most patients had a normal cognitive ability, whereas 20% (12/62) required special education. Serious intellectual impairment was observed in only two patients.

Mortality and survival

In this cohort of 66 patients, 15 individuals had reached the age of at least 35 years. Only two patients had died. Both had infantile disease onset, rapid disease progression and death before the age of 2 years.

Magnetic resonance imaging characteristics

Except for three patients, all patients in the databases fulfilled all major and one or more minor MRI criteria for LBSL (Steenweg et al., 2012). Two siblings (Patients LBSL160/161) and Patient LBSL223 lacked the major criterion of signal abnormalities throughout the pyramidal tracts; there was no involvement of the pyramids at the medulla oblongata and the lateral corticospinal tracts of the spinal cord. Patient LBSL223 lacked abnormalities in cerebellum and brainstem at the age of 2 years (Supplementary Fig. 1). Patient LBSL229 showed additional signal abnormalities in the anterior funiculus over the entire length of the spinal cord. In general, in late onset mildly affected patients, the cerebral white matter abnormalities were less profound than in severely affected patients (Supplementary Fig. 2).

Correlation between age of onset and disease severity

Forty-two patients were available for the comparison between age of onset and disease severity after 10 years. We divided the patients in age of onset categories that are in common use in medicine: infantile (0 to <2 years), early juvenile (2 to <6 years), late juvenile (6 to <12 years), teenage (12 to <18 years), and adolescent and adult (18 years and older). When comparing these patient categories, earlier onset was related to a more severe neurological deterioration in the first 10 years after disease onset ($P = 0.00011$, Fig. 1).

When studying the relationship between the continuous covariate age of onset and the time at loss of ambulation, only the time that patients became fully wheelchair dependent correlated with the age of onset (cox regression analysis, $P = 0.014$). Based on the time at full wheelchair dependency, evidence was found for the categorization into two groups of patients (age of onset 0–1.5 years and age of onset >1.5 years; $P$-value $= 0.022$). Patients with an infantile onset lost the ability to walk without support ($P = 0.0001$) soonest after disease onset and became fully wheelchair dependent sooner ($P < 0.0001$, Fig. 2). All the adult onset patients were still ambulant, without or with support, at the last clinical evaluation.

Genotypic variation

Since the discovery of DARS2 mutations in 38 patients with LBSL in 2007 (Scheper et al., 2007), we have confirmed the diagnosis of LBSL by DNA analysis in 40 additional patients (39 families). Of the mutations found, 13 have not previously been published. In addition, 42 patients with two pathogenic mutations have been
published by others (Mikhaylova et al., 2009; Galluzzi et al., 2011; Labauge et al., 2011; Miyake et al., 2011; Synofzik et al., 2011; Huang et al., 2012; Moore et al., 2012; Tzoulis et al., 2012).

An overview of all 60 known mutations is shown in Fig. 3 and the mutations of our patients are listed in Supplementary Table 3. The mutations are spread over the entire DARS2 gene (Fig. 3). Ninety-four per cent (113/120) of the patients had an intron 2 mutation in the polypyrimidine tract just upstream of exon 3. Thirteen different mutations were found in this region with c.228-21_-20delTTinsC being the most common (88/120). In all Amsterdam database patients, these intron 2 mutations co-segregated with a single nucleotide polymorphism (c.228-20 T>C) on the same allele, which resulted in a modified description of some earlier published mutations (Supplementary Table 3). All patients in our database were compound heterozygous. Four patients (two families) have been described with homozygous mutations (Miyake et al., 2011; Synofzik et al., 2011). In Patient LBSL44 three mutations were found that were predicted to be pathogenic. For this patient, no parental DNA was available to investigate the allelic distribution.

Genotype-phenotype relation

Of 66 phenotyped patients with LBSL, 25 patients had a private mutation and 31 patients had a unique combination of mutations. The fact that only 22 patients had a combination of DARS2 mutations shared by other unrelated patients hampered the study of a genotype-phenotype correlation.

In the 45 patients who had the common c.228-21_-20delTTinsC mutation and different mutations on the second allele, the clinical severity ranged from childhood onset disease with loss of ambulation before adulthood to very slowly progressive adult onset disease. There was no evident relationship between the location or type of the second mutation and disease severity. Interestingly,
the four patients with the most severe phenotype did not have the common mutation, but had another intron 2 mutation.

Two groups could be formed with multiple patients sharing the same combination of mutations: c.228-21_-20delTTinsC together with c.455 G>T (n = 9) or with c.492 + 2 T>C (n = 11). All patients in these groups had a benign phenotype characterized by an onset in childhood up to young adulthood followed by mildly progressive neurological deterioration. There was no significant difference in clinical outcome between these groups or between these groups and the rest of the patients. Two additional patients with the same genotype (Patients LBSL103 and LBSL166, c.228-21_-20delTTinsC, c.397-2 A>G) had a mild disease with an adult onset.

Only four patients did not have an intron 2 mutation affecting the splicing of exon 3. This number was too small to conclude whether their phenotypes are different. It was, however, striking that two of these patients (Patients LBSL160/161) lacked some major MRI criteria, as described above. Another patient who lacked some of the classical MRI characteristics (Patient LBSL223) had a unique intron 2 mutation and a rather severe disease course.

In seven of eight affected sibling pairs, the first symptoms occurred within the same phase of life for both patients, with a maximum difference of 3 years; in Patient HEL2 the symptoms occurred 13 years later than in his brother (Patient HEL2). Among five affected sibling pairs, mild differences in clinical severity of motor dysfunction or cognitive problems were observed.

### Decreased mitochondrial aspartylation in patients with LBSL

Mitochondrial aspartylation activity was measured in mitochondrial extract from lymphoblasts of four patients with LBSL (Patients LBSL8, LBSL44, LBSL160 and LBSL266) and four control subjects. The patients had different combinations of mutations and their phenotypes ranged from infantile onset disease with severe neurological deterioration (Patient LBSL266) up to adult onset, mild disease (Patient LBSL44) (Supplementary Table 3). The results show a substantial loss of activity of mitochondrial aspartylation in all patient cells compared to control subjects (Fig. 4 and Supplementary Fig. 3). The level of residual enzyme activity was in the same range for all patients with overlap in values for the most severely and mildest affected patients.

### Compounds altering DARS2 exon 3 splicing

We have previously developed a yellow fluorescent protein (YFP) reporter construct, in which increased splicing efficiency of intron 2 is reflected by a decrease in the YFP signal (Van Berge et al., 2012). These results were validated at both the RNA and protein level. In the present study, we used this construct with the most common splice site mutation to screen compounds for their ability to modify the splicing efficiency of intron 2. Of the 2000 compounds ranked by the rank product method, the five compounds causing the largest decrease in the EYFP/mCherry ratio in both screens were used for further studies: gentian violet, cantharidin, pyrithione zinc, celastrol and alanyl-DL-leucine (Supplementary Table 4). Effects of all compounds except for cantharidin could be confirmed in separate experiments using the reporter construct. The two additional reporter constructs with different splice site mutations in the same region showed similar effects (Fig. 5A). Therefore, the identified compounds affect the splicing efficiency at the intron 2/exon 3 boundary independent of the exact mutation.

To directly assess the effect on messenger RNA splicing, reverse transcriptase PCR was performed to detect the messenger RNA with and without the third exon of DARS2. In addition to sodium orthovanadate, one of the selected compounds, cantharidin, showed a direct effect on the splicing of the third exon. Both compounds increased the amount of correctly spliced product (Fig. 5B). There was a concentration-dependent effect up to 10 μM for cantharidin (Fig. 5C). In experiments with a smaller number of different concentrations, we showed that the concentration-dependent effects were reproducible (Supplementary Fig. 4A) and all three mutant reporter constructs respond in a similar concentration-dependent manner (Supplementary Fig. 4B).

Cantharidin had a clear effect on splicing of intron 2 on the messenger RNA level (Fig. 5B) and was chosen to study the effect on lymphoblasts from patients with LBSL. Two patient-derived lymphoblast cell lines and two control cell lines were treated with 10 μM cantharidin for 24 h. Quantitative reverse transcriptase PCR on RNA purified from these cells showed that cantharidin increased the correct splicing of DARS2 exon 3 in these patient-derived cells. With the addition of cantharidin, the percentage of messenger RNA without exon 3 was decreased on average from 11.3% to 5.4% (Fig. 5D).
Figure 5. Compounds affecting DARS2 exon 3 splicing. (A) The effects of selected compounds on three different reporter constructs are shown. The effect of DMSO was set at a YFP/mCherry-ratio of 1. This ratio is decreased with all selected compounds except alanyl-DL-leucine. The three different mutations in the reporter constructs show similar results. (B) Reverse transcriptase PCR was performed on cells transfected with the reporter construct and treated with the indicated compound. After isolation of total RNA and complementary DNA synthesis, the inclusion or exclusion of exon 3 was visualized by PCR with primers 2Fb and GFP-R (Supplementary Table 1) before agarose gel electrophoresis. The expected sizes of the bands are 615 bp (without exon 3) and 682 bp (with exon 3) as indicated on the right.
Discussion

In this study, we investigated the phenotypic variation in a relatively large number of patients with LBSL. LBSL was originally described as a juvenile onset disorder with a reliable, slow progression (Van der Knaap et al., 2003). Our study confirms that LBSL is a neurological disorder with generally slow progression and low mortality, with some cases even later onset and slower disease course than initially described. Complete wheelchair dependency is rare, especially before the age of 18 years. Life expectancy may be normal for most patients; the oldest patient in this cohort is 59 years old. Patients with LBSL have in general a better prognosis than most other hereditary leukoencephalopathies, but the infantile onset cases are an exception. They form a distinct category characterized by a more rapid neurological deterioration and—in the most severe cases—early death. The implication of our observations is that both paediatric and adult neurologists may meet patients with LBSL and need to be aware of this disease.

The patients included in the present study had been selected for analysis of the DARS2 gene on the basis of specific MRI findings. A limitation to this approach is that, possibly, LBSL patients with an atypical MRI pattern and asymptomatic patients have been missed. So far, one index patient (Patient LBSL236) and three siblings of patients with LBSL with the same pathogenic mutations are known that are asymptomatic (LaBauge et al., 2011 and personal observations). We suspect that until now most early-onset patients have remained undiagnosed because the severe phenotype is not widely known. Consequently, the clinical spectrum of the disease may still be wider and the distribution over ages of onset may be different to what is currently described.

Almost all patients with LBSL have compound heterozygous DARS2 mutations. Mitochondrial aspartyl-tRNA synthetase is an enzyme that is essential for life and patients never have two null mutations. These observations suggest that the range of permissive mutations resulting in mitochondrial aspartyl-tRNA synthetase activity that is high enough not to be lethal, but below a certain threshold for symptoms to appear, is narrow. The c.228-21_-20delTTinsC mutation has not been reported in the homozygous state in any LBSL patient, which is striking considering the relatively frequent occurrence of this mutation in LBSL patients, and the high carrier rate of 1:95 that has been described in the Finnish population (Isohanni et al., 2010). It is also striking that there is no patient with LBSL who is compound-heterozygous for two different intron 2 splice site mutations. The intron 2 splice site mutations are leaky and allow the production of some normal protein. It is possible that the presence of an intron 2 splice site mutation on both alleles leads to another pathology, but we suspect that this may not lead to a disease. We postulate that the remaining mitochondrial aspartyl-tRNA synthetase activity in patients’ cells is largely derived from the allele with the ‘leaky’ intron 2 mutation, whereas the second mutation probably is a functional null allele. Patient LBSL44 had three predictively pathogenic mutations; it is most likely that the intron 2 mutation is located on one allele, and the two other mutations on the other allele, leading to one null allele.

We found that a known single nucleotide polymorphism (c.228-20T>C) invariably co-segregates with mutations in the polyuridine tract in intron 2 in patients from the Amsterdam database. It is unknown whether this is also the case in other patients. Our finding raises the possibility that a single mutation in this intron 2 tract may only be pathogenic when occurring in combination with this single nucleotide polymorphism.

Unfortunately, the heterogeneous nature of the genotypes seriously hampered the genotype–phenotype correlation study. For a proper genotype–phenotype correlation study much larger groups of patients are required. Interestingly, four severe patients do not have the common c.228-21_-20delTTinsC mutation seen in 45 milder patients. Patient LBSL263 has the same mutations as a patient described in the literature who was also severely affected (Galluzzi et al., 2011) (the presence of the above mentioned single nucleotide polymorphism was not mentioned). Two groups of patients with similar mutations and a mild disease course could be formed, suggesting that these genotypes are related to a benign phenotype. Striking intrafamilial differences were absent. Outside our study, only one family has been described with three affected siblings (Miyake et al., 2011) and these displayed remarkable interindividual differences. Our observations support the hypothesis that there is a genotype–phenotype correlation. Intrafamilial differences are most likely explained by the influence of environmental and other genetic factors.

We developed an enzyme assay to assess mitochondrial aspartyl-tRNA synthetase activity and for the first time confirmed its decreased activity directly in patient cells. Decreased activity of mutant enzymes has been shown previously in in vitro amination and expression studies (Schepers et al., 2007; Van Berge et al., 2013), but it is difficult to extrapolate these results to patient cells, where the activity is almost always determined by a combination of two different mutant alleles. We demonstrate that the tested patient cells have a significantly reduced mitochondrial aspartyl-tRNA synthetase activity. We found no clear correlation between enzyme activity and severity of the phenotype. Possibly, the assay we used was not sensitive enough to detect subtle differences. It could also be that lymphoblasts are not the most suitable cells to show the differences; they might be better detectable in other cell types, such as neuronal cells.

Figure 5 Continued

M represents a marker lane containing the Invitrogen 1Kb Plus DNA ladder and the size of the band is indicated on the left. (C) The effect of increasing concentrations of cantharidin on the YFP/mCherry-ratio is shown. (D) Relative expression of mitochondrial aspartyl-tRNA synthetase messenger RNA without exon 3 in control and patient lymphoblasts with and without treatment of 10 μM cantharidin for 24 h. The expression of the variant without exon 3 is shown as the mean percentage of total amount of mitochondrial aspartyl-tRNA synthetase messenger RNA ± standard error of the mean. The asterisk indicates P < 0.05.
The high occurrence of splice site mutations makes the splicing process a promising target for therapy. We have previously demonstrated that antisense oligonucleotides can alter the splicing efficiency at the intron 2/exon 3 boundary (van Berge et al., 2012). Despite the great progress in the field of antisense oligonucleotides in recent years, development of a therapy based on the successful delivery of antisense oligonucleotides to the CNS will probably not be possible through systemic administration (Southwell et al., 2012). Therefore, we used a compound library containing FDA-approved and natural compounds, many of which are known to be able to cross the blood–brain barrier. With this library, a screen was performed to find compounds that influence the intron 2/exon 3 splicing event. After validation of hits from this screen, cantharidin was identified as the most effective compound. It increased the inclusion of exon 3 in our reporter constructs and in lymphoblasts from patients with LBSL. Cantharidin is a protein phosphatase 1 and 2 A inhibitor. It has previously been found to increase exon 7 inclusion in the SMN2 gene, relevant for spinal muscular atrophy (Novoyatleva et al., 2008). Reducing protein phosphatase 1 activity promotes usage of numerous alternative exons, indicating that protein phosphatase 1 activity plays a role in splice site selection (Novoyatleva et al., 2008). Cantharidin is too toxic for use in patients (Moed et al., 2001), but this study provides proof-of-concept that influencing the splice site mutations is possible and a highly promising therapeutic target for LBSL. Future research should be directed at less toxic variants of cantharidin (Zhang et al., 2011) or other protein phosphatase 1 or 2 A inhibitors.

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Supplementary material

Supplementary material is available at Brain online.

References


**Appendix 1**

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