Short Report

The c.65-2A>G splice site mutation is associated with a mild phenotype in Danon disease due to the transcription of normal LAMP2 mRNA

Danon disease (DD) is a rare X-linked multisystem disorder caused by mutations of the LAMP2 gene and characterized by intellectual disability, skeletal myopathy and cardiomyopathy. The survival time is severely reduced. Contrasting with the usual disease course, we report on a family with an exceptionally mild phenotype of DD despite having two potentially damaging LAMP2 mutations. Using RNA-Seq analysis, we showed that a c.65-2A>G splice site mutation results in the tissue-specific production of four different transcripts including the full-length mRNA in muscle tissue but not in leukocytes. We confirmed our results by immunohistochemistry and immunoblotting, showing the detection of LAMP2 protein only in muscle. The second mutation (c.586A>T, p.T196S) has been reported before to have an uncertain clinical significance. In our patients, however, neither of the two mutations seem to have a high enough functional impact to cause a severe phenotype. Overall, our study reveals that alternative splicing is a potential mechanism in DD with underlying splice site mutations of the LAMP2 gene in order to rescue the full-length mRNA. Moreover, our report of a mild phenotype complements the DD spectrum, which is of great importance for a rare disease suspected to be underdiagnosed.

Conflict of interest

The authors have no conflicts of interest to declare.

Danon disease (DD; MIM# 300257) is a X-linked domin‐
at metabolic disorder with multisys‐tem manifestations among which the clinical triad of cardiomyopathy, skele‐tal myopathy and intellectual disability are the most common. Men are affected earlier and more severely than women and die at an average age of approximately 20 years because of cardiac failure (1). The disorder is caused by the defects of the lysosome-associated membrane protein-2 (LAMP2; MIM# 309060), which is involved in the maturation of (auto)phagosomes and regulates their intracellular trafficking (2, 3). To date, 17 splice site mutations have been reported in the literature (4), most of which result in truncated mRNA products. Although the majority of those tran‐scripts are proposed to undergo post-transcriptional nonsense-mediated decay, some studies using immunoassays have detected traces of truncated LAMP2 protein (5, 6). The age at disease onset and the age of death appear not to differ between patients with splice site mutations and other mutations (4), suggesting that a full-length LAMP2 protein is crucial for its proper function. Patients with missense mutations of exon
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9 (affecting the LAMP2B isoform only) experience a milder phenotype with a significantly later disease onset (7).

In this study, we report a family with an unusually mild form of DD caused by a splice site mutation in intron 1 (c.65-2A>G) enabling the tissue-specific production of normal LAMP2 mRNA and, presumably, LAMP2 protein.

Materials and methods

Ethics statement

As a retrospective case report, this study does not require ethics committee approval at the Medical University of Vienna. Written informed consent was obtained from included patients to conduct the clinical and biological investigations and to publish the results.

Morphological studies and western blot analysis

The biopsy was obtained from the vastus lateralis muscle. LAMP2 protein expression was analyzed with two different antibodies, a mouse monoclonal anti-LAMP2 (H4B4, sc-18822, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a rabbit polyclonal antibody raised against amino acids 70–237 of human LAMP2 protein (ab101325, 1:50, Abcam, Cambridge, MA, USA). The same antibodies were used as primary antibodies for western blot analyses (details provided as Supporting information).

Molecular analysis

Genomic DNA was extracted from leukocytes using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). Primers were designed to amplify all coding exons of LAMP2 including adjacent exon/intron boundaries. The polymerase chain reaction (PCR) fragments were purified by agarose gel electrophoresis and sequenced in forward and reverse direction using the dideoxy Cy5/Cy5.5 Dye Primer cycle sequencing kit (Visible Genetics, Toronto, ON, Canada) and the OpenGene DNA sequencing system (Visible Genetics) (8). RNA-Seq analysis was performed with RNA from leukocytes (patient II-2, III-2 and control person) and from muscle tissue (patient III-2) (details provided as Supporting information).

Results

Index patient

The 40 year old male index patient (patient III-2, Fig. 1) was initially referred to the neuromuscular outpatient clinic at the age of 33 because of elevated levels of serum creatine kinase (CK) and a generalized fatigability. The physical examination revealed no specific abnormalities, especially no muscle weakness. For the following 7 years, no deterioration was observed. Laboratory testing during this period confirmed consistently elevated serum CK (up to 1100 U/l; normal <190 U/l), aspartate aminotransferase (AST) (171 U/l; normal <35 U/l) and alanine aminotransferase (ALT) (213 U/l; normal <45 U/l) levels. All other laboratory parameters were normal. A liver biopsy had been performed on the suspicion of a hepatopathy at the age of 32 but no abnormalities could be detected. Electromyography and nerve conduction studies were normal. Magnetic resonance imaging of the brain and the muscles of the lower limbs were also normal. Cardiac ultrasound revealed concentric hypertrophy of the left ventricle with normal systolic function. A 24-h holter recording showed premature atrial and ventricular contractions and non-sustained runs of atrial tachycardia. Bradycardic episodes have also been reported. Neuropsychological examination revealed no relevant deficits; the patient’s IQ was 85.

Mother of patient III-2

The 56 year old mother (patient II-2, Fig. 1) reported no symptoms and, correspondingly, the physical examination revealed no weakness or other deficits.
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Laboratory testing (including CK, AST and ALT), electromyography, nerve conduction studies and a magnetic resonance imaging of the brain were normal. Cardiac ultrasound revealed mild concentric hypertrophy of the left ventricle with normal systolic function. The 24-h holter recording showed premature atrial and ventricular contractions and non-sustained runs of atrial tachycardia. The patient had an IQ of 75. Her mother (patient I-2, Fig. 1) had deceased due to heart failure at the age of 35. Unfortunately, there were no reports available.

Muscle biopsy of patient III-2

The specimen showed myopathic changes with minor variability in muscle fiber size. Many fibers contained multiple cytoplasmic autophagic vacuoles that appeared as solid basophilic granules on hematoxylin and eosin staining. Muscle fibers lacked LAMP2 immunoreactivity using a monoclonal LAMP2 antibody. However, they retained positive immunoreactivity using a polyclonal antibody (Fig. 2a–f). We found a predominant sarcolemmal staining and a distribution within the vacuoles.

Western blot analysis

Immunoblotting was performed on leukocyte extracts from the patient and his mother, on skeletal muscle just from the patient and on controls (Fig. 2g–h). The monoclonal LAMP2 antibody could not detect any protein using both leukocyte and skeletal muscle extracts from the patient. In contrast, LAMP2 bands between 100 and 150 kDa were present with about the same intensity in leukocytes from the patient’s mother and in all controls. Performing experiments with the polyclonal LAMP2 antibody, we surprisingly detected corresponding protein bands at 105 kDa in skeletal muscle extracts from the patient and in control samples, whereas there was no binding in leukocyte extracts from the patient. LAMP2 bands were present in leukocyte extracts from both his mother and controls.

DNA studies

After extracting DNA from leukocytes exons 1–8, 9A and 9B of the LAMP2 gene were sequenced. We detected a c.65-2A>G splice site mutation (rs397516743) in intron 1 affecting the splice acceptor site. In addition, two other mutations were found: the c.156A>T in exon 2 (p.V52V, rs12097) with a minor allele frequency (MAF) of 0.3809 designated a silent mutation and the c.586A>T in exon 5 (p.T196S, rs138991195) with a MAF of 0.0003 a missense mutation. The c.586A>T affects a position that reveals high amino acid conservation and is predicted to be ‘possibly damaging’ for the protein with a PolyPhen-2 score of 0.896 (http://genetics.bwh.harvard.edu/pph2). However, the variant is categorized as ‘likely benign’ and with ‘uncertain significance’ according to the ClinVar database. (http://www.ncbi.nlm.nih.gov/clinvar/variation/44432/). All three mutations have also been detected in the patient’s mother.

RNA studies

Performing RNA-Seq analysis from blood and muscle tissue, we found four different transcripts in the patient (Fig. 3): (i) full-length normal transcripts detected in muscle but not in leukocytes, (ii) transcripts with an in-frame deletion in exon 2 leading to a predicted protein lacking 13 amino acids in blood and muscle, (iii) transcripts with skipped exon 1 and 2 and a frameshift (p.Gly22Glufs*14) in blood and muscle, and (iv) transcripts with skipped exons 1 and 2 in blood and muscle with an unknown effect on the protein level. The mother of the index patient processed the (i) full-length transcript and transcripts (ii) and (iii) in blood (muscle has not been examined). Control lymphocytes from a healthy male person just expressed the full-length transcript.

Discussion

DD is a serious lysosomal storage disorder characterized by cardiac and skeletal myopathy and a severely reduced survival time. The mean age for heart transplantation and death in male patients was reported to be 18 and 19 years, respectively. The average life expectancy in women was 35 years (1). In our study, we report on a family with an exceptionally mild phenotype although the underlying presence of two potentially hazardous mutations in the LAMP2 gene. The c.65-2A>G splice site mutation results in the tissue-specific production of four different transcripts including the full-length mRNA. To our knowledge, this is the first report on a splice site mutation of the LAMP2 gene being associated with transcripts of full-length protein and, consecutively, an unusually mild phenotype. However, similar findings on splice site mutations resulting in the production of small amounts of full-length mRNA, and mild phenotypes have also been published in the context of cystic fibrosis (9) and dystrophic epidermolysis bullosa (10).

A single patient with the c.65-2A>G mutation has been published before (5). The clinical information provided was limited, but the male patient seemed to suffer from hypertrophic cardiomyopathy without any musculoskeletal deficits or mental retardation. Using RT-PCR the mutation was reported to result in an exon 2-skipped transcript and a frameshift. This was confirmed by immunoblotting, which did not show any reaction between protein extracts from lymphocytes and fibroblasts and polyclonal LAMP2 antibodies. Muscle tissue had not been examined. These results somehow contrast with the milder phenotype of the patient in our study, which is all the more remarkable given the apparent ‘null allele’ mutation. To elucidate this inconsistency, we performed RNA-Seq analyses from leukocytes and muscle tissue and identified four different transcripts in the patient. Surprisingly, we also found full-length normal transcripts, but only in muscle tissue and not in leukocytes. We confirmed our results by immunohistochemistry and western blotting, showing LAMP2 detection with predominant sarcolemmal and vacuolar distribution just in muscle tissue by a polyclonal
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Fig. 2. Muscle biopsy and western blot analysis. The muscle displayed moderate variation in fiber size with striking vacuoles in several muscle fibers [(a) Hematoxylin and eosin staining, ×40 and insert ×60; the bar represents 50 μm]. These vacuoles stain for glycogen [(b) PAS ×60] and are lined by membranes, which contain sarcomemmal proteins [(c) alpha-sarkoglycan, ×40]. Immunohistochemistry displays virtual absence of LAMP2 protein [(d) ×40, monoclonal anti-LAMP2] compared with non-diseased muscle [(e) ×40], whereas protein expression is retained and showed a sarcomemmal and vacuolar distribution using a polyclonal anti-LAMP2 [(f) ×40]. This was reproduced in the western blot analysis. Using a polyclonal antibody, we could not detect protein in leukocytes (g) but in muscle tissue (h) from the index patient (patient III-2), whereas the monoclonal antibody failed to show a reaction. However, the exact epitope of the monoclonal antibody is unknown. Both antibodies detected LAMP2 protein in leukocytes of patient II-2 (mother). All controls showed a positive reaction. Myosin heavy chain served as a loading control.

antibody. Interestingly, the vacuolar LAMP2 staining was reported before in another case of DD with a mild phenotype (with a missense mutation in exon 9 affecting the LAMP2B isoform only) (11). Overall, it rather seems that the c.65-2A>G splice site mutation results in the tissue-specific expression of full-length LAMP2 protein contributing to the mild phenotype of DD presented in our study.

In contrast, the index patient’s grandmother died of heart failure at the age of 35 without being tested for DD. If she was a carrier, this would rather be suggestive of a severe phenotype. However, her genetic status has not been confirmed and other factors could have contributed to her early death.

There was a relatively high protein level detected by the polyclonal LAMP2 antibody in the western blot analysis (Fig. 2h) and a relatively low ratio of normal transcript in the RNA-Seq analysis of muscle tissue from the patient (5.1% of total reads, Fig. 3). A potential explanation for this apparent discrepancy might be that the RNA transcripts (ii) and (iv) are translated into detectable proteins. The polyclonal LAMP2 antibody used in our study was directed against an amino acid sequence corresponding to exons 3–5 and thus would
have also detected transcripts (ii) and (iv) (which lacked 39 bp in exon 2 and exons 1 and 2, respectively). Moreover, as a semi-quantitative method NGS does not measure the absolute amount of a specific transcript but rather displays the relative amount of one transcript to another. Thus, even though the normal full-length transcript constitutes for just about 5% of all NGS reads, this might suffice for adequate amounts of full-length protein.

Surprisingly, we have not detected any protein by immunohistochemistry or western blotting using a commercial monoclonal LAMP2 antibody, for which the specific epitope is not known. This could be explained by the fact that the family presented in this study had another mutation in exon 5 (c.586A>T), which might have been affecting the interaction between the LAMP2 protein with the monoclonal antibodies in our experiments.

In conclusion, our study shows that the c.65-2A>G splice site mutation produces different transcripts including the full-length mRNA. The pattern of transcription seems to be tissue-specific with normal protein being expressed in muscle tissue. This might explain the exceptionally mild phenotype in our patients. Our findings also convey important clinical implications. In patients with splice site mutations, prognostic predictions seem to be more accurate using muscle tissue instead of leukocytes for genetic analyses. Moreover, our report of a mild phenotype complements the DD spectrum, which is of great importance for a rare disease suspected to be underdiagnosed (12).

Supporting Information
Additional supporting information may be found in the online version of this article at the publisher’s web-site.

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