Short Communication

Characterization of a rare Unverricht–Lundborg disease mutation

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ARTICLE INFO

ABSTRACT

Cystatin B (CSTB) gene mutations cause Unverricht–Lundborg disease (ULD), a rare form of myoclonic epilepsy. The previous identification of a Portuguese patient, homozygous for a unique splicing defect (c.66G>A; p.Q22Q), provided awareness regarding the existence of variant forms of ULD. In this work we aimed at the characterization of this mutation at the population level and at the cellular level. The cellular fractionation studies here carried out showed mislocalization of the protein and add to the knowledge on this disease.

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1. Introduction

First described in 1891, Unverricht–Lundborg disease (ULD, progressive myoclonic epilepsy type 1, ULD, OMIM254800) is an autosomal recessive disorder, and the most common cause of progressive myoclonic epilepsy (PME) [1]. Clinically, ULD is characterized by myoclonus action, tonic–clonic seizures and an onset at age 6–15 with gradual worsening. Cystatin B (CSTB; ID: 1476) gene mutations cause ULD [2], and CSTB lesions often lead to abnormal RNA processing [3,4]. The CSTB gene is located on the region 21q22.3 and encodes a protein from the cystatin superfamily. Cystatins are reversible competitive inhibitors of C1 cysteine proteases in which the cathepsins are included [5]. Cystatin B protein (Cstb, Stefin B), an unglycosylated inhibitor of ~11 kDa, tightly binds cathepsins B, H, L and S. The main function of cathepsins is nonselective degradation of intracellular proteins, but they also participate in antigen processing, oxidative stress and apoptosis [6,7]. In ULD, the levels of cystatin B are decreased while the levels of cathepsins B, L and S are significantly increased [8], which can have a great impact on the disease development. Although CSTB has been characterized in vitro, its physiological function is still unclear. The fact that it is known to have a protective role against leaking lysosomal proteases [9] links it to the puzzling group of disorders that affect lysosomal

function. Furthermore, it may also have regulatory functions in osteoclasts [10] and presents neuroprotective function [11]. Cystatin B can play an important role in regulating the proteolytic activity of cathepsin L in the nucleus, protecting substrates, such as transcription factors, from proteolytic processing [12]. Thus, the balance between cystatins/cathepsins is responsible for normal cell function.

Finland presents the highest prevalence of ULD (4:100,000) [13], but Mediterranean and North American areas are also believed to have a higher prevalence [1]. To date only 13 mutations have been described in this gene. The most common mutation, in all populations, accounts for about 90% of the alleles and is an unstable dodecamer repeat expansion in the promoter region of CSTB (13) which leads to reduced mRNA levels and decreased protein levels [14]. Mostly due to the difficult diagnosis of ULD, the disease is believed to be underdiagnosed [15]. In Portugal the cases identified are extremely rare and the finding of a distinct patient led to the need to expand the molecular and cellular characterization in order to provide the means to improve methods, procedures and possible treatments. The patient had a late onset and presented a slow progressive form [4] in addition to homozygosity for a rare mutation. Since no consanguinity was acknowledged, a situation of underdiagnosis and increased risk for that particular mutation was envisaged. A population screening was carried out on a large sample tested by allelic discrimination (AD). Studies concerning the cellular involvement of Cstb protein were also carried out in order to better characterize this variant while expanding the methods available. Skin fibroblasts from the patient were used in the work here reported. The use of skin fibroblasts presents a great advantage in relation to other types of cells, not only are they easier to obtain and maintain in culture but, since they bear the patient's genetic background, their phenotypic

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features reflect the disease dysfunction and allow the validation of their use as models for neurologic disorders [16].

2. Material and methods

2.1. Biological samples

Following ethical guidelines, cell lines and other biological samples were obtained for analysis with the patient's and family members' written informed consent. The consent was obtained by the physician in agreement with local Ethics Committee. The experiments of the present study comply with the current law and are in accordance with the Declaration of Helsinki. Furthermore, the samples were used in codified form and devoid of personal data in order to protect confidentiality.

For the study of the c.66G>A patient, a skin biopsy was obtained by the accompanying physician. Dermal fibroblasts were also obtained from a normal control. Fibroblasts were cultured and expanded and the genotype was confirmed as described elsewhere [4].

For the screening analysis, a total of 717 samples of individuals born in Portugal were randomly selected. The samples consisted of surplus dried blood remainder samples on filter paper (Guthrie cards) from the National Program of Neonatal Screening (Instituto Nacional de Saúde Dr. Ricardo Jorge). Genomic DNA was automatically extracted using a Maxwell 16 System (Promega) following the manufacturer's instructions. Cell cultures were expanded following standard methods. Protein was prepared from freshly scraped cultured fibroblasts, according to a method described by Suzuki and collaborators in 2010 [17] that makes rapid obtaining of nuclear and cytoplasmic cell fractions possible. The method was used as described but with minor adjustments.

2.2. Allelic discrimination of the c.66G>A mutation of the CSTB gene

The AD method combines Real-Time PCR (RT-PCR) quantification and mutation detection of both alleles in a single step. We used the Custom TaqMan® Assay Design Tool and SNP Genotyping Assays (Life Technologies). The assay consisted of unlabeled PCR primers (sequence 5′–3′: CSTB forward primer: GCCGAGACCCAGCACATC; CSTB reverse primer: CGCGCCCTGAGGCTAAG), and TaqMan MGB probes FAM™ and VIC® dye-labeled probes (G allele probe: VIC—GGTGGGTGGACC AGC; A allele probe: FAM—GGTGGGTGAACCAGC). All assays were performed as 5 μL reactions using TaqMan Universal PCR Master Mix (Life Technologies), 40 × TaqMan SNP Genotyping Assay Mix (Life Technologies), and 2.5 ng genomic DNA using iCycler (Bio-Rad). The RT-PCR thermal cycling and analysis parameters were standard.

2.3. Western Blot analysis

Western Blot (WB) was performed in order to see if there was any difference in cytoplasmatic and nuclear Cstb localization between patient and normal control cells. After protein extraction [17], 10 μL and 5 μL of whole cell lysate, cytoplasmic and nuclear fractions, respectively, were loaded and electrophoresed on NUPAGE Bis-Tris Midi Gel 4–12%, using the Novex Midi Gel System (Life Technologies) followed by semi-dry transfer onto the nitrocellulose membrane. Protein fractions were detected by incubation with the anti-Stefin B antibody (ab54566, mouse monoclonal, Abcam) followed by incubation with anti-mouse IgG-HRP as the secondary antibody (sc-2005, Santa Cruz Biotechnology). Cellular fractionation was controlled by incubation of protein fractions with the anti-alpha tubulin antibody (T6199, mouse monoclonal, Sigma) as a cytoplasmic marker or anti-nucleoporin (NUP98 A13983, rabbit monoclonal, Life Technologies) as a nuclear marker, followed by incubation with HRP-conjugated anti-mouse (sc-2005, Santa Cruz Biotechnology) and anti-rabbit (sc-2004, Santa Cruz Biotechnology) secondary antibodies, respectively. The protein signal was developed using the Enhanced Chemiluminescence System (GE Healthcare).

2.4. Fluorescence microscopy

Immunofluorescence (IF) with the same primary antibodies used in WB was performed in the patient's and normal control cells except for the anti-CSTB antibody (sc-33275, Santa Cruz Biotechnology) since, in our experience, the anti-Stefin B antibody (ab54566) was not suitable for IF. The secondary antibodies used were chicken anti-mouse IgG-TR (sc-3924, Santa Cruz Biotechnology), and anti-rabbit IgG-FITC (sc-2012, Santa Cruz Biotechnology). A standard protocol procedure was used. Additionally, LysoTracker® Blue (L-7525, Life Technologies) staining, was used to test lysosome stability. Fluorescence imaging was observed with a Leica DM4000 microscope with a DFC345 FX camera. Images were recorded using the Leica Application Suite software.

3. Results and discussion

A total of 717 samples, 1434 alleles, were analyzed by AD. Strong fluorescent signals were obtained for each allele, resulting in clear separation between the four cluster points corresponding to the genotypes GG (homozygous wild-type), GA (heterozygous mutant), and AA (homozygous mutant) and with no amplification for nontemplate controls (NTC). After sample analysis no positive results, heterozygous mutant or homozygous mutant, were detected (data not shown). As a Portuguese mutation, it was interesting to know that c.66G>A is absent from the general population and, therefore, as a unique mutation it is not expected to raise the overall risk for ULD. Since the dodecamer repeat is the major CSTB mutation, it would be interesting to study its prevalence in Portugal in order to improve genetic counseling and the development of new therapeutic strategies.

As can be seen in Fig. 1-A, WB results show that in ULD cells Cstb is clearly present in the cells' total fraction and in the nuclear fraction, although with a marked decrease in relation to normal. In normal control cells we can observe the homogenous presence throughout the cell compartments. However, in the cytoplasm, the ULD Cstb decrease is clear when compared with the normal control which could suggest a lack of protective anti-protease function and subsequent compromise of cellular integrity.

Immunofluorescence results (Fig. 1-B) were in accordance with the WB results, suggesting a different distribution for the patient's Cstb protein. IF with the Cstb antibody indicated the increased presence of Cstb in the nuclei of the patient's cells and confirmed the decrease of Cstb in the cytoplasm. Nevertheless, experimental results obtained with alpha-tubulin and NUP98 showed no relevant differences between normal control and ULD patient cells. In other studies, Cstb was also localized throughout the cytoplasm [18] and nucleus [19]. More recently, Joensuu et al., described an ULD patient with a p.G50E missense mutation with a slight decrease in the amount of Cstb; subcellular localization of this mutant showed a strong signal in the nucleus and diffuse representation in the cytoplasm [3], in accordance with our findings, which represent partial loss of lysosomal localization. This finding supports the physiological importance of Cstb–lysosome association: cystatins are crucial for maintaining controlled proteolysis caused by the target cysteine proteases and any mechanism reducing this subtle regulated balance may result in substantial pathological problems [20]. Cumulatively to the pathogenic effect of the release of cathepsins into the cytoplasm, the increased retention of Cstb in the nucleus may result in delay in cell cycle progression [12], a fact that we observed during ULD fibroblast culture expansion since cell multiplication was much slower comparing with normal control fibroblasts. Cstb present in the nucleus has been described to bind to histones and indirectly regulate the cell cycle through inhibition of cathepsin L [12], and this increase in the expression of Cstb in the nucleus delays caspase activation and apoptosis, although not preventing cell death [19]. Recently Cstb protein mutants have been described as having a tendency to aggregate in cells [14]. Nevertheless, the cause of the degree of disease progression and neurodegeneration remains unclear. It is not known whether it may be due to altered/missing
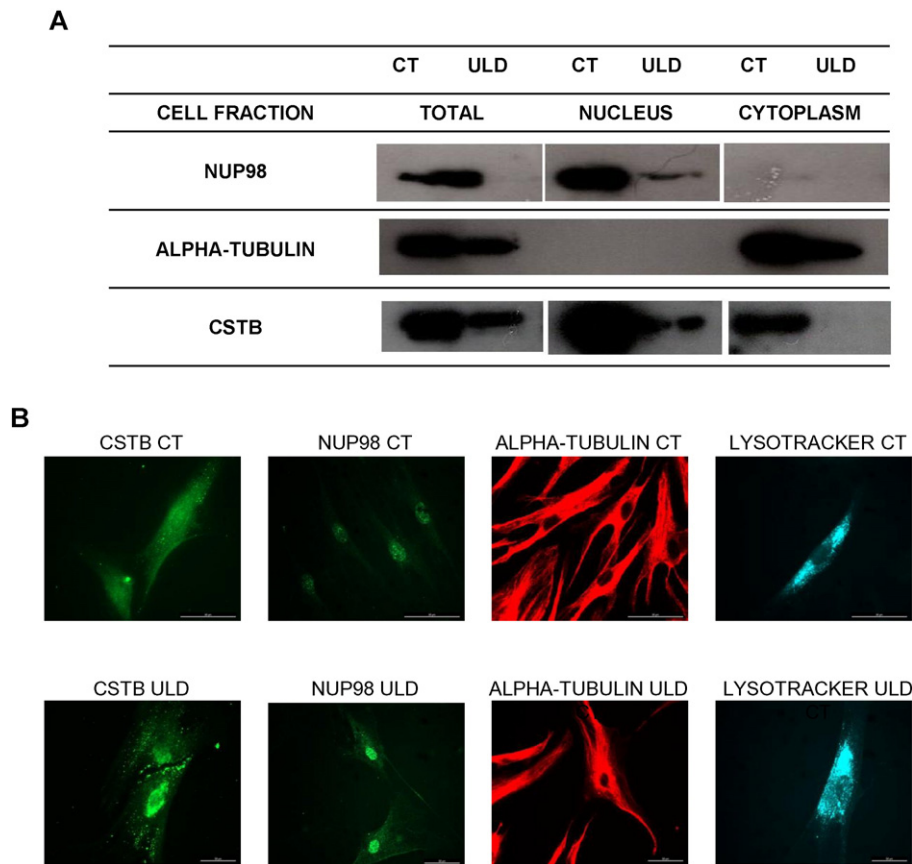
Fig. 1. Comparison between normal and mutant dermal fibroblasts. A — Western Blot of different cellular fractions: CT — normal control; ULD — ULD patient. NUP98 antibody has 98 kDa, alpha-tubulin antibody has 50 kDa, and CSTB has 11 kDa. B — Immunofluorescence images of control and ULD patient fibroblasts. Bar: 50 μm.

protein function or to protein abnormal misfolding and aggregation or a combination of both [14]. Recently, Cstb protein mutants have been described as having a tendency to aggregate in cells [14]. As observed in the figure, the distribution of protein detected in the patient cells is clearly different from the control. In the patient, Cstb seems to concentrate in its nuclear location and the lysosomes show a slightly different distribution. The putative aberrant protein resulting from the abnormal transcript (c. 66G>A) might be more prone to aggregate formation and lead to a toxic gain of function. Although we did not check the possible formation of aggregates, or the aggresome–autophagy pathway, it is possible that, as it happens with missense mutants [14], formation of toxic aggregates may contribute to disease development and neurotoxicity.

4. Conclusion

In this work we established that mutation c.66G>A is, very likely, unique and as such the parents of the homozygous patient must be related. Furthermore, this patient may also display unique characteristics and the cellular characterization should be further explored. The study of protein fractionation demonstrated that cystatin B does exist in the fibroblasts of the homozygous patient and is clearly mislocated in relation to the protein of normal cells. Since the abovementioned mutation has a milder effect, leading to a small amount of normal transcript, in addition to the abnormal transcript [4], we confirmed that the normal form is likely to ensure partial minimal function, in agreement with the slower progressive form of disease presented by the patient. However, the aberrant transcript is probably responsible for the different Cstb distribution observed by IF in the patient's cells. The putative aberrant protein associated with mutation c.66G>A might lead to a toxic gain of function. The fact that this patient has a unique genotype, being homozygous for a splicing mutation and not presenting the dodecamer expansion, adds value to our findings. Further investigation will be needed in order to determine the contribution of abnormal protein misfolding and aggregation to the pathogenesis of ULD.

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