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Autosomal recessive Bethlem myopathy: A clinical, genetic and functional study

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Abstract

Bethlem myopathy represents the milder form of the spectrum of Collagen VI-related dystrophies, which are characterized by a clinical continuum between the two extremities, the Bethlem myopathy and the Ullrich congenital muscular dystrophy, and include less defined intermediate phenotypes. Bethlem myopathy is mainly an autosomal dominant disorder and the causing mutations occur in the *COL6A* genes encoding for the α1 (*COL6A1*), α2 (*COL6A2*) and α3 (*COL6A3*) chains. However, few cases of recessive inheritance have been also reported. We here describe clinical, genetic and functional findings in a recessive Bethlem myopathy family harbouring two novel pathogenic mutations in the *COL6A2* gene. Two adult siblings presented with muscle weakness and wasting, elbows and Achilles tendon retractions, lumbar hyperlordosis, waddling gait and positive Gowers' sign. Muscle biopsy showed a dystrophic pattern. Molecular analysis of the *COL6A2* gene revealed the novel paternally-inherited nonsense p.Gln889* mutation and the maternally-inherited p.Pro260_Lys261insProPro small insertion. Fibroblast studies in both affected patients showed the concomitant reduction in the amount of normal Collagen VI (p.Gln889*) and impairment of Collagen VI secretion and assembly (p.Pro260_Lys261insProPro). Each of the two variants behave as a recessive mutation as shown by the asymptomatic heterozygous parents, while their concomitant effects determined a relatively mild Bethlem myopathy phenotype. This study confirms the occurrence of recessive inherited Bethlem myopathy and expands the genetic heterogeneity of this group of muscle diseases.

Keywords: Collagen VI; Bethlem myopathy; Ullrich congenital muscular dystrophy; COL6A2.

1. Introduction

Collagen VI-related dystrophies represent a clinical continuum ranging from the milder Bethlem myopathy (BM) and the severe Ullrich congenital muscular dystrophy (UCMD). The phenotypical spectrum includes an intermediate UCMD-BM phenotype, limb-girdle variants and myosclerosis [1–4].

These rare conditions share a typical clinical presentation characterized by weakness and hypotonia, associated with laxity of distal joints, contractures and skeletal deformities [5].

In UCMD the onset of the disease occurs typically at the birth or during the first year of life with delay in the acquisition of motor skills [1]. The patients never start walking or, if they learn it, lose ambulation by approximately 10 years [6,7]. Decline in pulmonary function occurs early and mechanical ventilation at night is necessary from the first two decades on average [8].

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Patients with intermediate phenotypes lose ambulation ability by approximately 19 years. The respiratory insufficiency begins slightly later than UCMD but proceeds at a similar rate [8,9].

BM represents the milder form of the spectrum of Collagen VI-related dystrophies [10]. The disease arises during childhood with mild proximal weakness and notable distal joint laxity. Hip dysplasia, equinovarus deformity and torticollis can be early observed. Commonly the contractures set in towards the end of the first decade of life. Walking is maintained until adulthood; however, two-thirds of patient need walking aid by 60 years [11]. The decline in pulmonary function is variable and does not occur until adulthood [8].

Collagen VI-related dystrophies are caused by mutations in one of the three genes (*COL6A1*, *COL6A2* and *COL6A3*) encoding for the three subunits (α1, α2 and α3) of Collagen VI [12]. Unlike UCMD which notoriously displays both an autosomal dominant and recessive inheritance, BM has been considered exclusively an autosomal dominant disease for a long time. Just in the last years, few cases of recessive inheritance were described [13–17].

We here report the findings from a clinical, genetic and functional analysis in a recessive BM family carrying two novel pathogenic mutations in the *COL6A2* gene.

2. Patients and methods

2.1. Patients

The proband (P1) is a 40-year-old man having a history of proximal muscle weakness since childhood. Early motor milestones were normal, however he experienced some difficulties in activities that other children of the same age can do well. He underwent multiple Achilles tendons lengthening surgeries at age of 8, 16 and 19.

Neurological evaluation showed waddling gait and positive Gowers' sign, severe and diffuse muscle atrophy and weakness, elbows and Achilles tendon retractions and lumbar hyperlordosis (Fig. 1). Keratosis pilaris along the extensor surfaces of the arms and legs was also noted.

Creatine kinase levels were always moderately increased (about 500–1000 U/L). Electromyography showed a myopathic pattern.

The proband's 23-year-old sister (P2) presented similar clinical history and a milder phenotype: moderate diffuse muscle weakness and atrophy with proximal predominance, bilateral winging scapula, elbows and Achilles tendon retractions and mild spinal rigidity.

Creatine kinase levels were mildly increased (about 400 U/L). Electromyography showed myopathic changes. Muscle magnetic resonance imaging (MRI), performed with axial T1-weighted and STIR sequences, revealed a pattern suggestive for BM characterized by mainly peripheral adipose substitution of the vasti muscles and central adipose substitution of the rectus femoris at the level of both thighs (Fig. 2A). At the level of the leg a predominant adipose substitution of the peripheral portions of the gastrocnemii muscles was evident (Fig. 2B). No muscle edema could be detected.

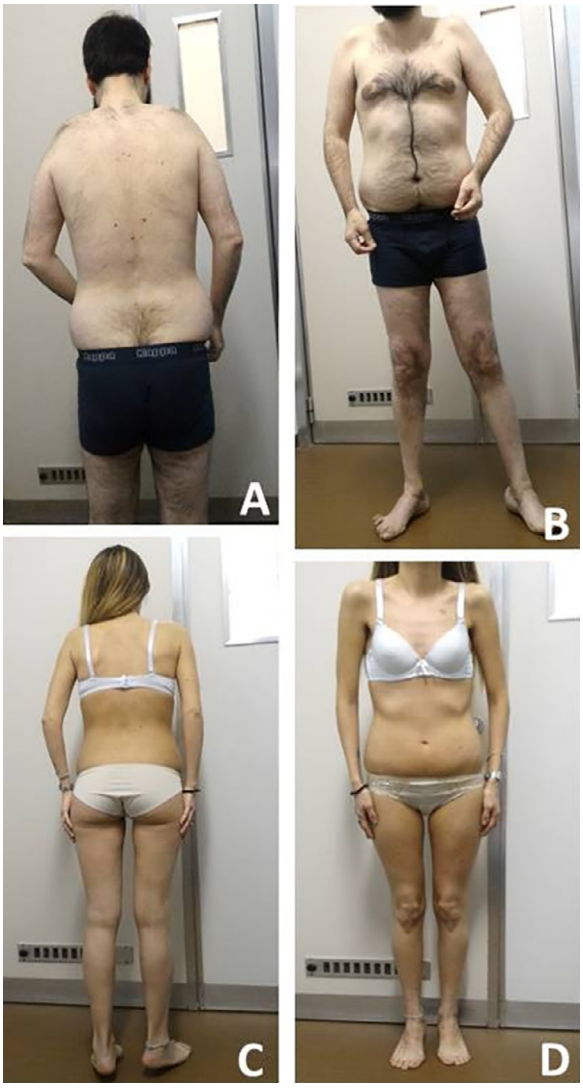

Fig. 1. Limb muscle atrophy and elbow tendon retractions are evident in patient 1 (A,B) and patient 2 (C,D).

Their non-consanguineous parents, both 63 years old, and a 33-year-old brother did not have any symptom or clinical sign of neuromuscular diseases.

2.2. Muscle biopsy

An open muscle biopsy was performed on the right quadriceps muscle in both patients. The tissue was frozen and then cut for routine histological staining. Morphological studies were performed as described [18].

2.3. Mutation analysis

Genetic analysis was performed on genomic DNA purified from peripheral blood lymphocytes by QIA-Symphony Automated Platform (QIAGEN) after informed consent being collected. A custom NGS panel

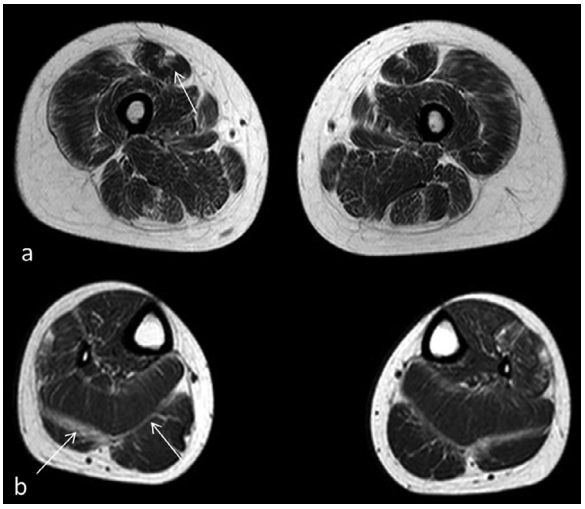

Fig. 2. Axial T1-weighted images at the level of the thigh (a) and of the leg (b). At the level of both thighs a predominant peripheral adipose substitution is evident at the level of the vastus lateralis muscles; central involvement of the rectus femoris- the so called «central shadow» in ultrasound - is also documented (white arrow on the right). At the level of the leg, a peripheral involvement is evident in gastrocnemii muscles, giving the typical «bat wings» appearance (white arrows on the right).

was used, consisting of 188 amplicons (250 bp length each, specific for 77 target regions) covering 100% of the three *COL6A* gene coding regions, spreading over 28.544 bp of genomic DNA (Illumina FC-134–2002-TruSeq Custom Amplicon Low Input (16SMP) COL6PANEL 041,116–1). Runs were performed on a MiSeq^{TM}Dx Instrument, according to manufacturer instructions. Identified mutations were confirmed by conventional Sanger sequencing on an ABI3130xl automated Sequencer (Applied Biosystems, Foster City, CA).

2.4. Primary cell cultures

Skin biopsy was obtained from both patients and a culture of fibroblasts was established according to standard procedures.

Cells were seeded in 6-well plates or glass coverslips and cultured in DMEM supplemented with 10% foetal bovine serum (FBS). Cells were kept in culture for three days upon confluency. During the last 24 h of cultures, cells were either treated with 0.25 mM of L-ascorbic acid, to enhance Collagen VI deposition, or left untreated in DMEM without serum. After treatments, cells were either fixed with 4% paraformaldehyde or lysed to extract proteins. Cell media were kept and proteinase inhibitor (complete EDTA free, Roche) were added.

2.5. Western blotting

Cells were lysed in RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 1% IGEPAL, 0.5% Na-deoxycholate, 0.1% SDS) supplemented with protease (complete EDTA free, Roche) and phosphatase (Cocktail II, Sigma) inhibitors using a cell scraper. Protein extracts from both cell layers and media were quantified by BCA Protein assay kit (Pierce) and separated by either SDS-PAGE or electrophoresis in non-reducing conditions in 2.5% acrylamide/0.5% agarose gel. Proteins were blotted onto PVDF membranes (Millipore) and blocked with 5% milk in TBS-T. Membranes were probed overnight at 4 °C with the following primary antibodies: anti-α2(VI) (HPA007029, Sigma); anti-Collagen VI (70R-CR009X, Fitzgerald); anti-β-actin (MAB1501, Chemicon). After TBS-T washes, with horseradish peroxidase-conjugated secondary antibodies (Bethyl Laboratories). Detection was performed by chemiluminescence.

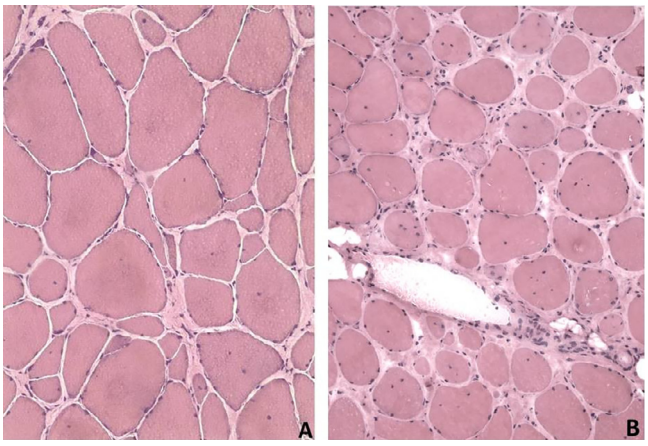

Fig. 3. Muscle biopsy of patient P1 (A) and P2 (B) showed a dystrophic pattern with remarkable endomysial fibrosis, increased variation in fiber size, internal nuclei and atrophic fibers.

2.6. Immunofluorescence

Samples for immunofluorescence labeling were fixed in cold methanol at −20 °C for 10 min, washed in PBS supplemented with 10% goat serum (Sigma) and incubated overnight at 4° with primary antibodies diluted in 5% PBS-Goat serum. The following antibodies were used: COL6A1-2–3 (MAB1944, Chemicon); COL6A2 (HPA007029, Sigma); Fibronectin (Sigma). After washing three times with PBS, secondary antibodies were applied. Slides were finally washed three times in PBS and mounted in 80% PBS-glycerol.

3. Results

3.1. Muscle biopsy

Muscle biopsy of patient P1, performed at age of 31 years, showed a dystrophic pattern with remarkable endomysial fibrosis, marked variability of fiber diameter, internal nuclei and atrophic fibers (Fig. 3A).

In patient P2, muscle biopsy performed at age of 19 years, revealed a similar, but more severe, histological pattern (Fig. 3B).

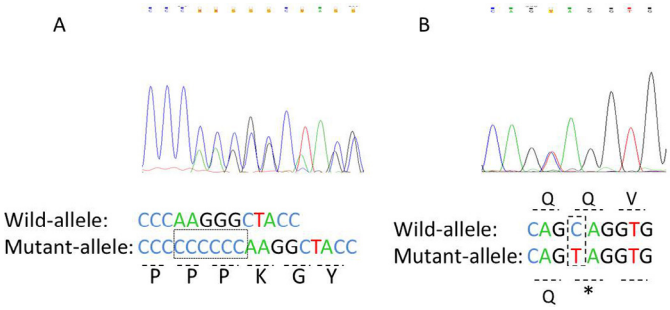

Fig. 4. COL6A2 mutational analysis of the patient 2. A and B: exon 5 and exon 28 electropherogram and relative alignments. The inserted and mutated bases are included in dashed rectangles.

3.2. Mutational analysis

Both patients were compound heterozygous for two previously unreported *COL6A2* changes: the nonsense mutation p.Gln889*, (c.2665C>T within exon 28), and the insertion p.Pro260_Lys261insProPro (c.780_781insCCCCCC within exon 5) (Fig. 4 and Supplemental figure 1). Both variants were absent from control population databases (ESP6500, 1000Genome, ExAc, gnomAD). Healthy mother and father were heterozygous for the p.Gln889* and p.Pro260_Lys261insProPro mutations respectively. The asymptomatic brother presents none of the two genetic variations.

3.3. Analysis of collagen VI in dermal fibroblast cultures

Western blot analysis in reducing conditions showed in both patients very low levels of the canonical 1019 amino acid long α2(VI) chain (C2 isoform with a predicted molecular weight of 140 kDa) with respect to the control sample. An alternative non-pathological 918 amino acid long splice variant was also detected in patients and control, corresponding to a molecular weight of about 120 kDa (C2A isoform).

Patients displayed a mutant polypeptide product, corresponding to the truncated α2(VI) chain, containing 889 amino acids and missing 130 amino acids at the C-terminal domain, with a molecular weight lower than that of the C2A isoform (Fig. 5A).

Western blot analysis under non-reducing conditions revealed normal amounts of Collagen VI dimers and tetramers in the cell layer while they were almost absent in cell media in both patients, thus pointing at a correct intracellular assembly of the protein but high instability of the secreted protein (Fig. 5B).

Immunofluorescence confirmed the markedly reduced expression of Collagen VI, which was scarcely detected and poorly organized in the extracellular matrix (Fig. 6A). Furthermore, patients' fibroblasts displayed an abnormal deposition of fibronectin in the extracellular matrix (Fig. 6B).

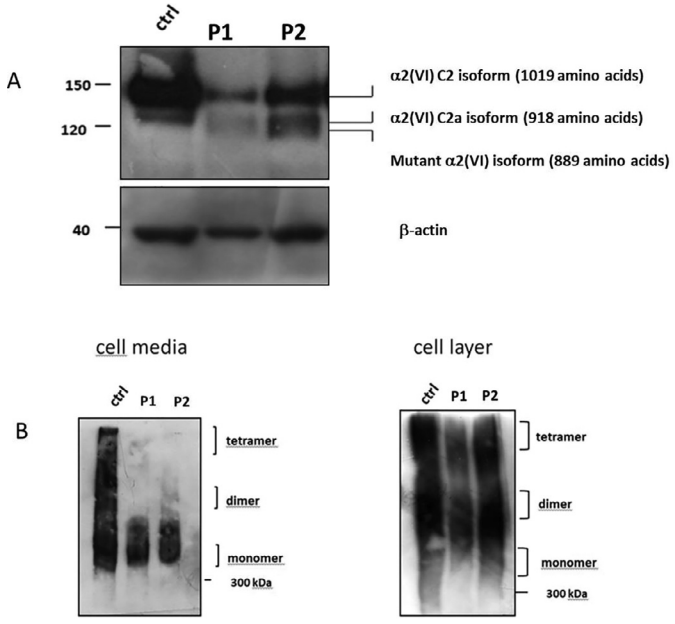

Fig. 5. A- Electrophoretic analysis of the α2(VI) subunits produced by control and BM fibroblasts. Protein extracts from cell layers were analyzed under reducing conditions on a 3–8% gradient polyacrylamide gel. The migration position of α2(VI) isoforms is indicated on the right. While in the control sample the antibody recognizes two bands, related to the canonical α2(VI) splicing isoforms (C2 and C2A), P1 and P2 samples display a third band corresponding to the mutated truncated α2(VI) polypeptide, with a molecular weight slightly lower than the C2A isoform. B- Analysis of Collagen VI produced by control and BM fibroblasts. Protein extracts from culture media and cell layers were analyzed in non-reducing conditions on a composite 0.5% agarose and 2.5% polyacrylamide gel. Collagen VI monomers and disulfide bonded dimers and tetramers are indicated on the right. Tetramers and dimers are not detectable in P1 and P2 cell media extracts.

4. Discussion

While BM usually displays an autosomal dominant inheritance, some cases of autosomal recessive inherited BM were recently described [13–17].

The first report dates back to 2009 when Foley et al. reported two adult siblings with a history of slowly progressive muscle weakness, elbows, ankles and long finger flexor contractures and a compound heterozygote genotype in the *COL6A2* gene (the already known UCMD c.1770delG mutation resulting in frameshifting and premature termination and the c.2488C>T variant causing a change from Arg-to Trp-at position 830) [5,12,14].

In the same year, Gualandi et al. reported two unrelated subjects exhibiting mild limb-girdle and axial weakness, contractures and hypermobility of the distal joints and carrying out a truncating mutation (Q819X; R366X) associated with missense variants on the other allele (D871N; R843W-R830Q) [13]. Decreased Collagen VI in the basal lamina of muscle fibers and in dermal fibroblast cultures and altered stability of the Collagen VI tetramers were demonstrated [13].

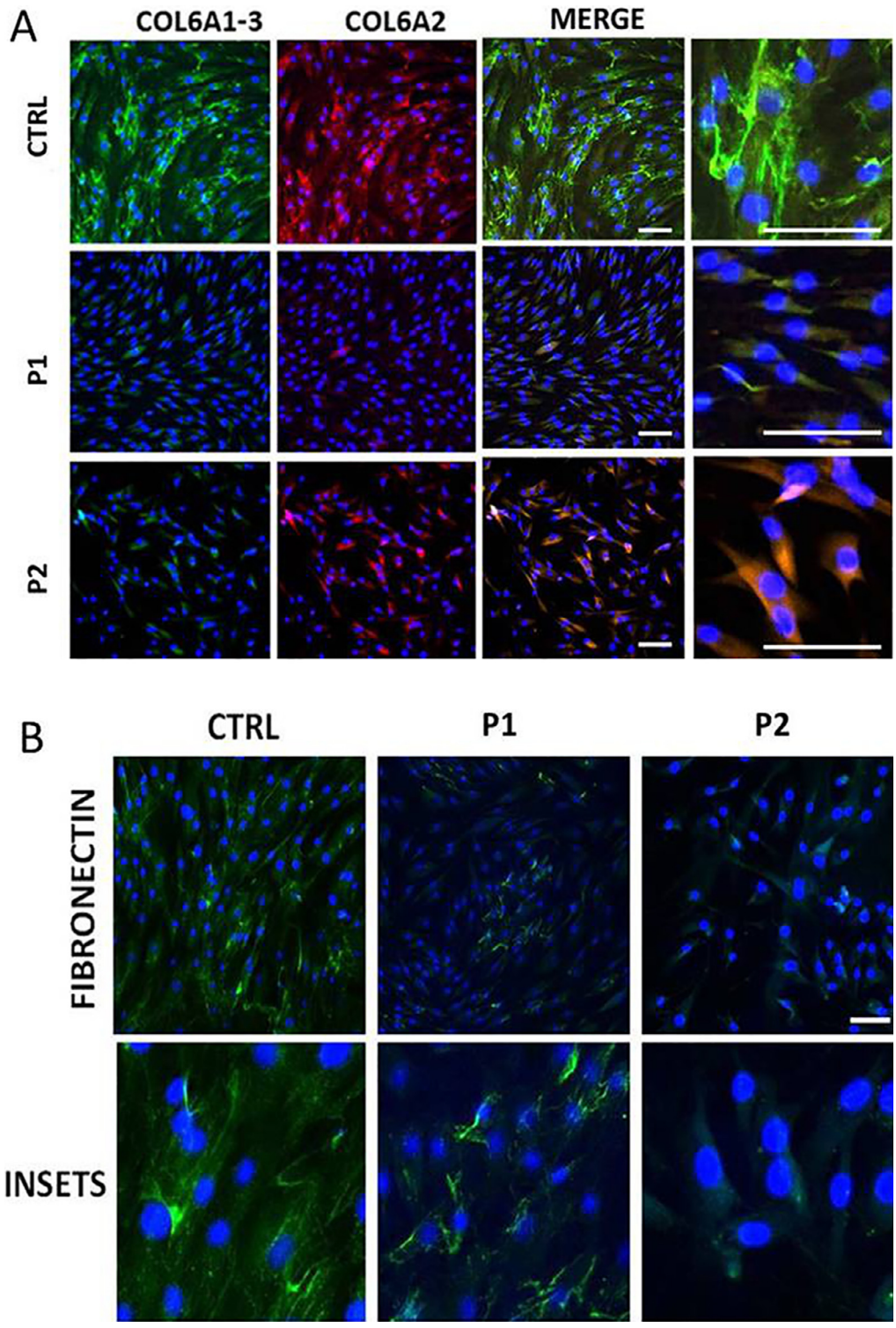

Fig. 6. A: Immunofluorescence analysis of Collagen VI produced by control and BM fibroblasts. Fibroblasts grown on slides were fixed and then stained with antibodies recognizing either whole Collagen VI (COL6A1-3) or the α2(VI) chain (COL6A2). Collagen VI staining appears highly reduced in P1 and P2 extracellular matrix, compared to the control. Cell nuclei were stained with DAPI; scale bar 100 μm. B- Immunofluorescence analysis of fibronectin produced by control and BM fibroblasts. Fibroblasts grown on slides were fixed and then stained with an anti-fibronectin antibody. Fibronectin secretion appears highly reduced in P1 and P2 extracellular matrix, compared to the control. Cell nuclei were stained with DAPI; scale bar 100 μm.

A few others cases of recessive BM have been described since then but recessive inheritance remains a rare occurrence, being about 13% and 21% of the total patients in the large BM cohorts reported by Deconinck et al. and Sframeli et al. respectively [15–17].

Our study further supports the occurrence of a recessive inheritance in the classic phenotype of BM.

Each of our two patients presented features of a classic BM phenotype [19,20] and were compound heterozygous for two variants in the *COL6A2* gene. Their heterozygous parents are healthy.

The mutations here reported have not yet been described in the literature. Specifically, the p.Gln889* mutation introduces a premature termination codon, truncating the amino acid sequence within exon 28 coding for the C2 domain of the α2(VI) chain. The second mutation is an insertion which causes the introduction of two proline residues within exon 5 coding for the triple helical domain of the α2(VI) chain.

We studied the effects of these two changes by Western Blot in both reducing and non-reducing conditions.

Analysis in reducing conditions allowed us to gather information on the weight of the Collagen VI chains and showed that the Gln889* mutation causes the production of a truncated polypeptide missing 130 amino acid residues at the C-terminal end of the α2(VI) chain.

We also detected the canonical 1019 amino acid long α2(VI) chain together with the alternative splicing C2A variant which is about 100 amino acid residues shorter than the canonical chain, as also reported in previous studies [21].

Therefore, in patients' fibroblasts, we detected three bands: the canonical α2(VI) chain, although at a markedly reduced amount, the alternative splicing product α2(VI) C2A isoform and the truncated α2(VI) polypeptide missing 130 amino acids at the C-terminal end as expected from the non-sense mutation.

Western blot in non-reducing conditions allowed us to evaluate the possible effect of the p.Pro260_Lys261insProPro mutation on the quaternary structure, since the procedures in which the protein extracts were prepared preserved the disulfide bridge interactions between the Collagen VI chains and their assembly into monomers, dimers and tetramers.

In these conditions, we detected normal amounts of dimers and tetramers in the cell layer. Differently, the culture medium, which contains secreted proteins, and, therefore, should display Collagen VI tetramers in large amounts, showed the presence of Collagen VI monomers while dimers and tetramers were substantially missing, thus suggesting a higher instability of Collagen VI assemblies, once secreted.

This was also confirmed by immunofluorescence analyses of cultured fibroblasts showing that Collagen VI is scarcely able to form the typical network structures within the extracellular matrix.

The concurrent reduction of the fibronectin network in the matrix of patients' fibroblasts compared to the control supports a dysfunction of secreted Collagen VI. Indeed, the extracellular organization of fibronectin is known to be influenced by Collagen VI whose deficiency has an effect on the amount of secreted and deposited levels of fibronectin [22].

Each of the two mutations, alone, is not able to cause the disease, as shown by the asymptomatic heterozygous parents. Differently, the concurrent (i) reduction in the amount of normal protein (mutation c.2665C> T) and (ii) impairment of protein secretion and stability as well as assembly with fibronectin (p.Pro260_Lys261insProPro mutation) displayed by both two affected siblings leads to a mild phenotype attributable to a BM picture.

Finally, our findings confirm that recessive inheritance may be a feature of BM. This evidence, although limited to few cases so far, leads to relevant implications in genetic counselling and in genotype-phenotype correlation in Collagen VI disorders.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2019.07.007.

References

[1] Ullrich O. Kongenitale, atonisch-sklerotische Muskeldystrophie. Monatsschr Kinderheilkd 1930;47:502–10.
[2] Bethlem J, Wijngaarden GK. Benign myopathy, with autosomal dominant inheritance. A report on three pedigrees. Brain 1976;99:91–100. doi:10.1093/brain/99.1.91.
[3] Scacheri PC, Gillanders EM, Subramony SH, Vedanarayanan V, Crowe CA, Thakore N, et al. Novel mutations in Collagen VI genes: expansion of the Bethlem myopathy phenotype. Neurology 2002;58:593–602. doi:10.1212/WNL.58.4.593.
[4] Merlini L, Martoni E, Grumati P, Sabatelli P, Squarzoni S, Urciuolo A, et al. Autosomal recessive myosclerosis myopathy is a Collagen VI disorder. Neurology 2008;71:1245–53. doi:10.1212/01.wnl.0000327611.01687.5e.
[5] Bertini E, Pepe G. Collagen type VI and related disorders: Bethlem myopathy and Ullrich scleroatonic muscular dystrophy. Eur J Paediatr Neurol 2002;6:193–8. doi:10.1053/ejpn.2002.0593.
[6] Nadeau A, Kinali M, Main M, Jimenez-Mallebrera C, Aloysius A, Clement E, et al. Natural history of Ullrich congenital muscular dystrophy. Neurology 2009;73:25–31. doi:10.1212/WNL.0b013e3181aae851.
[7] Briñas L, Richard P, Quijano-Roy S, Gartioux C, Ledeuil C, Lacène E, et al. Early onset Collagen VI myopathies: genetic and clinical correlations. Ann Neurol 2010;68:511–20. doi:10.1002/ana.22087.
[8] Foley AR, Quijano-Roy S, Collins J, Straub V, McCallum M, Deconinck N, et al. Natural history of pulmonary function in Collagen VI-related myopathies. Brain 2013;136:3625–33. doi:10.1093/brain/awt284.

[9] Bönnemann CG. The Collagen VI-related myopathies: muscle meets its matrix. Nat Rev Neurol 2011;7:379–90. doi:10.1038/nrneurol.2011.81.
[10] Mohire MD, Tandan R, Fries TJ, Little BW, Pendlebury WW, Bradley WG. Early-onset benign autosomal dominant limb-girdle myopathy with contractures (Bethlem myopathy). Neurology 1988;38:573–80. doi:10.1212/WNL.38.4.573.
[11] Jöbsis GJ, Boers JM, Barth PG, de Visser M. Bethlem myopathy: a slowly progressive congenital muscular dystrophy with contractures. Brain 1999;122:649–55. doi:10.1093/brain/122.4.649.
[12] Lampe AK, Bushby KM. Collagen VI related muscle disorders. J Med Genet 2005;42:673–85. doi:10.1136/jmg.2002.002311.
[13] Gualandi F, Urciuolo A, Martoni E, Sabatelli P, Squarzoni S, Bovolenta M, et al. Autosomal recessive Bethlem myopathy. Neurology 2009;73:1883–91. doi:10.1212/WNL.0b013e3181c3fd2a.
[14] Foley AR, Hu Y, Zou Y, Columbus A, Shoffner J, Dunn DM, et al. Autosomal recessive inheritance of classic Bethlem myopathy. Neuromuscul Disord 2009;19:813–17. doi:10.1016/j.nmd.2009.09.010.
[15] Zamurs LK, Idoate MA, Hanssen E, Gomez-Ibañez A, Pastor P, Lamandé SR. Aberrant mitochondria in a Bethlem myopathy patient with a homozygous amino acid substitution that destabilizes the Collagen VI α2(VI) chain. J. Biol Chem 2015;290:4272–81. doi:10.1074/jbc.M114.632208.
[16] Deconinck N, Richard P, Allamand V, Behin A, Lafôret P, Ferreiro A, et al. Bethlem myopathy: long-term follow-up identifies COL6 mutations predicting severe clinical evolution. J Neurol Neurosurg Psychiatry 2015;86:1337–46. doi:10.1136/jnnp-2013-307245.
[17] Sframeli M, Sarkozy A, Bertoli M, Astrea G, Hudson J, Scoto M, et al. Congenital muscular dystrophies in the UK population: clinical and molecular spectrum of a large cohort diagnosed over a 12-year period. Neuromuscul Disord 2017;27:793–803. doi:10.1016/j.nmd.2017.06.008.
[18] Bancroft JD, Cook HC. Manual of histological techniques. Edinburgh, London, Melbourne, New York: Churchill Livingstone; 1984.
[19] Mercuri E, Lampe A, Allsop J, Knight R, Pane M, Kinali M, et al. Muscle MRI in Ullrich congenital muscular dystrophy and Bethlem myopathy. Neuromuscul Disord 2005;15:303–10. doi:10.1016/j.nmd.2005.01.004.
[20] Mercuri E, Clements E, Offiah A, Pichiecchio A, Vasco G, Bianco F, et al. Muscle magnetic resonance imaging involvement in muscular dystrophies with rigidity of the spine. Ann Neurol 2010;67:201–8. doi:10.1002/ana.21846.
[21] Zhang RZ, Zou Y, Pan TC, Markova D, Fertala A, Hu Y, et al. Recessive COL6A2 C-globular missense mutations in Ullrich congenital muscular dystrophy: role of the C2a splice variant. J Biol Chem 2010;285:10005–15. doi:10.1074/jbc.M109.093666.
[22] Sabatelli P, Bonaldo P, Lattanzi G, Braghetta P, Bergamin N, Capanni C, et al. Collagen VI deficiency affects the organization of fibronectin in the extracellular matrix of cultured fibroblasts. Matrix Biol 2001;20:475–86. doi:10.1016/S0945-053X(01)00160-3.