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Collagen VI-related myopathy caused by compound heterozygous mutations of
COL6A3 in a consanguineous Kurdish family

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Abstract

Collagen VI related myopathies are caused by mutations of *COL6A1*, *COL6A2* and *COL6A3* and present with a wide phenotypic spectrum ranging from severe Ulrich congenital muscular dystrophy (UCMD) to mild Bethlem myopathy (BM). Here we report a consanguineous Kurdish family with three siblings affected by autosomal-recessive BM caused by compound heterozygous mutations of *COL6A3*. We found the previously described missense mutation

c.7447A>G / p.(Lys2483Glu) and a novel large deletion encompassing the exon 1 to 39 of the *COL6A3* gene. Apart from the classical clinical symptoms, all patients had keratoconus, which expands the phenotype of the collagen VI related myopathies.

Key words: collagen VI, Ulrich congenital muscular dystrophy (UCMD), Bethlem myopathy (BM), keratoconus

Introduction

Mutations in each of the collagen VI genes cause two types of muscle diseases: Ulrich congenital muscular dystrophy (UCMD), a severe phenotype, and a mild to moderate phenotype known as Bethlem myopathy (BM). Recently, two additional phenotypes including limb-girdle muscular dystrophy (LGMD) and autosomal recessive myosclerosis were reported [1]. However, there is extreme variability with intermediate phenotypes designated as mild UCMD or severe BM [2].

Here we report a consanguineous family with three BM siblings compound heterozygous for mutations of *COL6A3*. All patients had keratoconus, which has not been reported yet.

Patients and methods

The family is of Kurdish ethnic origin. The asymptomatic parents are second degree cousins. A maternal aunt is also affected.

Detailed clinical, electrophysiological, imaging and pathological examinations were performed.

After obtaining informed consent, genomic DNA from patients and their parents was extracted from blood. Molecular-genetic testing with Next Generation Sequencing (NGS) using a congenital muscular dystrophy (CMD) panel was done using the TruSight One Sequencing Kit

(Illumina, Inc). The library was sequenced on a MiSeq (Illumina, Inc.) with 2x150bp reads. Coverage was 98.48% >20x for the targeted panel where only coding exons were selected. Bioinformatic pipeline and analysis was performed using SophiaDDM (Sophia Genetics SA). Variant classification and interpretation were done according to the ACMG guidelines [3]. CNV analysis of the NGS data was performed using the NxClinical software (BioDiscovery, Inc.). Exon 36 of the *COL6A3* was amplified and sequenced. SYBR Green I was used as intercalating dye and the PCR products were run on the LightCycler 480 (F. Hoffmann-La Roche Ltd). Array-CGH was run with Microchip Agilent 180K according to manufacturer instructions and analysed using Agilent CytoGenomics v3.0 software (Agilent Technologies, Inc.).

Results

Phenotype

All patients were born after an uneventful pregnancy and had normal early psychomotor development. At the most recent examination all had no cognitive, cranial nerves, cerebellar, **autonomic** or sensory abnormalities.

Patient 1, male

First walking difficulties were noted at 5 years. At 12 years surgery for Achilles tendons contractures was performed. The patient was still able to walk with crutches at 18 years and became wheelchair dependent at 30 years. At age 32 years he presented with mild scoliosis and pronounced lumbar lordosis. Limb muscle atrophy and bilateral scapular winging was noted. There was general muscle weakness including axial musculature with thigh adductors and iliopsoas being most severely affected. There was keratosis pilaris and keloids at the sites of surgery. The patient was operated for bilateral keratoconus. Creatine kinase (CK) levels

(reference ranges <190) were 451 U/l and 216 U/l at 17 and 28 years, respectively. Heart ultrasound was unremarkable. The needle electromyography was myopathic.

Brain MRI was normal. MRI of the thighs showed severe symmetrical atrophy of the proximal muscles, especially the adductor magnus and semitendinosus with relative sparing of the gracilis and sartorius (Figure 1A).

Muscle biopsy disclosed myopathic changes with fiber type 1 predominance (Figure 2A). Immunohistochemistry showed normal staining for dystrophin, sarkoglycans, alpha- and beta-dystroglycan, collagen VI (Figure 2B), caveolin, emerin und dysferlin. Calpain-3 Western Blot was unremarkable. The 300kDa N-terminal fragment of the alpha-2 chain of laminin 211 was reduced while the 80kDa fragment showed normal expression (Figure 2D and 2C). There was diminished expression of the beta-1 and gamma-1 chain of laminin-211 (Figure 2E and 2F).

Patient 2, male

The patient presented with progressive walking difficulties at 5 years. Achilles tendons contractures were repeatedly operated. At 30 years he presented with waddling gait, general muscle weakness including axial musculature and pronounced weakness of thigh adductors and iliopsoas. He had mild kyphoscoliosis and finger contractures. Limb muscle atrophy with bilateral scapular winging were found. He had keratosis pilaris. CK was 258 U/l. Muscle MRI demonstrated symmetrical atrophy of the postural muscles, the thigh and pelvic girdle muscles, adductor magnus, biceps, semitendinosus with relative sparing of sartorius and gracilis and a “central shadow” in rectus femoris (Figure 1B).

Ophthalmological evaluation revealed bilateral keratoconus, more pronounced on the right (Figure 3 and 4). Heart ultrasound and pulmonary function tests were normal. However, obstructive sleep apnea was reported.

Patient 3, female

The patient presented at 6 years with walking difficulties and Achilles tendon contractures. At 24 years she presented with overweight, waddling gait, general muscle weakness more pronounced of the thigh adductors and iliopsoas. She had finger contractures. She had keratosis pilaris and was operated for bilateral keratokonus. CK was 927 U/l. Muscle MRI showed fatty degeneration of paraspinal muscles, gluteus maximus and thigh adductors (especially adductor magnus), with relative sparing of sartorius and gracilis (Figure 1C).

Molecular-genetic testing

Based on the phenotype we suspected collagen VI related myopathy and analyzed patient 2 with NGS CMD panel.

We found the previously described missense mutation NM_004369.3:c.7447A>G / p.(Lys2483Glu) of *COL6A3* and confirmed it with Sanger sequencing in all patients. With no wild type allele and consanguinity we suggested that the patients are homozygous for this mutation (Figure 5A and 5C).

We proceed with testing both unaffected parents. Surprisingly p.(Lys2483Glu) was heterozygously found only in the mother (Figure 5C). Paternity was confirmed by STR analysis.

Quantitative RT-PCR disclosed approximately 50% reduction of exon 36 of *COL6A3* in all siblings and in the father, which explains the apparent c.7447A>G/p.(Lys2483Glu) “homozygosity” observed in the three patients (Figure 5A and 5C). The high-resolution array-CGH showed that exons 1 to 39 of *COL6A3* were absent on the paternal allele (Figure 5D). This deletion was again confirmed by analyzing the NGS data for CNVs with the bioinformatic

tool NxClinical (Figure 5B). In more details, array-CGH showed that the observed deletion was around 90'000bp: arr[hg19] 2q37.3(238253619_238344510)x1 pat.

Discussion

All patients shared BM phenotype with onset of toe walking because of Achilles tendons contractures at 5 years, predominant proximal and paraspinal muscle involvement, contractures with classical “Bethlem sign”, mildly elevated CK, keratosis pilaris, keloids, mild scoliosis but no rigid spine.

However there is intrafamilial variability with the oldest brother being more severely affected losing ambulation at 30 years. The other male sibling was still able to walk with support at 30 years, while the 24-year-old sister can still walk independently.

In contrast to classical BM the eldest brother lost ambulation in early adulthood and should therefore be classified as an “intermediate” or severe BM phenotype.

Because of the consanguinity, we suspected a homozygous disease-causing mutation. Surprisingly we found that the siblings are compound heterozygous for a missense mutation and a large deletion of *COL6A3*. The missense c.7447A>G / p.(Lys2483Glu) mutation has already been reported in homozygous or compound heterozygous state [4-8]. Our data together with the previously reported cases showed that it is not deleterious mutation and behave as a recessive one causing recessive mild BM or mild LGMD [4-8]. This mutation was found in a patient with Canadian descent [4], in Polish patients [4], in patients from UK [7] and in Spain [8], suggesting that p.(Lys2483Glu) has a worldwide distribution and probably arose independently. In contrast to previously reported patients [4], none of our patients demonstrated malnourishment, the female sibling is overweight.

The novel 90kb deletion is the largest one in *COL6A3* identified so far. It leads to complete absence of the protein. The unaffected father carries it heterozygously further supporting that haploinsufficiency does not cause BM.

Muscle biopsy showed normal Collagen VI immunohistochemistry, in line with previous reports in a patient, who was compound heterozygous for p.(Lys2483Glu) and a frameshift mutation [4]. Reduced expression of beta-1 laminin with normal gamma-1 and alpha-2 laminin in BM has previously been reported [9]. However, we observed reduced expression of all three chains of laminin-211, including beta-1, gamma-1 and the 300 kDa fragment of the alpha-2 chain. The newly described large deletion is predicted to cause a complete absence of protein. We speculate that this leads to partial lack of connection of collagen VI and basement membrane thus explaining the secondary deficiency of laminin-211. Analysis of collagen VI production in dermal fibroblast cultures can be helpful, as it is expected to be completely absent or severely reduced in UCMD or demonstrating more subtle abnormalities in BM [10]. However it was not performed in our patient. Alternatively reverse transcription PCR of mRNA extracted from muscle or dermal fibroblasts should be performed in order to confirm the complete lack of collagen VI production from the deletion and detection of the missense cDNA mutant only. We did not performed mRNA analysis though.

Despite the lack of functional analysis, it is feasible that p.(Lys2483Glu) does not significantly affect tetramer assembly and may modulate the deleterious effect of the large, novel deletion, which would probably cause UCMD in homozygous state.

Recessive truncating mutations in the triple helical domain of collagen VI have been associated with the most severe phenotype [5]. However, intrafamilial variability in our and other families points to the existence of additional factors that shape the phenotype. Since collagen VI has a multiple interacting partners it is plausible that polymorphisms in many genes may contribute to the phenotype variability.

Of note, all affected siblings suffer from bilateral keratokonus. Eye involvement is frequently observed in α -dystroglycanopathies [11]. However, it has not been reported in UCMD or BM and *COL6A1* had been excluded as keratokonus causative gene [12]. Keratoconus is a bilateral asymmetric, progressive ectatic condition in which cornea becomes conical in shape and that can lead to significant visual impairment. The etiology of keratoconus is not fully understood with multiple studies suggesting a multifactorial disorder resulting from genetic predisposition and triggered by environmental factors [13]. Two studies in Asians reported two collagen related genes (*COL5A1* and *COL8A2*) associated with central corneal thinning. Given that collagen VI is also localized in corneal stroma [14,15] it is not surprising that the mutated protein could lead to keratokonus. Since many patients with collagen VI-associated myopathy lack evidence of keratokonus additional genetic factors may contribute to the development of keratokonus in our patients. Further studies are necessary to support this observation.

In conclusion, we report a family with autosomal-recessive BM caused by mutations of *COL6A3*. We provide further evidence that *COL6A3* p.(Lys2483Glu) is a worldwide recessive mutation and reported for the first time a novel large 90kb deletion in *COL6A3*. All affected siblings had keratokonus, thus expanding BM phenotype. Our data further demonstrated the value of quantitative RT-PCR, CNV analysis and segregation analysis in identification of mutations even in case of consanguinity and apparent homozygous mutation. Worldwide studies of multiple families with intrafamilial variability may identify additional genetic factors that modify the phenotype in collagen VI-related myopathies.

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Figure legends:

Figure 1

A. Left: axial T1-weighted gradient-echo in-phase image with Dixon two-point technique (thighs) Right: axial contrast enhanced T1-weighted gradient echo fat-saturated image with Dixon two-point technique (thighs). Note: Severe symmetrical atrophy of the proximal muscles

of the legs, especially adductor magnus and semitendinosus with relative sparing of gracilis, sartorius and “central shadow” in rectus femoris (arrow).

B. Left: axial contrast enhanced T1-weighted gradient echo fat-saturated image with Dixon two-point technique (hip) Right: axial contrast enhanced T1-weighted gradient echo fat-saturated image with Dixon two-point technique (thighs) Note: susceptibility artifacts around right hip from total hip replacement (asterisk). Marked atrophy of the thigh and pelvic girdle muscles, adductor magnus, biceps and semitendinosus muscles with relative sparing of sartorius and gracilis and “central shadow” in rectus femoris (arrow).

C. Left: axial contrast enhanced T1-weighted gradient echo fat-saturated image with Dixon two-point technique (hip) Right: axial contrast enhanced T1-weighted gradient echo fat-saturated image with Dixon two-point technique (thighs). Note: fatty infiltration of gluteus maximus and thigh muscles, e.g. adductors with relative sparing of sartorius and gracilis (arrow).

Figure 2. Muscle biopsy.

A. HE stained cross section of skeletal muscle showing dystrophic changes including increased fiber size variation with atrophic and hypertrophic muscle fibers, internal nuclei and increased endomysial connective tissue. B. Immunohistochemistry showing normal amounts and extracellular localization of ColVI. C. Normal staining of 80 kDa C-terminal fragment of the alpha 2 chain of laminin-211. D. Reduced sarcolemmal expression of the 300 kDa N-terminal fragment of laminin-211. E. Reduced sarcolemmal expression of the beta-1 chain of laminin-211. F. Reduced sarcolemmal expression of the gamma-1 chain of laminin-211. Scale bar = 50um.

Figure 3 and 4. Corneal topography performed with Pentacam rotating camera of the left and right eye, respectively. A. Overview. Upper: a cross-sectional image showing the cornea,

anterior chamber, iris, and lens. Lower left: a 3-D representation of the patient's corneal shape; the anterior corneal surface is shown in red, posterior corneal surface in green, and iris in blue. Lower right: a pachymetry map indicating corneal thickness; cooler colors are thicker and warmer colors are thinner (numeric scale on right). B. Pentacam 4 Map Report. Representation of the curvature map on the left and elevation map on the right. Warmer colors represent steeper corneal curvature while cooler colors represent flatter areas. For the elevation maps (upper and lower right, anterior and posterior float, respectively), warmer colors denote where the cornea is elevated above the best fit sphere and cooler colors denote where the cornea is depressed below the best fit sphere. A pachymetry map (lower left) indicating corneal thickness; cooler colors are thicker and warmer colors are thinner. C. Belin-Ambrósio enhanced ectasia display (BAD) showing altered indices with a final D index (BAD-D) higher than 2.1 confirming keratoconus

Figure 5.

(A) IGV view of the mutation found with NGS in Patient 1. Only a small subset of the reads are shown as horizontal bars. The mutation is written in blue as reverse complement (cytosine, C) and the 97% variant frequency (VF) is represented by the blue rectangle above the mutation.

(B) CNV calling for patient 1 performed on the basis of the NGS data showing deletion of a major part of the *COL6A3* gene, with the exons described on the first line and the deletion with a red horizontal bar. The coverage for each exon is shown in the middle of the panel in black. The normalized coverage (calculated copy number) is shown as grey virtual probes on the bottom of the panel, where the called deletion is highlighted in light red.

(C) Traces of the Sanger sequencing for each patient. Only the forwards sequence is shown. The mutation location is highlighted with the red arrow for each patient and the parents.

(D) CNV calling by microarray analysis for patient 1 and the father. The *COL6A3* gene is shown on the first line with the deletion highlighted with a red bar. The array probes are shown with gray points and the CNV calling is highlighted with light red.