A novel *de novo COL6A1* mutation emphasizes the role of intron 14 donor splice site defects as a cause of moderate-progressive form of ColVI myopathy – a case report and review of the genotype–phenotype correlation

Agnieszka A. Koppolu[1,2], Agnieszka Madej-Pilarczyk[3], Małgorzata Rydzanicz[1], Joanna Kosińska[1], Piotr Gasperowicz[1], Jolanta Dorszewska[4], Wojciech Kozubski[5], Barbara Steinborn[6], Andrzej M. Kochański[3], Rafał Płoski[1]

[1]Department of Medical Genetics, Medical University of Warsaw, Warsaw, [2]Postgraduate School of Molecular Medicine, Warsaw, [3]Neuromuscular Unit, Mossakowski Medical Research Centre Polish Academy of Sciences, [4]Laboratory of Neurobiology, Department of Neurology, Poznan University of Medical Sciences, Poznan, [5]Chair and Department of Neurology, Poznan University of Medical Sciences, Poznan, [6]Departments of Developmental Neurology, Poznan University of Medical Sciences, Poznan, Poland

Abstract

Collagen VI-related myopathy is a group of disorders affecting skeletal muscles and connective tissue. The most common symptoms are muscle weakness and joint deformities which limit the movement and progress over time. Several forms of collagen VI-related myopathies have been described: Bethlem myopathy, an intermediate form and Ullrich congenital muscular dystrophy, which is the most severe. Here we report a novel de novo c.1056+3A>C substitution in intron 14 of the COL6A1 gene encoding alpha-chains of collagen VI in a 13-year-old girl suffering from collagen VI (ColVI) myopathy. Analysis performed on cDNA generated from the RNA obtained from the patient's blood cells showed that the reported variant leads to the entire exon 14 skipping and probably results in an in-frame deletion of 18 amino acids of the COL6A1 protein. Clinical presentation, abnormal secretion of the collagen demonstrated in muscle biopsy and the COL6A1 c.1056+3A>C mutation justify classification of the presented case as ColVI myopathy with moderate-progressive course. Analysis of the literature indicates that the donor splice site of COL6A1 intron 14, associated with the phenotype of Bethlem myopathy or intermediate form, is a hot spot for ColVI myopathies.

Key words: COL6A1, RNA splicing, Bethlem myopathy.

Introduction

Collagen VI myopathies encompass a spectrum of skeletal muscle disorders clinically manifesting as muscle weakness, joint contractures and distal laxity. Pathological features include defects in collagen VI alpha-chains, which are encoded by three genes: *COL6A1, COL6A2* and *COL6A3*. Collagen VI is expressed in connective tissue, tendons and skeletal muscle, where it contributes to cell matrix adhesion.

Communicating author

Prof. Rafał Płoski, Department of Medical Genetics, Medical University of Warsaw, 3c Pawinskiego St., 02-106 Warsaw, Poland, phone: +48 22 572 06 06, e-mail: rploski@wp.pl

Two allelic entities – relatively mild Bethlem myopathy (BM) (OMIM # 158810) and severe Ulrich congenital muscular dystrophy (UCMD) (OMIM # 254090) – are extremes within the clinical continuum which also includes intermediate forms of various severity [3,5]. In the classic form BM is a slowly progressing muscular dystrophy with limb-girdle distribution and onset in infancy, joint contractures and hyperextensibility of distal joints. The first comprehensive clinical descriptions were published by Bethlem and Wijngaarden in 1976 [4] and by Arts *et al.* in 1978 [1], who reported three multigenerational families of Dutch origin and one family of Polish descent, respectively. The mode of inheritance is autosomal dominant. The clinical picture of UCMD, clinically characterized by Ullrich in 1930 [19], includes onset in early infancy, muscle wasting, weakness and hypotonia, proximal joint and spine contractures, distal joint laxity and possible respiratory failure. The mode of inheritance is autosomal recessive (the majority of cases) or autosomal dominant [7].

In this study we report on a patient suffering from Bethlem myopathy resulting from a novel *de novo COL6A1* mutation: c.1056+3A>C.

Material and methods

Peripheral blood samples

Peripheral blood samples were collected from the proband and proband's relatives (mother, father and sister) and genomic DNA was extracted from all the samples.

Gene panel sequencing and verification

Libraries for gene panel sequencing of the proband's DNA obtained from peripheral blood was prepared using TruSight One Sequencing Panel (Illumina, San Diego, California, USA) target enrichment. Libraries were pair-end sequenced (2 × 100 bp reads) on the platform Illumina HiSeq1500 (Illumina). The 20× and 10× target coverage was 95.3% and 97.9%, respectively. To verify and/or confirm the results Sanger sequencing was performed. To confirm the parenthood the NGM Kit (Applied Biosystems (AB), Foster City, CA, USA) was used.

RNA extraction and cDNA synthesis

RNA was isolated from blood cells using the Animal Tissue RNA Purification Kit (Norgen Biotek Corp.,

Thorold, ON, Canada) according to the manufacturer's instructions. RNA quantity and quality were measured using the NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) and 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) RNA 6000 Nano. RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, California, USA).

Polymerase chain reaction and sequencing

In order to amplify the cDNA sequence containing the entire exon 14, specific forward and reverse primers were designed in exons 13 and 15 respectively: 5'-CTGTTGGGTACCAGGGAATG-3' and 5'-ACCGTCAGGTCCTGGGTTC-3'. Thermal conditions of the PCR reaction are available on request.

PCR products were visualized on 2% agarose gel containing ethidium bromide and purified. Libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) and pair end sequenced (2 × 100 bp) using Illumina HiSeq 1500 (Illumina). The results were visualized on an Integrative Genomics Viewer (IGV) [17,18].

Bioinformatic predictions

The effect of c.1056+3A>C in the *COL6A1* gene on the splicing at the donor site was evaluated by NetGene2 [6], Humans Splicing Finder [8], Splice Site Prediction by Neural Network (BDGP: NNSplice) [16] and Alternative Splice Site Predictor (ASSP) [20].

Results

Case report

Here we present a 13-year-old girl with inherited myopathy associated with collagenopathy. The girl was the second child of unrelated parents. She was born at 39 weeks of gestation by elective caesarean section due to oligohydramnios and breech presentation. Her birth weight was 2940 g (< 3 c), length was 57 cm, head circumference was 34.5 cm, and the Apgar score was 9. During the neonatal period weak sucking, decreased muscle tone, joint laxity and hyperextension of elbows, knees and wrists were observed. In addition, hip dysplasia was found. Motor milestones were delayed: sitting – 9-10 m, crawling – 12 m, walking – 17 m. Since early childhood she had presented a waddling gait, imbalance,
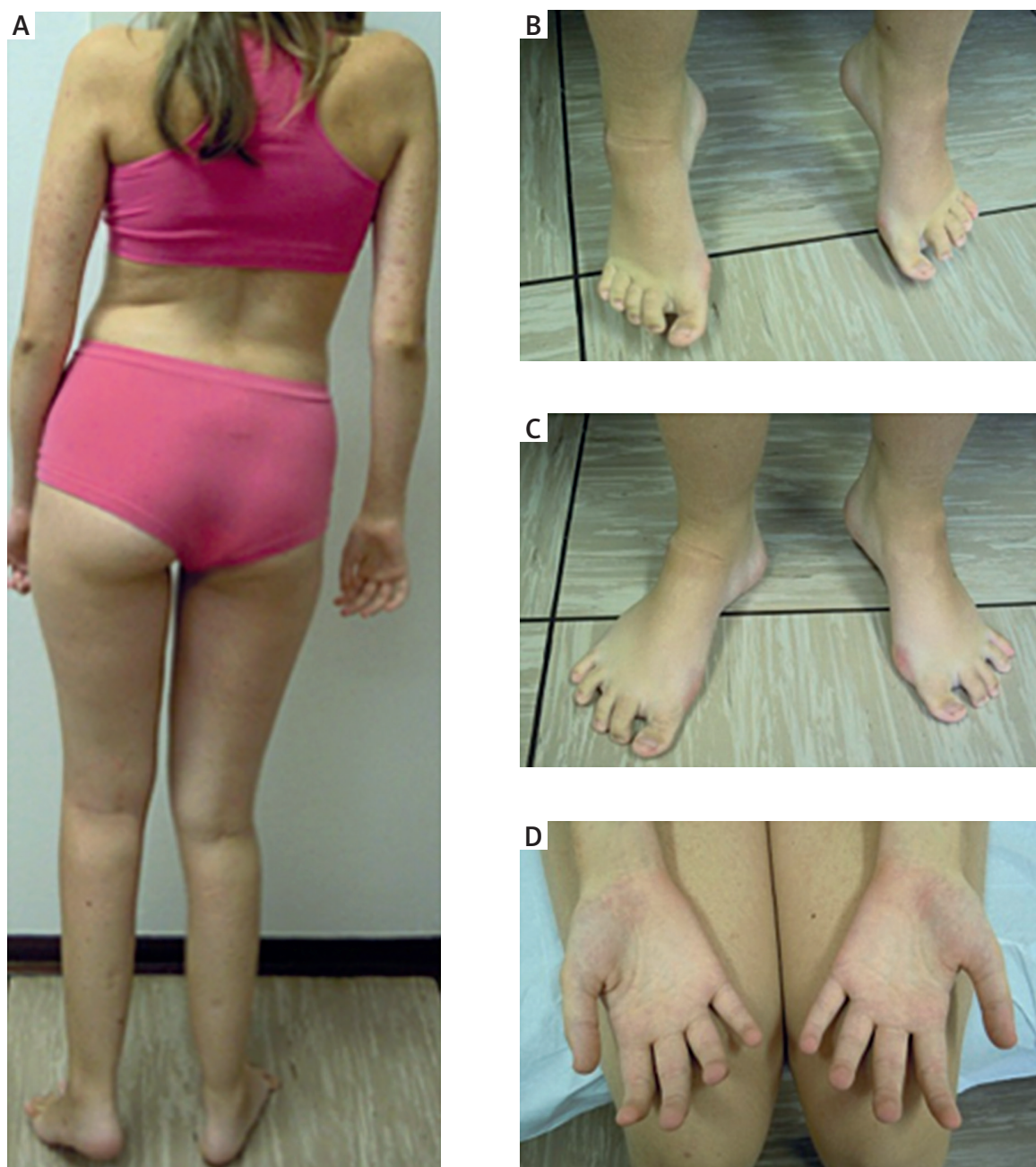
Agnieszka A. Koppolu, Agnieszka Madej-Pilarczyk, Małgorzata Rydzanicz, Joanna Kosińska, Piotr Gasperowicz, Jolanta Dorszewska, Wojciech Kozubski, Barbara Steinborn, Andrzej M. Kochański, Rafał Płoski

Fig. 1. A 13-year-old girl with collagenopathy. **A)** Note shoulder and hip asymmetry and scoliosis, calf atrophy and Achilles tendon contractures. **B)** Foot deformation visible in relaxed position with feet dangling. **C)** Distal hypotonia: while standing there are evident gaps between toes. **D)** Slim hands with long fingers.

problems with climbing stairs and standing up from a sitting and kneeling position, and frequent stumbling and falling. No joint dislocations or sprains occurred. Mental development, including speech, was normal. Comorbidities included food allergy, manifesting as atopic dermatitis and treated with

a gluten-free diet and antihistaminic drugs. Examinations performed at the age of 9 years revealed persistent gait abnormalities despite regular physical therapy, with imbalance and disturbed motor coordination, probably as a result of marked hypotonia of distal muscles. The girl required assistance

during walking, and was not able to run, jump or squat. In addition to generalized muscle weakness, there was compensating left-sided scoliosis within the Th-L spine and lack of full extension of the hips as well as restricted motor function. Joint reflexes were weak, knee reflexes absent. Isolated Achilles tendon contractures and clubfeet developed. At the age of 10 years surgical correction of the ankle equinus contracture was done by the Vulpius procedure, which included superficial gastrocnemius-soleus recession with an intramuscular lengthening of the soleus muscle, which resulted in moderate clinical improvement. At the age of 13 years the patient was able to walk only with calf orthoses. She presented a waddling gait on a wide base, with compensating spine hyperlordosis. Physical examination findings also included: head dropping when standing up from the lying position due to weakness of axial muscles, atrophy of shoulder girdle muscles, normal spine mobility, marked distal hypotonia with finger and toe laxity, hyperflexion of small hand joints, clubfeet, ankle contractures and Gower's sign (Fig. 1). She has been treated with deflazacort (Calcort) 6 mg with a good subjective effect with respect to global muscle strength.

Initial diagnostic work-up before panel sequencing excluded large chromosomal aberrations, the most common chromosomal microdeletions/microduplications, Pompe disease, GM1 gangliosidosis, other metabolic myopathies, spinal muscular atrophy, limb-girdle muscular dystrophy 2A (mutations in exon 4 and 11 of the CAPN3 gene) and laminopathy. Clinical evolution of the disease could not support the initial diagnosis of Ehlers-Danlos syndrome, considered within the scope of the differential diagnosis.

Creatine kinase (CK) was 210 IU (n: 26-140 IU). Electromyography showed no evident myopathic pattern. Electroneurography showed normal sensory and motor conduction. Head MRI did not show abnormalities. On echocardiography mild aortic and mitral valve regurgitation was found.

Conventional microscopy of a quadriceps muscle specimen showed small clumps of two types of fibers with normal diameter and of very small fibers within hypertrophic adipose tissue. Fiber division to metabolic type was abnormal, with a huge predominance of type 1 fibers. On ultrastructural examination there were atrophic fibers and very small uninuclear fibers,

resembling myotubes (immature cells). These cells were surrounded by overgrown collagen (Supplementary Fig. 1).

DNA study

We identified a heterozygous *de novo* splice donor site mutation at c.1056+3A>C of the *COL6A1* gene as a putative cause of the proband's symptoms (Supplementary Fig. 2). Sanger sequencing confirmed presence of the mutation in the proband. Analysis of the family members showed that the mutation was a de novo event (it was not found in the mother, father or sister of the proband) (Fig. 2). The parenthood was confirmed by dedicated short tandem repeat (STR) analysis.

RNA study

cDNA analysis revealed, that, as expected, the shorter PCR product (322 bp) was observed only in patient 1 (proband). The full length PCR product (376 bp) was observed in the proband, parents and the control (wild type). The results of sequencing of the PCR products revealed abnormal splicing events resulting in exon 14 skipping (Supplementary Figs. 3 and 4).

Discussion

In this study we report on a patient suffering from Bethlem myopathy resulting from a novel *de novo COL6A1* mutation: c.1056+3A>C. Mutations located at donor splice sites cause formation of shorter/longer products due to the cryptic donors used by the spliceosome or the entire exon skipping when a donor located in the previous exon is used. In keeping with this we confirmed by laboratory analysis that the *COL6A1* c.1056+3A>C mutation leads to abnormal mRNA splicing. The RNA/cDNA study revealed an additional, shorter PCR product present in the proband but absent in her parents and the control. Sequencing of the amplified cDNA revealed the absence of the entire exon 14. Other *COL6A1* mutations affecting the same splice site and similarly leading to exon 14 skipping have previously been reported in patients with Bethlem myopathy: c.1056+1G>A [2, 14], c.1056+2T>C [15] and c.1056+5G>T [9]. These mutations were shown to lead to formation of an abnormal collagen VI protein with an in-frame deletion of 18 amino acids from the triple helical domain of the α1 chain [2]. Thus, it

Agnieszka A. Koppolu, Agnieszka Madej-Pilarczyk, Małgorzata Rydzanicz, Joanna Kosińska, Piotr Gasperowicz, Jolanta Dorszewska, Wojciech Kozubski, Barbara Steinborn, Andrzej M. Kochański, Rafał Płoski
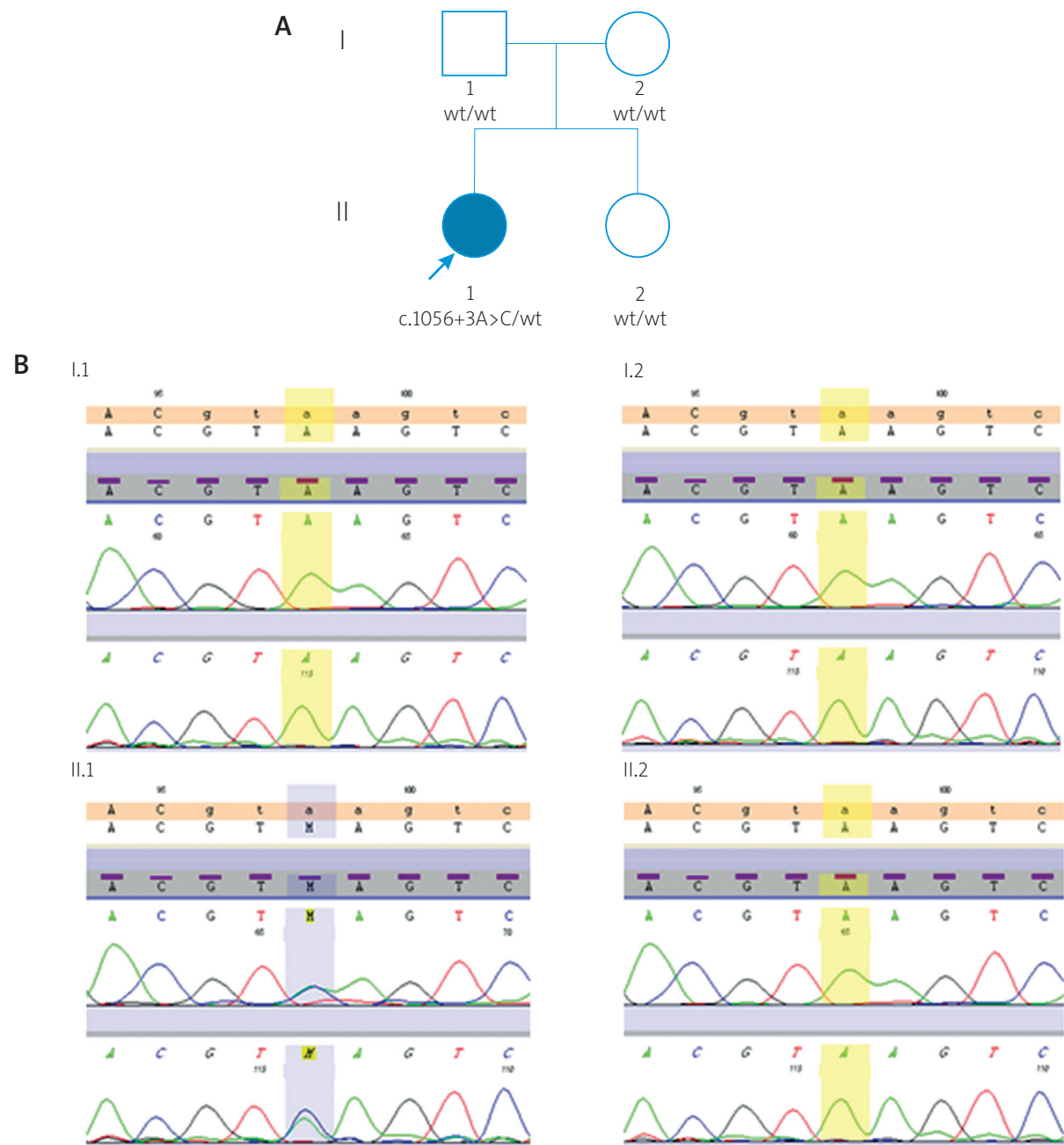
Fig. 2. A) Pedigree of the proband and the family. **B)** Sanger sequencing results confirmed the *de novo* pattern of inheritance of c.1056+3A>C mutation. Subjects I.1, I.2, II.2. (all unaffected: father, mother and sister) present the wild type genotype. Proband presents the heterozygous variant of c.1056+3A>C mutation.

is likely that in our patient the formation of abnormal collagen causes the clinical phenotype. Due to numerous reports of detected variants in the exon 14–intron 14 splice site in patients with Bethlem myopathy, we also speculate that this particular splice site is a hot spot for mutations underlying this disease.

Little is known about phenotype–genotype correlations and predictors of progression in Bethlem myopathy. In an elegant study of a large cohort of

patients harboring mutations within COL6A(1-3) genes three phenotypes were defined: (i) early severe, (ii) moderate progressive and (iii) mild [5], which might correspond to UCMD, intermediate form and BM, respectively. At the age of 13 years our patient is still ambulant, but she is able to walk only with calf orthoses. At this moment we cannot predict the progression of her disease. However, hip contractures, scoliosis, head dropping and pronounced distal laxity might indicate an uncertain prognosis regarding preservation of independent movement in adulthood [10]. If this was the case, we would classify the patient in the intermediate group, with a moderate-progressive course. We cannot exclude that dry, rough and reddish skin on the arms and thighs, attributed to atopic dermatitis, might be consistent with keratosis follicularis, often seen in patients with ColVI-associated disorders [5]. Slight elevation of CK and histopathological features such as variation of fiber diameter, fiber atrophy, increase of fatty tissue and overgrown collagen have all been previously described in BM [1,4].

The analysis of patients with ColVI-related myopathy reported by Brinas shows that heterozygous *COL6A1* splice-site mutations, which caused exon skipping, are found more frequently in patients with intermediate, i.e. moderate-progressive phenotype. The *COL6A1* c.1056+3A>C mutation found by us is located within the triple-helical domain (THD) of the COL6A1 protein [13]. The vast majority of THD affecting mutations are associated with moderate to severe phenotype [5]. There are several reports on *COL6A1* splice-site variants, which produced the same effect on mRNA and protein as in our patients, i.e. skipping of exon 14 and loss of 18 amino acids of the final protein. The c.1056+1G>A variant was described first by Lamande *et al.* in 1999 [11] in a family with slowly progressive limb-girdle myopathy with prominent joint contractures with autosomal dominant inheritance. This variant was also found in two patients by Baker *et al.* [2] with a milder phenotype than that observed in our proband: neither patient had congenital hip dislocation, motor capacity was not affected or was only mildly reduced, distal hyperlaxity and weakness were seen in one patient, scoliosis in the other, and in neither of them did head dropping occur. The family described by Park *et al.* [14] was affected with slowly progressive muscle weakness without sig-

nificant disability until old age. Contrary to our patient there was no laxity of distal joints and weakness was more pronounced in proximal muscles. Lampe *et al.* [12] reported that 4 patients with the mutation c.1056+1G>A had classical Bethlem myopathy. Of 3 members of a 3-generation family with the *COL61A* mutation c.1056+2T>C, reported by Pepe *et al.,* the male proband and his mother presented with stable muscle weakness, allowing preservation of independent walking, Achilles tendon contractures treated by tenotomy and shortening of finger flexors and distal joint hyperlaxity were observed in the boy. However, the proband's grandfather had been wheelchair-bound since the age of 35 due to motor disability and multijoint contractures. The phenotype in a patient with variant c.1056+5G>T, reported by Foley *et al.* in the context of pulmonary function in ColVI-related myopathies, was classified as intermediate; however, no detailed clinical description was provided (Supplementary Table I).

Acknowledgments

Disclosure

Authors report no conflict of interest.

References

1. Arts WF, Bethlem J, Volkers WS. Further investigations on benign myopathy with autosomal dominant inheritance. J Neurol 1978; 217: 201-206.
2. Baker NL, Mörgelin M, Pace RA, Peat RA, Adams NE, Gardner RJ, Rowland LP, Miller G, De Jonghe P, Ceulemans B, Hannibal MC, Edwards M, Thompson EM, Jacobson R, Quinlivan RC, Aftimos S, Kornberg AJ, North KN, Bateman JF, Lamandé SR. Molecular consequences of dominant Bethlem myopathy collagen VI mutations. Ann Neurol 2007; 62: 390-405.
3. Bertini E, Pepe G. Collagen type VI and related disorders: Bethlem myopathy and Ullrich scleroatonic muscular dystrophy. Eur J Paediatr Neurol 2002; 6: 193-198.
4. Bethlem J, Wijngaarden GK. Benign myopathy, with autosomal dominant inheritance. A report on three pedigrees. Brain 1976; 99: 91-100.
5. Brinas L, Richard P, Quijano-Roy S, Gartioux C, Ledeuil C, Lacène E, Makri S, Ferreiro A, Maugenre S, Topaloglu H, Haliloglu G, Pénisson-Besnier I, Jeannet PY, Merlini L, Navarro C, Toutain A, Chaigne D, Desguerre I, de Die-Smulders C, Dunand M, Echenne B, Eymard B, Kuntzer T, Maincent K, Mayer M, Plessis G, Rivier F,

Agnieszka A. Koppolu, Agnieszka Madej-Pilarczyk, Małgorzata Rydzanicz, Joanna Kosińska, Piotr Gasperowicz, Jolanta Dorszewska, Wojciech Kozubski, Barbara Steinborn, Andrzej M. Kochański, Rafał Płoski

Roelens F, Stojkovic T, Taratuto AL, Lubieniecki F, Monges S, Tranchant C, Viollet L, Romero NB, Estournet B, Guicheney P, Allamand V. Early onset collagen VI myopathies: Genetic and clinical correlations. Ann Neurol 2010; 68: 511-520.

6. Brunak S, Engelbrecht J, Knudsen S. Prediction of human mRNA donor and acceptor sites from the DNA sequence. J Mol Biol 1991; 220: 49-65.

7. Camacho Vanegas O, Bertini E, Zhang RZ, Petrini S, Minosse C, Sabatelli P, Giusti B, Chu ML, Pepe G. Ullrich scleroatonic muscular dystrophy is caused by recessive mutations in collagen type VI. Proc Natl Acad Sci U S A 2001; 98: 7516-7521.

8. Desmet FO, Hamroun D, Lalande M, Collod-Béroud G, Claustres M, Béroud C. Human Splicing Finder: an online bioinformatics tool to predict splicing signals. Nucleic Acids Res 2009; 37: e67.

9. Foley AR, Quijano-Roy S, Collins J, Straub V, McCallum M, Deconinck N, Mercuri E, Pane M, D'Amico A, Bertini E, North K, Ryan MM, Richard P, Allamand V, Hicks D, Lamandé S, Hu Y, Gualandi F, Auh S, Muntoni F, Bönnemann CG. Natural history of pulmonary function in collagen VI-related myopathies. Brain 2013; 136: 3625-3633.

10. Jobsis GJ, Boers JM, Barth PG, de Visser M. Bethlem myopathy: a slowly progressive congenital muscular dystrophy with contractures. Brain 1999; 122: 649-655.

11. Lamande SR, Shields KA, Kornberg AJ, Shield LK, Bateman JF. Bethlem myopathy and engineered collagen VI triple helical deletions prevent intracellular multimer assembly and protein secretion. J Biol Chem 1999; 274: 21817-21822.

12. Lampe AK, Dunn DM, von Niederhausern AC, Hamil C, Aoyagi A, Laval SH, Marie SK, Chu ML, Swoboda K, Muntoni F, Bonnemann CG, Flanigan KM, Bushby KM, Weiss RB. Automated genomic sequence analysis of the three collagen VI genes: applications to Ullrich congenital muscular dystrophy and Bethlem myopathy. J Med Genet 2005; 42: 108-120.

13. Okada M, Kawahara G, Noguchi S, Sugie K, Murayama K, Nonaka I, Hayashi YK, Nishino I. Primary collagen VI deficiency is the second most common congenital muscular dystrophy in Japan. Neurology 2007; 69: 1035-1042.

14. Park HJ, Choi YC, Kim SM, Kim SH, Hong YB, Yoon BR, Chung KW, Choi BO. Molecular Genetic Diagnosis of a Bethlem Myopathy Family with an Autosomal-Dominant COL6A1 Mutation, as Evidenced by Exome Sequencing. J Clin Neurol 2015; 11: 183-187.

15. Pepe G, Giusti B, Bertini E, Brunelli T, Saitta B, Comeglio P, Bolognese A, Merlini L, Federici G, Abbate R, Chu ML. A heterozygous splice site mutation in COL6A1 leading to an in-frame deletion of the alpha1(VI) collagen chain in an italian family affected by bethlem myopathy. Biochem Biophys Res Commun 1999; 258: 802-807.

16. Reese MG, Eeckman FH, Kulp D, Haussler D. Improved splice site detection in Genie. J Comput Biol 1997; 4: 311-323.

17. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP. Integrative genomics viewer. Nat Biotechnol 2011; 29: 24-26.

18. Thorvaldsdottir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinform 2013; 14: 178-192.

19. Ullrich O. Kongenitale, atonisch-sklerotische Muskeldystrophie, ein weiterer Typus der heredodegenerativen Erkrankungen des neuromuskulären Systems. Zeitschrift für die gesamte Neurologie und Psychiatrie 1930; 126: 171-201.

20. Wang M, Marin A. Characterization and prediction of alternative splice sites. Gene 2006; 366: 219-227.