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Exclusion of biglycan mutations in a cohort of patients with neuromuscular disorders

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Abstract

Biglycan has been considered a good candidate for neuromuscular disease based on direct interactions with collagen VI and α-dystroglycan, both of which are linked with congenital muscular dystrophy (CMD). We screened 83 patients with CMD and other neuromuscular disorders and six controls for mutations and variations in the biglycan sequence. We identified a number of novel sequence variations. After family analysis and control screening we found that none of these polymorphisms were disease-causing mutations. Thus mutations in biglycan are not a common cause of neuromuscular disorders in our cohort.

Keywords: Congenital muscular dystrophy; Biglycan; α-Dystroglycan; Collagen VI

1. Introduction

Congenital muscular dystrophy (CMD) is characterized by muscle weakness at birth or in the first few months of life. We have recently screened two large Australasian cohorts of CMD and limb girdle muscular dystrophy (LGMD) patients for known genetic causes. Despite recent diagnostic advances, a definite diagnosis was reached in only 24% of CMD patients [1] and 22% of LGMD patients [2], thus the underlying cause of disease in the majority of CMD and LGMD cases remains unknown. Most of the known CMDs and LGMDs are due to deficiencies in proteins which are localized at the membrane or in the extracellular matrix of muscle. Therefore, proteins that interact with these members or have a similar function are excellent candidates for muscular dystrophy.

Biglycan is a small leucine rich repeat proteoglycan [3,4] widely distributed in connective tissues, including the extracellular matrix of muscle [5]. Biglycan is considered a good candidate for muscular dystrophy because it interacts with α-dystroglycan [6], α- and γ-sarcoglycan [7] and collagen VI [8], which are all associated with muscular dystrophy [9]. Biglycan also interacts with fibrillar collagens and is an

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important determinant of fibrillar collagen assembly and structure *in vivo* [10,11]. In addition, biglycan is able to organize collagen VI microfibrils into hexagonal-like networks *in vitro* via its core protein [8]. It has therefore been postulated that biglycan could mediate interaction between collagen VI and the fibrillar collagens to aid in the formation of the structurally critical fibrillar collagen networks [12]. These multiple interactions of biglycan raise the possibility that biglycan may mediate linkage of the cell surface molecules α-dystroglycan and α- and γ-sarcoglycans to collagen VI in muscle and/or collagen VI to the fibrillar extracellular matrix surrounding each muscle fibre, thus contributing to muscle integrity.

The most compelling direct evidence for a role for biglycan in human muscular dystrophies comes from the phenotype of the biglycan null mouse. Biglycan deficient mice display a mild muscular dystrophy phenotype characterized by membrane disruption, cell death of a subpopulation of myofibres and central nuclei characteristic of regenerating fibres. These mice also have mild osteoporosis which is only evident from 3 months of age [13–15].

On this basis, we sought to determine if biglycan is a primary cause of neuromuscular disease by directly sequencing biglycan in muscular dystrophy patients in whom the genetic diagnosis is unknown.

2. Materials and methods

2.1. Patients

Sequencing of the X-linked biglycan gene (*BGN*) was performed for 83 patients and six controls. The majority of patients ($n = 51$) had a clinical phenotype typical of CMD (Table 1), previously described in Peat et al. [1]. These patients had been screened with antibodies to known CMD related proteins and we had demonstrated no staining abnormalities in 41 of 51 CMD patients. In addition, eight of 15 patients with abnormal immunofluorescence for α-dystroglycan, and one of seven patients with abnormal collagen VI immunofluorescence, were screened for mutations in the six glycosyltransferase genes and the *COL6* genes, respectively, but no mutations were found. We also screened patients with other congenital or childhood onset myopathies including four patients with congenital fiber-type disproportion (CFTD), displaying a clinical appearance typical of a selenoproteinopathy, and in whom *SEPN1* mutations were excluded, and eight patients with clinical symptoms of Bethlem myopathy without mutations in their *COL6* genes.

Table 1
Controls and patients in whom biglycan was sequenced

Patient diagnosis	Males	Females
CMD – α-dystroglycan abnormal IF	7	2
CMD – collagen VI abnormal IF	1	–
CMD – no abnormal IF	25	16
Congenital fiber-type disproportion	2	2
Bethlem myopathy	6	2
Non-CMD neuromuscular disorders	16	4
Controls	3	3
Total	60	29

IF, immunofluorescence.

In addition, we screened the biglycan gene in 20 patients presenting with non-CMD neuromuscular disorders. A subset of these patients had abnormalities in the expression of the syntrophins or dystrobrevins by immunofluorescence [16] and so were included as biglycan has been shown to affect the expression of these proteins [15]. This group included patients with limb girdle muscular dystrophy who had been screened and excluded for known causes of LGMD by immunofluorescence, Western blot and genetic testing; patients with multi-minicore disease, in whom common causes of CFTD and multi-minicore disease were excluded by genetic analysis; and non-dystrophic patients with congenital onset of muscle weakness. The ethnic origin was known for 32 of the probands' parents, of those, approximately one-half were of Caucasian decent and the remainder were of multiple ethnicities reflecting the ethnic diversity of the Australian population (Australian Aboriginal, Chinese, Egyptian, Greek, Indian, Lebanese, Mauritian, Pakistani, Sri Lankan, and Vietnamese). For 25 of the probands, there was a known family history with affected family members and/or consanguinity, the remaining 58 probands were either sporadic cases with no known family history or did not have enough clinical information to determine an inheritance pattern. We also included females in our cohort in the event that mutations in biglycan were lethal for males. This study was approved by the Ethics Committees of The Children's Hospital at Westmead, The University of Sydney, and The Royal Children's Hospital, Melbourne.

2.2. PCR amplification of the biglycan gene

Nine sets of biglycan genomic DNA-specific primers were designed to produce nine fragments covering all eight *BGN* exons including non-coding exon 1 (Supplemental Table 1). For exon 1, PCR conditions were as follows; 94 °C 5 min, followed by 40 cycles of 94 °C 30 s, 64.2 °C 30 s, 68 °C 30 s, followed by 68 °C for 7 min. For exons 2–8, conditions were; 95 °C 5 min, followed by 40 cycles of 94 °C 30 s, 58 °C or 60 °C 30 s, 72 °C 30 s, followed by 72 °C for 5 min. Direct sequencing of PCR products was performed using the same amplification primers. Alignments of biglycan sequences from a variety of vertebrate species were performed using the protein alignment program ClustalW (www.ebi.ac.uk/clustalw/).

3. Results

3.1. Sequence variations identified in the biglycan cohort

All eight exons from the *BGN* gene including up to 100 bp of intronic regions flanking the exons were sequenced using the human biglycan gDNA sequence

Table 2
Sequence variations identified in patient cohort and controls

Detected in	Exon/Intron	gDNA change (ATG = 1)	A.A. position	SNP db	ESTs	SNP frequency
Control	5′ UTR	g.−9802 g>a*	–	–	No	36%
Control	Exon 1	g.−9558 G>T	–	rs5945197	No	36%
Patient	Intron 1	g.−83 g>a*	–	–	No	1%
Control	Exon 2	g.51 G>A*	p.L17L	–	No	36%
Control	Intron 2	g.289 g>a*	–	–	No	8%
Control	Intron 2	g.313 a>g	–	rs2980053	Yes	91%
Patient	Intron 2	g.569 t>c*	–	–	No	1%
Patient	Exon 3	g.625 A>G*	p.K86R	–	No	2%
Patient	Exon 4	g.1293 G>A*	p.R138Q	–	No	1%
Control	Exon 4	g.1420 C>T	p.S180S	rs1126499	Yes	51%
Patient	Exon 4	g.1444 C>T	p.I188I	rs2070933	No	1%
Patient	Exon 6	g.2200 C>T*	p.D226D	–	No	1%
Control	Intron 6	g.2384 c>t	–	rs2073479	No	36%
Patient	Intron 7	g.3599 c>t*	–	–	No	3%
Control	3′ UTR	g.3942 G>A	–	rs1042103	No	31%
Patient	3′ UTR	g.4065 A>T	–	rs743641	No	8%
Patient	3′ UTR	g.4132 G>T	–	rs743642	No	11%
Control	3′ UTR	g.4334 C>A	–	rs11538656	No	36%
Control	3′ UTR	g.4330 Variations of InDels in repeat region	–	rs34973897, rs6151400	–	75%
Control	3′ UTR	g.4656 A>G	–	rs2980054	Yes	100%
Control	3′ UTR	g.4754_4779 del25*	–	–	No	25%
Patient	100 bp after 3′ UTR	g.5011_5033 del23*	–	–	No	2%

UTR, untranslated region; A.A., amino acid; SNP db, single nucleotide polymorphism database; ESTs, expressed sequence tags; –, not applicable; InDels, insertions/deletions; bp, nucleotide base pair; *, unreported SNP; SNP frequency is calculated using all patients and control data.

NM_001711 (chrX:152413605–152428198) from the UCSC genome browser (http://genome.ucsc.edu/) as a reference sequence. All sequence changes were detected with forward and reverse strand sequencing and then confirmed by repeat PCR and sequencing. Changes found are listed in Table 2. All changes were checked against the reference human genome sequence using the UCSC genome browser and a single nucleotide polymorphism (SNP) number was recorded if the change had been published before. If there was no known SNP, then the change with 50 base pairs (bp) of sequence 5′ and 3′ was used to search the NCBI est_human database using a basic local alignment search tool (BLAST, www.ncbi.nlm.nih.gov/BLAST/) to identify published expressed sequence tags (ESTs) corresponding to this region of the *BGN* gene. ESTs represent sequenced cDNA clones obtained from tissue samples from multiple individuals, and can thus be used to identify some common genetic variations. If the change identified in a patient was also identified in a public EST sequence, it was considered unlikely to represent a pathogenic mutation.

From the sequencing data, we detected four synonymous and two non-synonymous coding region changes, and 16 intronic or non-coding changes in the biglycan gene. Three of the non-coding changes had a frequency of at least 25% in our patient group but were not reported in the SNP and EST databases; however, all of these changes were detected in control samples and thus are not likely to be pathogenic. Half of all changes detected in both control and patient samples were not found in either the SNP or EST databases. This is unsurprising since the information in both databases comes from small, ethnically restricted populations. We detected two single base changes that resulted in amino acid substitutions. Both changes occurred in highly conserved amino acid residues. The hemizygous change g.625A > G (p.K86R) was detected in exon 3 of *BGN* in two male patients and a hemizygous change g.1293G > A (p.R138Q) in exon 4 of *BGN* in one male patient.

For the K86R biglycan change, we sequenced 263 de-identified healthy controls (116 males, 147 females). The change was not present in any of the male controls, but was observed in a heterozygous state in three females. We went on to examine a further 300 male controls with mental retardation and 730 male controls with normal IQ [17] and detected the K86R substitution in four control subjects in the group with normal IQ, confirming that this change is a rare non-pathogenic polymorphism with a polymorphism frequency of 0.35% in males.

For the R138Q biglycan change, we screened 188 controls (49 males, 139 females) and the change was not detected. We later excluded R138Q as a disease-causing mutation when it was detected in the patient's healthy male sibling.

4. Discussion

We have excluded mutations in biglycan as a common cause of disease in a cohort of patients with neuromuscular disorders, and have identified common and rare polymorphisms in the biglycan gene. Although biglycan has been considered an excellent candidate gene for CMD, only one previous study had screened biglycan in a cohort of patients. Ishikawa and colleagues studied eight patients with typical Ullrich congenital muscular dystrophy presentation and

reduced amounts of collagen VI at the sarcolemma [18]. Collagen VI mutations were identified in only one patient. The biglycan and decorin genes were sequenced in the other seven patients but no mutations were found.

It is possible that biglycan mutations result in a later-onset myopathy with a phenotype that is less severe than CMD, as biglycan null mice exhibit a mild form of muscular dystrophy [10]. Biglycan has been shown to be secondarily abnormal in a number of muscle disorders [19,20] and direct injection of recombinant biglycan into the dystrophic muscle of biglycan null mice restores the expression of the syntrophin–dystrobrevin–nNOS dystrophin associated subcomplex [15] indicating a role for biglycan in the maintenance of normal muscle function. Thus, like α7-integrin which is secondarily abnormal in many patients with CMD but can also cause primary disease [1,21,22], biglycan remains a potential primary disease candidate for neuromuscular disorders. Alternatively mutations in biglycan may result in a different disease phenotype since it is highly expressed in bone and connective tissue [23] and at the neuromuscular junctions [6].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2008.05.013.

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