Next generation sequencing on patients with LGMD and nonspecific myopathies: Findings associated with ANO5 mutations

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Abstract

We studied 786 undiagnosed patients with LGMD or nonspecific myopathic features to investigate the role of ANO5 mutations in limb-girdle muscular dystrophies (LGMDs) and in nonspecific myopathies using the next generation sequencing (NGS) approach. In 160 LGMD patients, we first sequenced hotspot exons 5 and 20 and then sequenced the remaining part of the coding region. Another 626 patients, recruited using broader inclusion criteria, were directly analyzed by targeted NGS. By combining NGS and Sanger sequencing, we identified 33/786 (4%) patients carrying putative pathogenic changes in both alleles and 23 ANO5 heterozygotes (3%). The phenotypic spectrum is broader than expected, from hyperCKemia to myopathies, with lack of genotype/phenotype correlations. In particular, this is currently the largest screening of the ANO5 gene. The large number of heterozygotes for damaging mutations suggests that anoctaminopathies should be frequent and often nonpenetrant. We propose the multiple genetic testing by targeted NGS as a first step to analyze patients with nonspecific myopathic presentations. This represents a straightforward approach to overcome the difficulties of clinical heterogeneity of ANO5 patients, and to test, at the same time, many other genes involved in neuromuscular disorders.

Keywords: Next generation sequencing; Muscular dystrophy; LGMD2L; Anoctamin; NGS screening; Targeted resequencing; Limb girdle muscular dystrophy

1. Introduction

The diagnosis of autosomal recessive limb-girdle muscular dystrophies (LGMDs) is complex for the presence of a number of different conditions with similar clinical presentation [1]. The genetic studies have demonstrated the involvement of at least 23 different genes for the LGMD2 forms [2] and others that are involved in metabolic, congenital or other myopathies that can also present with a clinical LGMD-like phenotype [3]. For the correct diagnosis of specific forms four elements may be of pivotal importance: 1) the clinical picture; 2) the muscle biopsy; 3) the imaging; 4) the DNA results, with the last approach that is changed dramatically in the course of the present study. We have studied one of the most interesting forms of LGMD that is caused by recessive mutations in a gene coding for a calcium-activated chloride channel, known as
anoctamin 5 (ANO5) [4]. The ANO5 gene at 11p14.3 spans 90,192 bp and contains 22 exons, the coding sequence is 2.7 kb for 913 amino acids. This form, according to the order of mapping, has been defined as LGMD2L. Genetic studies in some countries have shown that LGMD2L may be a very common form of LGMD [5]. In place of the proximal limb-girdle presentation, some patients show Miyosh-like muscular dystrophy type 3 (MMD3) [6]. Dominant variations in the same gene have been associated to gnathodiaphyseal dysplasia (GDD) [7].

The LGMD2L phenotype was described for the first time in 2007 in 14 patients of French Canadian origin, showing atrophy and weakness of the quadriceps and biceps brachii muscles [8]. In 2010, ANO5 was identified as the causative gene [4]. More recently, it has been indicated as the third most common form of LGMD in the North of Europe and the c.191dupA mutation has been shown to be the most prevalent because of a founder effect [5,9].

Distinctive features of LGMD2L versus other LGMD forms are: 1) the sex imbalance, with females that are less frequently or severely affected than males [10]; 2) asymmetry of muscle involvement that is rare among the LGMD and frequent in FSH [4]; 3) the pain following exercise that is typical of metabolic or inflammatory conditions [11].

All the previous studies have evidenced the extreme heterogeneity of the observed phenotypes, comprising the asymmetric atrophy and weakness affecting primarily the quadriceps, hamstrings and biceps, an adulthood onset and a slow progression [4,5,10,12,13]. The weakness of both distal and proximal lower limbs, exercise intolerance, a so called "pseudometabolic" phenotype and also amyloid deposits in the muscles [11,14] are all features present in patients affected by anoctaminopathy or dysferlinopathy [15].

In this paper, we describe the results of a genetic screening in a subset of patients with a broad clinical phenotype of LGMD or generic myopathy. In total, we have fully sequenced the ANO5 gene in 786 patients using Sanger and/or Next Generation Sequencing (NGS): in this cohort, we have found 33 cases belonging to 28 families. Our data confirm the genetic heterogeneity of the ANO5 gene and highlight the weak genotype-phenotype correlation.

2. Methods

2.1. Sample collection

From a large collection of families with a clinical diagnosis of LGMD or with molecularly uncharacterized myopathy, we recruited 786 patients. In particular, 712/786 (90.6%) patients were from Italian families. Additional patients (n = 74) were from Belgium (39), France (8), Finland (4), Brazil (3), Turkey (3), Romania (3), Morocco (3), Germany (2), Russia (2), Greece (1), Israel (1), Uganda (1), Spain (1), Libya (1), Cyprus (1) and The Netherlands (1).

In all the cases, genomic DNA has been tested and, when available, a further analysis on mRNA from blood or from muscle has been performed.

2.2. Clinical and diagnostic criteria

Based on literature evidence, as a first step, we screened 160 patients for mutations in exon 5 and in exon 20. Because of the low mutation rate detected in these exons, we extended the analysis to all the other exons.

All the patients recruited for the first step have an LGMD or an LGMD-like phenotype, including a raised serum creatine kinase (CK), progressive muscle weakness affecting primarily the shoulder girdle and pelvic muscles and a muscle biopsy with dystrophic features. All of them show an autosomal recessive inheritance or are sporadic cases. Moreover, most (about 85%) of the 160 samples analyzed by PCR and Sanger sequencing had resulted negative for mutations in DYSF and CAPN3 genes.

Another 626 samples were recruited for NGS, including all the ANO5 exons and the 10 flanking nucleotides.

The inclusion criteria for the NGS screening were less stringent. Samples of still living patients, affected by an uncharacterized muscular dystrophy (65%) or myopathy (35%), were included. These patients had a wide spectrum of clinical phenotypes, ranging from an isolated hyperCKemia to mild or severe conditions with a variable age of onset and progression. A large portion of these patients (30%) had not been screened previously and less than 20% had been analyzed for mutations in LGMD recessive genes (in particular CAPN3, DYSF or sarcoglycan genes).

Moreover, DNA samples from 52 unaffected people were sequenced as a control group.

Written informed consent for DNA analysis was obtained from all the recruited patients or their caregivers when primary diagnostic procedures were performed, with explicit consent for future use for research purposes, according to the Declaration of Helsinki. Approval for the study was obtained by the Seconda Università di Napoli Ethics Committee.

2.3. Molecular analysis

Genomic DNA was extracted from peripheral blood by phenol/chloroform. All the ANO5 exons have been amplified by PCR using M13-tailed primers. M13 primers have been used to perform Sanger sequencing using an ABI PRISM 3130 XL automatic DNA Sequencer Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). We used a TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions to extract RNA from the muscle biopsies and the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany) to extract RNA from the blood.

The retrotranscription reaction was performed using 2 mg of total mRNA, according to the procedure described in the SuperScript III kit (Invitrogen).

We amplified the ANO5 cDNA in seven overlapping fragments. Supplementary Table S1 lists primers and PCR conditions.

For NGS screening, samples were enriched using HaloPlex Target Enrichment System (Protocol version D, August 2012, Agilent Technologies, Santa Clara, CA, USA) [16].
For each of the novel mutations identified, amino acid change, presence in dbSNP v137 [17], frequency in NHLBI Exome Variant Server (http://evs.gs.washington.edu/EVS) and 1000 genomes large scale projects (http://www.1000genomes.org) [18], conservation and causative effects, using different prediction algorithms [19–21], were evaluated.

To assess intronic and exonic mutations leading to splicing defects, a free bioinformatic tool (http://www.fruitfly.org/seq_tools/splice.html) [22] was consulted.

3. Results

To study 160 patients with undiagnosed LGMD, we first sequenced the hotspot regions at exons 5 and 20 of the ANO5 gene, but the screening was diagnostic in two families only (I and XVI, homozygotes for c.191dupA and c.2272 C>T, respectively). Ten additional patients were heterozygous for a single ANO5 mutation. We next extended the analysis to all the other exons by PCR and Sanger sequencing. By this exon-by-exon scanning, we were able to detect another fourteen other exons by PCR and Sanger sequencing. By this exon-by-exon scanning, we were able to detect another fourteen mutations, concluding the genetic diagnosis in other 15 patients (Table 1).

To profit from the extraordinary throughput of next generation sequencing [23], we included the ANO5 gene in a large and accurate screening of genes causing neuromuscular disorders [16]. In particular, the ANO5 exons and the ten flanking bases were >90% covered at no less than 100× (Supplementary Fig. S1) after a Haloplex-based enrichment. We studied further 626 patients with broader phenotypic presentation, ranging from classic LGMD phenotype to congenital myopathies, nonspecific myopathic features or hyperCKemia. In this way, we identified other 15 ANO5 patients. All the variants identified by NGS were then confirmed by the dideoxy method. Interestingly, we detected different ANO5 variants in both alleles in patients XVIII and XXIV. These were classified as affected by a congenital myopathy with a hypothesized dominant transmission. However, they did not show mutations in the other genes causing congenital myopathies. Considering their specific phenotype and the unavailability of other relatives’ samples to study the status and the segregation of their mutations, we were not able to correctly interpret them.

We also identified 23 patients with a single mutated allele, including one (XXXVIII) with two mutations in cis on the same chromosome.

To avoid the risk to miss mutations, the DNA samples from heterozygous patients were also resequenced exon-by-exon and no additional variations were detected. Array-CGH (Motorchip [24]) testing was also negative.

Finally, three normal control samples also showed heterozygous variants in ANO5, including a novel missense substitution (crt11).

By combining NGS and Sanger sequencing, we identified 33/786 individuals, from 28 different families, homozygous or compound heterozygous for mutations in the ANO5 gene. Forty-three mutations were detected in 16/22 exons. Twelve of them had already been detected and described in literature; on the contrary, thirty-one had never been described previously (Table 1).

3.1. Novel mutations

3.1.1. Missense and nonsense mutations

Twenty-four novel missense and nonsense mutations were detected. In particular, four of them (c.1207 C>T, c.1213 C>T, c.1261 C>T and c.1639 C>T) introduced a premature stop codon. All the other variants determined an amino acidic change and their clinical significance was evaluated by different bioinformatic tools.

The mutations identified in homozygosity (c.161 T>C in II and c.2498 T>A in VII) or in compound heterozygosity (c.2489 C>T in XV, c.2342 in XLV and c.2411 C>G in XLII) were all predicted to be causative by at least two out of three tools.

3.1.2. Point mutations modifying a splicing site consensus

Four point mutations (c.294 G>A; c.649-2 A>G; c.1119 +1 G>A and c.2235 +1 G>A) were expected to modify a splicing site, as suggested by bioinformatic tools. We analyzed ANO5 mRNA in the leukocytes (for patient XXII) or in the muscle (V), to confirm the splicing effect. As detected on control samples, the leukocyte ANO5 isofrom lacks exon 4, but maintains the reading frame and determines a predicted protein 14 amino acids smaller (Fig. 1).

In patient XXII, the mutation produces a shortened mRNA without exons 4 and 5 (Fig. 2A, B). The extra-skipping of exon 5 causes an in-frame deletion of 114 nucleotides encoding 38 amino acids. However, both DNA and RNA analyses did not allow us to detect a second mutation in this patient that remains formally undiagnosed.

The muscular mRNA of patient V revealed the activation of a cryptic splice site 20 nucleotides upstream the natural 3′ end of exon 11 (Fig. 2C–D). The frame-shift results in a premature stop codon after five amino acids. For splice site mutations in patients XIX and XX, we did not study muscle mRNA, but there is little doubt about their deleterious effect.

3.1.3. Small deletions and insertions

We found two small deletions in the same patient (VI). In particular, in exon 6 we detected a c. 304–308 delAAAGA, causing a frame-shift with the substitution of a Lysine with a Valine 102 and a premature stop codon after a single amino acidic residue.

In exon 19, we found a 3-nucleotide deletion c. 2102–2105 delATA, causing the loss of an Asparagine 701. This residue, evolutionarily conserved, is the first amino acid of the putative sixth cytoplasmatic loop and its loss is predicted to be damaging.

An insertion of a single nucleotide was found in one allele of patient XLVII and it determined an immediate premature stop codon.

3.2. Phenotypic spectrum of ANO5 patients

As already reported [10], also in our cohort of patients, the number of affected males (21 = 64%) was higher than that of
### Table 1
List of patients and controls with ANOS variants.

| Sample ID | Status | Mutations |
|-----------|--------|-----------|
| L1*       | hom    | c.191 dupA (exon 5) p.Asn64LysfsX15* |
| L2*       | hom    | c.191 dupA (exon 5) p.Asn64LysfsX15* |
| II*       | hom    | c.161T > C (exon 4) p.Phe54Ser |
| III*      | hom    | c.172C > T (exon 4) p.Arg58Trp |
| IV1*      | c.het  | c.173T > C (exon 16) p.Phe578Ser + c.2272C > T (exon 20) p.Arg758Cys |
| IV2*      | c.het  | c.173T > C (exon 16) p.Phe578Ser + c.2272C > T (exon 20) p.Arg758Cys |
| V9        | c.het  | c.1119 + 1 G > A (exon 11) p.Ser376IlefsX5 + c.2272C > T (exon 20) p.Arg758Cys |
| VI*       | c.het  | c.304–308 delAAAGA (exon 6) p.Lys102ValfsX1 + c.2102–2105 delATA (exon 19) p.Asn701 |
| VII*      | hom    | c.2498T > A (exon 21) p.Met833Lys |
| VIII*     | hom    | c.1639C > T (exon 16) p.Arg547X |
| IX,1*     | c.het  | c.191 dupA (exon 5) p.Asn64LysfsX15* + c.1733T > C (exon 16) p.Phe578Ser |
| IX,2*     | c.het  | c.191 dupA (exon 5) p.Asn64LysfsX15* + c.1733T > C (exon 16) p.Phe578Ser |
| X,1*      | c.het  | c.191 dupA (exon 5) p.Asn64LysfsX15* + c.2516T > G (exon 21) p.Met839Arg |
| X,2*      | c.het  | c.191 dupA (exon 5) p.Asn64LysfsX15* + c.2516T > G (exon 21) p.Met839Arg |
| XI*       | c.het  | c.191 dupA (exon 5) p.Asn64LysfsX15* + c.1261C > T (exon 13) p.Gln421X |
| XII*6*    | hom    | c.692G > T (exon 8) p.Gly231Val |
| XII*8*    | hom    | c.191 dupA (exon 5) p.Asn64LysfsX15* |
| XIV1*4*   | hom    | c.162dupA (exon 15) p.Met543AsnfsX10 |
| XIV2*     | hom    | c.162dupA (exon 15) p.Met543AsnfsX10 |
| XV*       | c.het  | c.191 dupA (exon 5) p.Asn64LysfsX15* + c.2489C > T (exon 21) p.Ala830Val |
| XV*       | c.het  | c.191 dupA (exon 5) p.Asn64LysfsX15* + c.2489C > T (exon 21) p.Ala830Val |
| XV*       | c.het  | c.1520delT (exon 15) p.Phe507SerfsX6 + c.1898-4A > G (exon 18) spl. |
| XVIII*7*  | het/c.het | c.616A > G (exon 7) p.Thr206Ala + c.1211G > T (exon 13) p.Arg404Leu |
| XIX*6*    | hom    | c.2235 + 1G > A (exon19) spl. |
| XX*5*     | hom    | c.649-2A > G (exon8) spl. |
| XXI*6*    | c.het  | c.191 dupA (exon 5) p.Asn64LysfsX15* + c.1213C > T (exon 13) p.Gln405X |
| XXII*     | het    | c.294G > A (exon 5) p.Ala98Glu |
| XXII*     | het    | c.294G > A (exon 5) p.Ala98Glu |
| XXIV*     | het/c.het | c.191 dupA (exon 5) p.Asn64LysfsX15* + c.2387C > T (exon20) p.Ser796Leu |
| XXV*      | het    | c.2698A > C (exon 22) p.Met900Leu |
| XXVI*5*   | het    | c.279C > G (exon 5) p.Asp93Glu |
| XXVI*7*   | het    | c.797C > T (exon 9) p.Pro266Leu |
| XXVIII*8*  | het    | c.428A > G (exon 7) p.Tyr143Cys |
| XXIX*8*   | het    | c.777G > T (exon 9) p.Lys259Asn |
| XXX*6*    | het    | c.2141C > G (exon 19) p.Thr714Ser |
| XXXI*6*   | het    | c.294G > A (exon 5) p.Ala98Glu |
| XXXII*7*  | het    | c.2141C > G (exon 19) p.Thr714Ser |
| XXXIII*6* | het    | c.604G > A (exon 7) p.Glu201Lys |
| XXXIV*    | het    | c.800C > G (exon 9) p.Thr267Ser |
| XXXV*8*   | het    | c.692G > T (exon 8) p.Gly231Val |
| XXXVI*6*  | het    | c.155A > G (exon4) p.Asn52Ser |
| XXXVI*7*  | het    | c.1646G > T (exon 16) p.Ser555Ile |
| XXXVII*8* | het    | c.259G > A p.Val87Ile (exon 5) + c.692G > T (exon 8) p.Gly231Val + [in cis] |
| XXXIX*6*  | het    | c.2387C > T (exon20) p.Ser796Leu |
| XL*6*     | het    | c.191 dupA (exon 5) p.Asn64LysfsX15* |
| XLII*8*   | c.het  | c.1207C > T (exon 13) p.Gln403X + c.2411C > G (exon 20) p.Cys804Ser |
| XLIII*7*  | c.het  | c.191 dupA (exon 5) p.Asn64LysfsX15* + c.1520delT (exon 15) p.Phe507SerfsX6 |
| XLIV*9*   | hom    | c.191 dupA (exon 5) p.Asn64LysfsX15* |
| XLIV*5*   | c.het  | c.692G > T (exon 8) p.Gly231Val + c.2342T > C (exon 20) p.Leu781Pro |
| XLV*6*    | c.het  | c.1213C > T (exon 13) p.Gln405X + c.2387C > T (exon20) p.Ser796Leu |
| XLVI*6*   | c.het  | c.729_730insT (exon 8) p.Asn244X + c.2387C > T (exon20) p.Ser796Leu |
| XLVII*6*  | het    | c.155A > G (exon4) p.Asn52Ser |
| XLVII*6*  | het    | c.1516A > G (exon15) p.Ser506Gly |
| XV*6*     | het    | c.2141C > G (exon 19) p.Thr714Ser |
| LI*6*     | het    | c.2516T > G (exon 21) p.Met839Arg |
| ctrl1*8*  | het    | c.1854G > C (exon 17) p.Met618Ile |
| ctrl2*8*  | het    | c.2387C > T (exon20) p.Ser796Leu |
| ctrl3*8*  | het    | c.2387C > T (exon20) p.Ser796Leu |

s = Sanger sequencing; f = familial; ngs = NGS analysis; hom = homozygous; het = heterozygous; c.het = compound heterozygous.

Novel mutations are in bold.
females (12 = 36%). The age of onset varied significantly (mean = 26.81; min = 13; max = 44). Interestingly, four ANO5 patients (IV.1; IV.2; X.2; XIV.2) are still asymptomatic even if they showed increased CK serum levels. The average CK values were 3200 IU/l ranging from 500 IU/l to 9800 IU/l.

The clinical signs of patients with LGMD2L presentation, such as the early asymmetric quadriceps weakness, the high CK and the slow progression, and the histological features, including mild myopathic changes, were in agreement with literature data (Table 2) [13,25–27]. All the patients are still

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Fig. 1. Alternative splicing of exon 4. ANO5 mRNA in muscle shows the full-length isoform containing the exon 4 (A). Blood isoform is differentially spliced, the exon 4 is removed and the exon 3 is directly joined with the exon 5 (B).

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Fig. 2. Intronic mutations affecting the splicing in patients XIX and V. In patient XIX, the G > A mutation in the last exonic nucleotide (A) causes the loss of the canonical splicing site (B). In blood cDNA, the sequence shows the normal splicing, which connects exon 3 and exon 5, and the abnormal splicing with the complete loss of exon 4. In patient V, the G > A mutation in the first intronic nucleotide (C) causes the activation of a cryptic splicing site twenty nucleotides upstream (D), as evidenced on muscular cDNA. The loss of the last twenty nucleotides of exon 11 produces a frame-shift and a premature stop codon five amino acids later.
| Patient ID | Sex | Current age | Age of onset | CK, IU/l, range | ECG and ultrasonography | Clinical phenotype | Biopsy | Loss of walking | Genotype |
|-----------|-----|-------------|--------------|----------------|-------------------------|-------------------|--------|----------------|----------|
| L1        | M   | 63          | 37           | 2800–9800      | Normal NA              | Hyper-Ck-emia, lower quadriceps pain and later mild weakness | Muscular dystrophy with regenerating and necrotic fibers, variation in fibers size (small and hypertrophic) | No     | c.191dupA, p.Asn64LysfsX15 + c.191dupA, p.Asn64LysfsX15 |
| L2        | F   | 35          | 32           | 2000–3000      | Normal NA              | Hyper-Ck-emia, followed by lower quadriceps pain doing stairs | Mild dystrophic changes | No     | c.161T>C; p.Phe54Ser + c.161T>C; p.Phe54Ser |
| III       | M   | 31          | 16           | 1000–2000      | Normal Normal          | Hyperotrophy of calves, calves, cramps and fatigkeit | Sparse rounded hypertrophic fibers and some splitting fibers | No     | c.172C>T; p.Arg54Trp + c.172C>T; p.Arg54Trp |
| IV        | J   | 30           | 19           | >5000          | Normal Normal          | Hyper-Ck-emia, still asymptomatic | NA | No | c.1733T>C; p.Phe578Ser + c.2272C>T; p.Arg758Cys |
| IV        | K   | 20           | 15           | 1000–2000      | Normal Normal          | Hyper-Ck-emia, still asymptomatic | NA | No | c.1119+1 G > A; p.Ser373ThrfsX1 + c.2162–2105 delATTA; p.Asn701Ile |
| V         | F   | 39          | 24           | 3000–4000      | PQ short Normal        | Distal weakness arm, hyper-Ck-emia, myalgia, painful contractures | Mitochondrial myopathy | No     | c.1639G>T; p.Arg547X + c.1639C>T; p.Arg547X |
| VI        | F   | 44          | 39           | 3000–4000      | Normal                 | Absence of weakness, mild calf hypertrophy | Central nuclei and increased fiber size | No     | c.616A>G; p.Thr206Ala + c.1211G>T; p.Arg394Leu |
| VII       | M   | 46          | 17           | 3500–8000      | Normal Normal          | Weakness of ankle dorsal and plantar flexors, hamstrings and adductors | Mild dystrophic changes | No     | c.2498T>A; p.Met831Lys + c.2498T>A; p.Met831Lys |
| VIII      | M   | 75          | 37           | 500–2500       | Normal Normal          | Symmetric proximo-distal lower limb weakness, quadriceps and calf atrophy, abdominal and neck flexors muscles weakness and dysphonia | No (walk with canes) | No     | c.1560A>G; p.Glu520ValX15 + c.1560A>G; p.Glu520Val |
| IX        | M   | 57          | 30           | 1000           | NA NA                  | Hyper-Ck-emia, lower quadriceps pain, possible congenital myopathy | Dystrophic changes | No | c.616dupA, p.Asn64LysfsX15 + c.2165T>G; p.Arg648Leu |
| X1        | M   | 47          | 28           | >3000          | Normal Normal          | Hyper-Ck-emia, cramps, myalgia | Mild fiber size variability | No | c.191dupA; p.Asn64LysfsX15 + c.2165T>G; p.Arg648Leu |
| X2        | F   | 50          | 50           | 500–1500       | NA NA                  | Hyper-Ck-emia, still asymptomatic | NA | No | c.191dupA; p.Asn64LysfsX15 + c.2165T>G; p.Arg648Leu |
| XI        | M   | 44          | 30           | 2200–3000      | Normal Normal          | Progressive atrophy and weakness of biceps brachii muscles, hamstrings and hip adductors | Myopathic changes and necrotic fibers | No | c.1639G>T; p.Arg547X + c.1639C>T; p.Arg547X |
| XII       | F   | 56          | 47           | 536            | NA                    | Autosomal dominant myopathy | No (but sporadic use of wheelchair) | No | c.191dupA; p.Asn64LysfsX15 + c.191dupA; p.Asn64LysfsX15 |
| XIII      | M   | 38          | 32           | 1500–3000      | Normal Normal          | Hyper-Ck-emia, cramps, myalgia | Minimal changes with increased fiber variability | No | c.191dupA; p.Asn64LysfsX15 + c.191dupA; p.Asn64LysfsX15 |
| XIV       | M   | 45          | 40           | >1000          | Normal Normal          | Hyper-Ck-emia, still asymptomatic | Minimal changes with increased fiber variability | No | c.1627dupA; p.Met543AsnfsX10; p.Met543AsnfsX10 |
| XIX       | M   | 54          | 44           | 2000–3000      | NA NA                  | Easy fatigability and difficulty walking | Dystrophic features with phagocytosed fibers | No | c.2235+1 G > A; p.E745fsX1 + c.2235+1 G > A; p.E745fsX1 |
| XX        | M   | 42          | 15           | 4800–6600      | NA NA                  | Hyper-Ck-emia, high-arched feet | Necrotizing myopathy | No | c.191dupA; p.Asn64LysfsX15 + c.1213C>T; p.Glu645X |
| XXI       | M   | 35          | 29           | 2830           | NA NA                  | Hyper-Ck-emia | Slight dystrophy | No | c.191dupA; p.Asn64LysfsX15 + c.1213C>T; p.Glu645X |
| XXII      | F   | 56          | 47           | 536            | NA                    | Autosomal dominant myopathy | Myopathic changes | NA | c.191dupA; p.Asn64LysfsX15 + c.191dupA; p.Asn64LysfsX15 |
| XLII      | F   | 37          | 18           | >1000          | Normal Normal          | LGMD | Myopathic pattern | NA | c.191dupA; p.Asn64LysfsX15 + c.191dupA; p.Asn64LysfsX15 |
| XLIII     | F   | 23          | 13           | >1000          | Normal Normal          | LGMD | Myopathic pattern | NA | c.191dupA; p.Asn64LysfsX15 + c.191dupA; p.Asn64LysfsX15 |
| XLIV      | F   | 33          | NA           | NA             | NA NA                  | LGMD | NA | c.191dupA; p.Asn64LysfsX15 + c.191dupA; p.Asn64LysfsX15 |
| XLV       | F   | 42          | NA           | NA             | NA NA                  | LGMD | NA | c.191dupA; p.Asn64LysfsX15 + c.191dupA; p.Asn64LysfsX15 |
| XLVI      | M   | 39          | NA           | NA             | NA NA                  | NA | NA | c.191dupA; p.Asn64LysfsX15 + c.191dupA; p.Asn64LysfsX15 |
| XLVII     | F   | 43          | NA           | NA             | NA NA                  | NA | NA | c.191dupA; p.Asn64LysfsX15 + c.191dupA; p.Asn64LysfsX15 |

Patients XV, XVI and XVII have been described elsewhere ([16]) and are not included in the table. For patients XLIV–XLVII, detailed clinical data were not available.
ambulant, but two that occasionally use walking aids (aged 71 and 75).

When assessed, cardiac and respiratory functions were normal with the exception of patient V, who showed a short PQ interval, and of patient XII, who is suffering from a restrictive respiratory insufficiency.

For 15 families, a brief summary of the phenotype was added as Supplementary appendix. Patients XV, XVI and XVII have been previously characterized elsewhere [13].

4. Discussion

By combining NGS and Sanger sequencing, we have carried out the largest screening of the ANO5 gene in 786 myopathic patients and 52 controls. In our cohort of patients, thirty-three are homozygous or compound heterozygous for causative mutations in ANO5. Interestingly only 18/33 are Italian (although they are 90% of the cohort), providing a further evidence of lower frequency of anoctaminopathies in this country [12] where dysferlinopathies and calpainopathies remain the most common form of LGMD [28]. In contrast, we have evidenced a single heterozygous variant in 3% (23/786) of patients. Some of these may be non-pathological rare variants, but others, such as c.191dupA, are well-known causative mutations. When fully studied, heterozygous patients show no hidden mutation on the second allele. Is this compatible with the disease prevalence? Previous published papers have evidenced the high prevalence of anoctaminopathies in Northern Europe: in particular, a prevalence of 0.27/100,000 has been estimated in the North of England [5] and of 2/100,000 in the Finnish population [13]. However, a rarer frequency of variants has been reported elsewhere [12].

To explain 3% of heterozygotes, we propose two hypotheses:

1 We would find a similar number of heterozygotes in any other cohort of subjects, because the frequency of pathological alleles is at least 10-fold higher than expected (>0.01 instead of 0.001). This immediately indicates that over 90% of cases with both ANO5 mutations should be quite healthy, in the absence of a second unknown hit.

2 We have found many heterozygous subjects, because they are true patients: this suggests that ANO5-myopathy could be transmitted as a dominant trait, in the presence of a second unknown hit. A point in favor of the first hypothesis is the long list of ANO5 variants present in the Exome Variant Server and in dbSNP: ANO5 is certainly a highly polymorphic gene. In fact, eighty variants with a frequency lower than 1.5% are listed in EVS (Supplementary Table S2) for a total of 959 carriers; 20 subjects (0.3%) are heterozygous for the well-known c.191dupA and 159 (2.4%) show a putative damaging variant (total 3%). The identification of a carrier of a single mutation in ANO5 gene is not to be considered an uncommon finding and it will be important to identify a second putative hit. Since deletorous copy number imbalances have been estimated in 5–10% of patients affected by neuromuscular disorders [24], copy number variants involving noncoding regulatory regions [29,30] could affect the ANO5 expression in some tissues.

Other explanations involve mutations in other genes belonging to the same pathway, the effect of modifier genes, epigenetic changes or environmental factors.

Interestingly, the phenotype–genotype analysis shows the absence of a correlation. All the asymptomatic patients have increased creatine kinase levels, supporting the hypothesis of the variable expressivity of ANO5 myopathy. In our cases, the expressivity seems to be independent of causative mutations and also unrelated to sex and age. In particular, the lack of a clear genotype–phenotype correlation is evident either comparing different families or even focusing on different patients within the same family (for example, the patient X,2, still asymptomatic, has a younger brother presenting with cramps and myalgia). The interfamilial variability could reflect a specific genetic background and the putative presence of a second hit, as postulated. On the contrary, the intra-familial heterogeneity could be due to different external factors, including lifestyle, sport activity and diet, which should be further investigated.

The anoctaminopathies are also characterized by an extremely high genetic heterogeneity [10,25]. Interestingly, among our patients with a complete molecular diagnosis of anoctaminopathy, c.191dupA and c.2272C>T, the most common variation described so far, represent less than 30% of identified mutations and they both account for only 20% of those detected in the Italian patients. On the contrary, we have identified 31 novel variants, confirming the genetic heterogeneity of ANO5 and demonstrating that the simple screening of one or two recurrent mutations cannot be considered effective in Southern European populations (Fig. 3).

Considering the power of next generation sequencing [23] and the clinical and genetic variability of muscular dystrophies, diagnostic approaches based on NGS are becoming increasingly frequent [31]. However, for clinical and diagnostic aims, a targeted resequencing of genes already known to be linked to the specific pathological condition is advisable [32] and ANO5 has to be included. Our data also demonstrate the utility of this approach, highlighting, however, how important the correct interpretation of the data generated by these approaches could be. Moreover, these NGS-based strategies are perfect to dissect the clinical variability [33], meeting, in this way, with the next challenges of research.

In conclusion, we suggest that the terms “anoctaminopathy” or “ANO5 myopathy” better define a heterogeneous disease caused by mutations in the ANO5 gene, irrespective of the first proximal (LGMD2L), distal symptoms (Miyoshi myopathy) or characterized by other myopathic features.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.nmd.2015.03.011.

References

[1] Eymard B, Levy N. Diagnostic strategy for limb-girdle muscular dystrophies. Rev Neurol (Paris) 2012;168:919–26.
[2] Nigro V, Savarese M. Genetic basis of limb-girdle muscular dystrophies: the 2014 update. Acta Myol 2014;33:1–12.
[3] Mercuri E, Muntoni F. Muscular dystrophies. Lancet 2013;381:845–60.
[4] Bolduc V, Marlow G, Boycott KM, et al. Recessive mutations in the putative calcium-activated chloride channel Anoctamin 5 cause proximal LGMD2L and distal MMD3 muscular dystrophies. Am J Hum Genet 2010;86:213–21.
[5] Hicks D, Sarkozy A, Muelas N, et al. A founder mutation in Anoctamin 5 is a major cause of limb-girdle muscular dystrophy. Brain 2011;134:171–82.
[6] Mahjaneh J, Jaiswal J, Lamminen A, et al. A new distal myopathy with mutation in anoctamin 5. Neuromuscul Disord 2010;20:791–5.
[7] Tsutsumi S, Kamata N, Vokes TJ, et al. The novel gene encoding a putative transmembrane protein is mutated in gnathodiaphyseal dysplasia (GDD). Am J Hum Genet 2004;74:1255–61.
[8] Jarry J, Rioux MF, Bolduc V, et al. A novel autosomal recessive limb-girdle muscular dystrophy with quadriceps atrophy maps to 11p13-p12. Brain 2007;130:368–80.
[9] Witting N, Duno M, Petri H, et al. Anoctamin 5 muscular dystrophy in Denmark: prevalence, genotypes, phenotypes, cardiac findings, and muscle protein expression. J Neurol 2013;260:2084–93.
[10] Sarkozy A, Hicks D, Hudson J, et al. ANO5 gene analysis in a large cohort of patients with anoctaminopathy: confirmation of male prevalence and high occurrence of the common exon 5 gene mutation. Hum Mutat 2013;34:1111–18.
[11] Milone M, Liewluck T, Pianosi PT. Amyloidosis and exercise intolerance in ANO5 muscular dystrophy. Neuromuscul Disord 2012;22:934–43.
[12] Penttila S, Palmio J, Suominen T, et al. Eight new mutations and the expanding phenotype variability in muscular dystrophy caused by ANO5. Neurology 2012;78:897–903.
[13] Liewluck T, Winder TL, Dimberg EL, et al. ANO5-muscular dystrophy: clinical, pathological and molecular findings. Eur J Neurol 2013;20:1383–9.
[14] Cacciottolo M, Numitone G, Aurino S, et al. Muscular dystrophy with marked Dysferlin deficiency is consistently caused by primary dysferlin gene mutations. Eur J Hum Genet 2011;19:974–80.
myopathic patients and in pools of DNA samples. Acta Neuropathol Commun 2014;2:100.

[17] Sherry ST, Ward MH, Kholodov M, et al. dbSNP: the NCBI database of genetic variation. Nucleic Acids Res 2001;29:308–11.

[18] Abecasis GR, Altshuler D, Auton A, et al. A map of human genome variation from population-scale sequencing. Nature 2010;467:1061–73.

[19] Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 2009;4:1073–81.

[20] Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. Curr Protoc Hum Genet 2013; Chapter 7: Unit 7.20.

[21] Schwarz JM, Rodelsperger C, Schuelke M, Seelow D. MutationTaster evaluates disease-causing potential of sequence alterations. Nat Methods 2010;7:575–6.

[22] Reese MG, Eeckman FH, Kulp D, Haussler D. Improved splice site detection in Genie. J Comput Biol 1997;4:311–23.

[23] Desai AN, Jere A. Next-generation sequencing: ready for the clinics? Clin Genet 2012;81:503–10.

[24] Piluso G, Dionisi M, Del Vecchio Blanco F, et al. Motor chip: a comparative genomic hybridization microarray for copy-number mutations in 245 neuromuscular disorders. Clin Chem 2011;57:1584–96.

[25] Penttila S, Palmio J, Udd B. ANO5-Related Muscle Diseases. 2012.

[26] Schessl J, Kress W, Schosser B. Novel ANO5 mutations causing hyper-CK-emia, limb girdle muscular weakness and Miyoshi type of muscular dystrophy. Muscle Nerve 2012;45:740–2.

[27] Sarkozy A, Deschauer M, Carlier RY, et al. Muscle MRI findings in limb girdle muscular dystrophy type 2L. Neuromuscul Disord 2012;22(Suppl. 2):S122–9.

[28] Fanin M, Nascimbeni AC, Aurino S, et al. Frequency of LGMD gene mutations in Italian patients with distinct clinical phenotypes. Neurology 2009;72:1432–5.

[29] Savarese M, Piluso G, Orteschi D, et al. Enhancer chip: detecting human copy number variations in regulatory elements. PLoS ONE 2012;7:e52264.

[30] Spielmann M, Klopocki E. CNVs of noncoding cis-regulatory elements in human disease. Curr Opin Genet Dev 2013;23:249–56.

[31] Valet N, Laporte J. Impacts of massively parallel sequencing for genetic diagnosis of neuromuscular disorders. Acta Neuropathol 2013;125:173–85.

[32] Rehm HL. Disease-targeted sequencing: a cornerstone in the clinic. Nat Rev Genet 2013;14:295–300.

[33] Nigro Y, Piluso G. Next generation sequencing (NGS) strategies for the genetic testing of myopathies. Acta Myol 2012;31:196–200.