CASE OF THE MONTH

ENTIRE *CAPN3* GENE DELETION IN A PATIENT WITH LIMB-GIRDLE MUSCULAR DYSTROPHY TYPE 2A

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ABSTRACT: Limb-girdle muscular dystrophy type 2A (LGMD2A) due to mutations in the *CAPN3* gene is one of the most common of autosomal recessive limb-girdle muscular dystrophies. We describe a patient who had a typical LGMD2A phenotype and posterior compartment involvement on MRI. Different genetic analyses were performed, including microarray analysis. There was an apparently homozygous mutation in exon 24, c.2465G>T, p.(*822Leuext62*), and a lack of correlation in the disease segregation analyses. This suggested the presence of a genomic rearrangement. In fact, a heterozygous deletion of the entire *CAPN3* gene was found. This novel deletion comprised the terminal region of the *GANC* gene and the entire *CAPN3* gene. This finding points out the need to reconsider and adapt our current strategy of molecular diagnosis in order to detect these types of genomic rearrangements that escape standard mutation screening procedures.

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Limb-girdle muscular dystrophy type 2A (LGMD2A) is characterized by progressive proximal muscle weakness. Loss of independent ambulation usually occurs after no more than 25 years of progression.[1–5] The molecular diagnosis is challenging due to overlapping phenotypes between different LGMDs, and routine techniques are not 100% sensitive. Indeed, in about 22% of patients with LGMD2A, only 1 pathogenic mutation in *CAPN3* gene has been found.[6–9] This may be because the second mutation is located in genomic regions outside the coding exons, such as promoters or introns.[10] The use of cDNA obtained from muscle or blood could solve some of the limitations of genomic DNA analysis, but this strategy is also limited by the phenomenon of nonsense-mediated decay.[11–13] The uncertainty of an incomplete or misleading molecular diagnosis could have major consequences for genetic counseling and for future therapeutic approaches. Therefore, additional techniques should be applied in selected cases.

CASE REPORT

We describe a young woman, age 21 years, who presented with a typical LGMD2A phenotype. Onset occurred when she was about 10 years of age. Her first symptom was muscle weakness in the lower extremities with difficulty running, climbing stairs, and frequent falls. Her serum creatine kinase (CK) levels ranged from 3000 to 9000 IU/L. On follow-up, she had progressive muscle weakness. Posterior thigh compartment involvement was observed on magnetic resonance imaging. At present, she is ambulant and, according to the modified Gardner-Medwin and Walton clinical severity score, is in grade 4.

Gene sequencing only identified an apparently homozygous mutation in exon 24, c.2465G>T, p.(*822Leuext62*) (Fig. 1A), not previously described, as well as 2 homozygous polymorphisms, c.495C>T and c.984C>T, in exons 3 and 7, respectively. The c.2465G>T mutation has not been described so far, but the c.2464T>C mutation, located in the same codon (Leiden Muscular Dystrophy pages, www.dmd.nl), eliminates the natural translation termination codon and produces a new

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Abbreviations: CK, creatine kinase; LGMD, limb-girdle muscular dystrophy; LOS, loss of heterozygosity; MLPA, multiplex ligation-dependent probe amplification; RT-PCR, reverse transcription polymerase chain reaction; SNP, single nucleotide polymorphism

Key words: CAPN3; deletion; homozygous mutation; genomic rearrangement; limb-girdle muscular dystrophy type 2A

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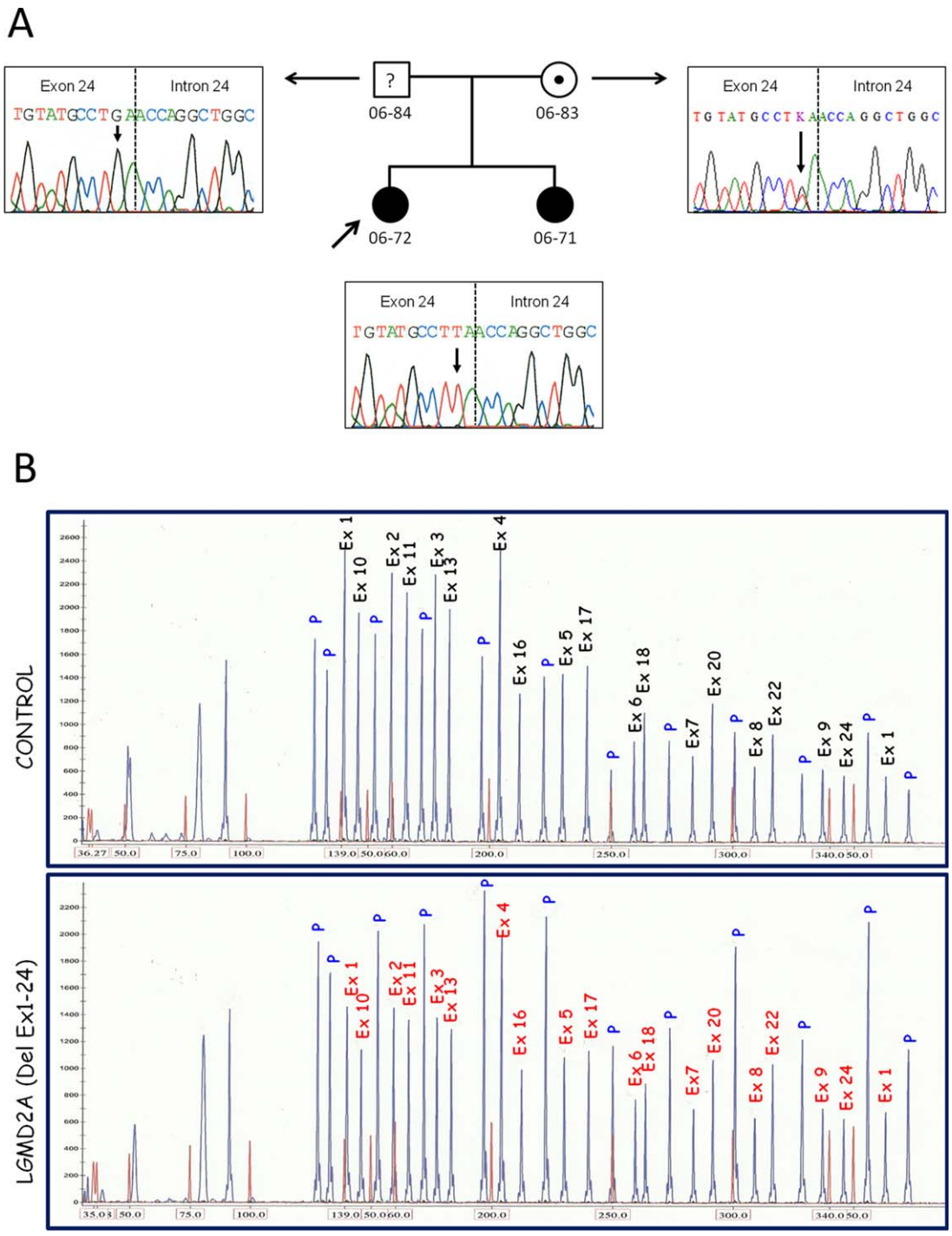

FIGURE 1. (A) Sequence from PCR amplification of blood cDNA from the patient showing the c.2465G>T, p.(*822Leuext62*) mutation in exon 24 of the *CAPN3* gene, apparently in the homozygous state. **(B)** MLPA image showing an approximately 50% decrease in all *CAPN3* exons in the patient. P: control probe (amplification controls of different chromosomes).

translation termination codon 62 amino acids 3′ later, exactly as observed in the case of the mutation described in this patient. Strikingly, when we analyzed parental segregation (performed routinely in our laboratory as part of other studies in healthy carriers), we found that this mutation as well as the 2 polymorphisms were present in the heterozygous state only in the mother but were absent in the father. Paternity analysis was performed and confirmed using a microsatellite analysis (D7S2506, D7S663, D13S263, D13S1246, 22-GATA46EO, D11S1298). Therefore, because we suspected this could be due to the presence of a deletion, multiplex ligation-dependent probe

Table 1. Illumina Human610-Quad Bead Chip results.

Index	Name	Chr	Position	Gene	4383643841_R02C01.TopAlleles
272791	rs621560	15	42,437,711	*PLA2G4F*	AG
255496	rs4923929	15	42,439,376	*PLA2G4F*	GG
122914	rs1619030	15	42,459,034	*VPS39*	AA
272837	rs622442	15	42,463,677	*VPS39*	AA
267532	rs598441	15	42,480,409	*VPS39*	AA
128516	rs1712391	15	42,484,890	*VPS39*	AA
158689	rs2138831	15	42,493,052	*VPS39*	GG
124538	rs1679012	15	42,528,676	*TMEM87A*	AA
255532	rs4924642	15	42,545,995	*TMEM87A*	AA
168659	rs2277533	15	42,565,588	*TMEM87A*	CC
337939	rs8024732	15	42,570,718	*GANC*	GG
57227	rs11070370	15	42,577,540	*GANC*	AA
255497	rs4923943	15	42,593,246	*GANC*	CC
126534	rs16973097	15	42,632,499	*GANC*	AA
126535	rs16973137	15	42,637,943	*GANC*	AA
305707	rs7180279	15	42,643,538	*GANC/CAPN3*	AA
135257	rs1801449	15	42,681,199	*CAPN3*	GG
206370	rs3115883	15	42,701,688	*CAPN3*	GG
66632	rs11639355	15	42,723,407	*ZFP106*	AA
82328	rs12440118	15	42,744,094	*ZFP107*	AA
352252	rs9302112	15	42,820,451	*SNAP23*	AA
50542	rs10851410	15	42,844,788	*HAUS2 = CEP27*	AA
7022	cnvi0007517	15	42,862,346	*CEP27/STARD9*	—
7021	cnvi0007516	15	42,864,793	*CEP27/STARD9*	—
82411	rs12443323	15	42,870,998	*STARD9*	AA
7024	cnvi0007520	15	42,871,051	*STARD9*	—
7023	cnvi0007519	15	42,872,639	*STARD9*	—
277569	rs6493054	15	42,886,504	*STARD9*	AA
127596	rs1705360	15	42,889,047	*STARD9*	AA
133765	rs17767270	15	42,898,612	*STARD9*	GG
66381	rs11632169	15	42,903,115	*STARD9*	AA
74604	rs1197547	15	42,905,340	*STARD9*	AA
338785	rs8043472	15	42,948,862	*STARD9*	AA
338183	rs8030587	15	42,981,806	*STARD9*	GG
213467	rs3742993	15	42,983,923	*STARD9*	AA
277570	rs6493061	15	42,988,412	*STARD9*	CC
45019	rs1058846	15	43,012,024	*STARD9*	GG
91609	rs12917189	15	43,023,482	*CDAN1*	AA
305803	rs7182141	15	43,062,548	*TTBK2*	AA
277571	rs6493065	15	43,064,772	*TTBK2*	AG

The heterozygous alleles confirm the presence of the 2 chromosomes, whereas the observation of a homozygous allele does not really one allow to state that it comes from only 1 or both chromosomes. Dark shading: heterozygous SNPs; light shading: CAPN3 gene locus.

amplification (MLPA; MRC-Holland, Amsterdam, The Netherlands) analysis was performed subsequently. MLPA, a high-throughput technique for molecular analysis of copy number variation, revealed an approximately 50% decrease in all *CAPN3* exons in the patient and the father, clearly indicating the presence of a heterozygous deletion of the entire *CAPN3* gene (Fig. 1B). The patient had a younger affected sister who had the same mutation in exon 24 and the same pattern of *CAPN3* deletion in the MLPA analysis.

Next, we attempted to identify the genomic break points of the deletion. For this purpose, we first used a DNA analysis system (Human610-Quad Bead Chip; Illumina, San Diego, California; www.Illumina.com) analyzing 610,000 selected tag single nucleotide polymorphisms (SNPs) and markers. We looked for regions of homozygous SNPs around the *CAPN3* gene and found a large loss-of-heterozygosity (LOH) area that could harbor the deletion (Table 1). This large LOH area was assessed further by direct Sanger sequencing of its flanking regions (from SNP rs621560 to rs4923929 in the *PLA2G4F* gene and from SNP rs7182141 to rs6493065 in the *TTBK2* gene; Fig. 2, higher magnification portion). However, no mutations or alterations were detected in these regions. As the LOH area between SNPs rs4923929 and rs7182141 was still too long to be sequenced, alternative techniques were applied.

Quantitative reverse transcription polymerase chain reaction (RT-PCR) was done to determine

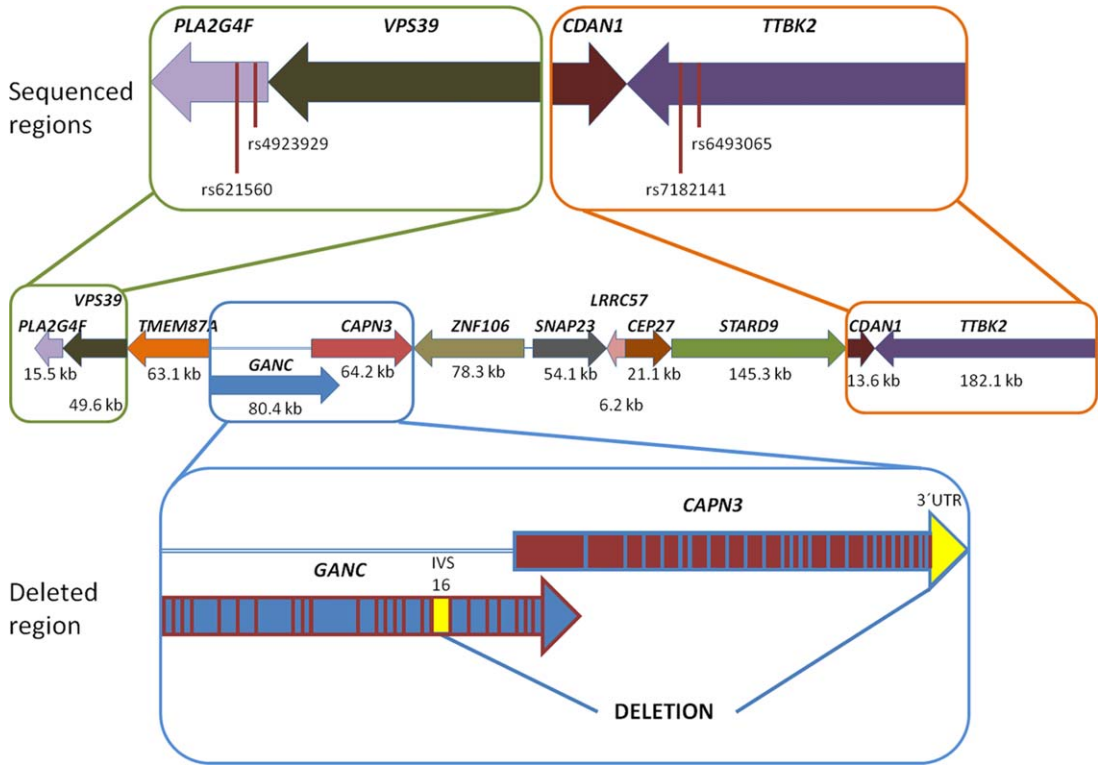

FIGURE 2. Genomic region flanking the *CAPN3* gene between the *PLA2G4F* and *TTBK2* genes. Higher magnification portion shows sequenced regions between heterozygous and homozygous SNPs located within the *PLA2G4F* and *TTBK2* genes, based on Illumina Human610-Quad Bead Chip results. Lower magnification portion shows deleted region indicating the break points in the *GANC* (IVS16) and *CAPN3* (3′UTR) genes. Vertical bars represent exons of both genes. Arrow lengths and exons not drawn to scale.

the deleted area by means of different gene dose of *CAPN3* surrounding genes. RNA was extracted from the blood of all family members and healthy controls, and *GAPDH* was used as the endogenous calibrator. This analysis revealed a similar gene dose of *TMEM87A* and *CEP27* in all individuals (Fig. 3). To narrow down this region, *GANC* (exons 15–16, exons 23–24), *SNAP23*, and *ZNF106* genes, which are closer to *CAPN3* in the genome, were quantified. Only *GANC* (exons 23–24) showed down-regulation in all subjects carrying the heterozygous deletion, thus delimiting the deleted area to the *GANC–CAPN3* gene region. In contrast, *SNAP23* expression was elevated slightly (>1.5 fold-change) in the same subjects (Fig. 3). Finally, to confirm our results and establish the specific break points in the chromosome, a custom comparative genomic hybridization microarray (Nimblegen,

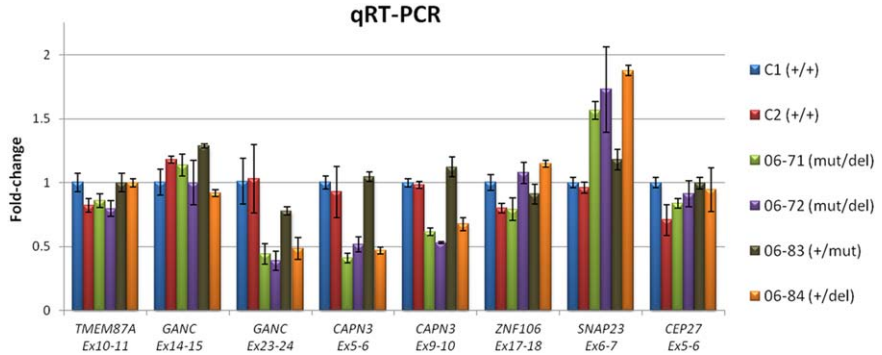

FIGURE 3. Quantitative RT-PCR gene dose. Expression of *CAPN3* surrounding genes, ordered according to the position along the genome. *GANC* in exons 23–24, but not in exons 14–15, showed half-dose in all subjects carrying the heterozygous deletion as in the case of the *CAPN3* gene. A slight increase in *SNAP23* gene expression (>1.5-fold change) was observed in the same individuals. The remaining genes had normal expression levels, indicating that these regions were not deleted. Mut: c.2465G>T, p.(*822Leuext62*) mutation; del: deletion of *GANC* IVS16–*CAPN3* 3′UTR; C1 and C2: healthy controls; 06-72: patient; 06-71: patient's affected sister; 06-83: patient's mother; 06-84: patient's father.

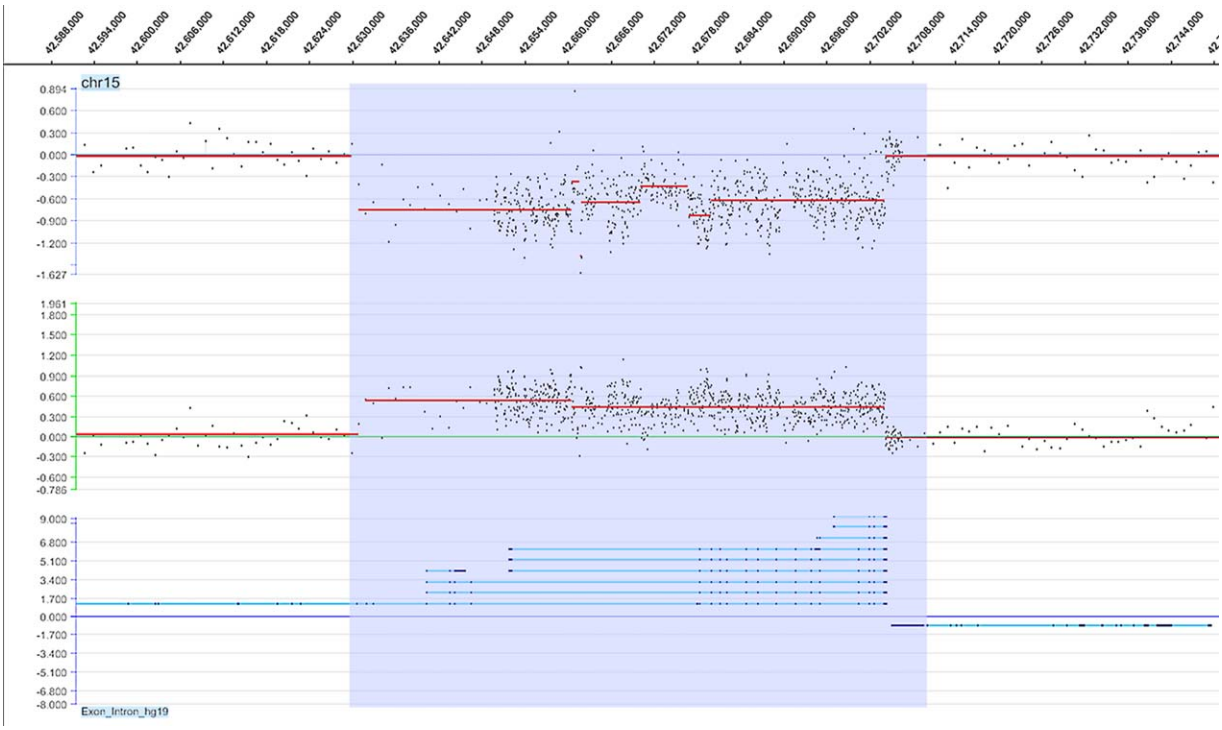

FIGURE 4. Custom comparative genomic hybridization (NimbleGen, Roche) microarray analysis with a 20-bp probe tiling covering the *CAPN3* genomic locus. Visualization of differential fluorescence intensities between the patient and control samples using SignalMap software (NimbleGen) at a threshold (Log2 ratio) set to 0.2 shows a heterozygous deletion comprising the entire genomic *CAPN3* locus and the terminal region of the *GANC* gene, with break points determined with an average precision range of 100 bp, as chr15: 42,630,844–42,704,125. Upper portion shows results of Cy3 (patient) / Cy5 (control) Log2 ratio, and lower portion the corresponding dye-swap confirmation.

Madison, Wisconsin) was used.[14] Using this method, which allows detection of copy number differences between test and reference samples, the break points of the deletion were determined finally on chromosome 15 (HG19, chr15: 42,630,844–42,704,125, with an average precision range of 100 bp) (Fig. 4). Therefore, the deletion was shown to comprise part of the *GANC* gene (starting point in intron 16) and the entire *CAPN3* gene locus (endpoint in the 3′UTR region) (Fig. 2, lower magnification portion).

Subsequently, analyses were performed in 2 patients of an additional family who met the clinical criteria of a typical LGMD2A phenotype and carried the same apparently homozygous c.2465G>T, p.(*822Leuext62*) mutation. Interestingly, the same heterozygous *CAPN3* locus deletion was identified in these patients. As both families came from the same region in the south of Spain, this suggests the presence of a founder mutation in this population.

DISCUSSION

This case illustrates that the standard molecular diagnosis performed in LGMD2A, usually performed by examining the genomic DNA or cDNA through direct Sanger sequencing, does not always detect large exon rearrangements such as deletions or duplications.[15]

In this study, an apparently homozygous *CAPN3* mutation was identified in a patient with LGMD2A. Because of the lack of concordant segregation in 1 progenitor after paternity confirmation, we suspected this could be due to the presence of a possible heterozygous deletion.

By applying different approaches, we detected and delimited the deleted region of the *CAPN3* locus. Interestingly, gene dose performed by quantitative RT-PCR showed reduction in *GANC* gene expression (exon 23–24) (Fig. 3), which corresponds to the deleted region (42,630,844–42,704,125 region of chromosome 15). It is worthwhile to mention that the last 4 exons of the *GANC* gene (exons 21–24) overlap the promoter region of the *CAPN3* gene.[16] The deleted area goes beyond this overlapping region, from intron 16 of the *GANC* gene to the 3′UTR region of the *CAPN3* gene.

In contrast, although *SNAP23* is located outside the deleted region, its expression was slightly increased. It has been described previously that gene expression changes are not always directly correlated with copy number, which suggests an underlying complexity that might involve the size of the deletion, altered chromatin structure, a

dose-compensation mechanism, or a combination of these factors.[17] Although we do not know whether or not *SNAP23* up-regulation is related to the deletion, it is tempting to suggest that a region that could regulate *SNAP23* gene expression may be included in the deleted area.

Although some exonic deletions have already been reported in the *CAPN3* gene[14,18] (Leiden Muscular Dystrophy pages, www.dmd.nl), deletion of the entire *CAPN3* gene has not been reported. These events are quite common in other muscular dystrophies such as Duchenne muscular dystrophy (www.dmd.nl) and other diseases. Recently, the first *TRIM32* total gene deletion was reported in an LGMD2H patient, and the investigators reported that cases considered to be homozygous could hide the presence of gross rearrangements on the other allele.[15]

Had we not systematically performed progenitor carrier analysis, we would have assumed it was a real homozygous patient and missed the entire gene deletion. Missing such rearrangements could have important consequences in diagnostic practices such as prenatal assessment, and on future therapeutic trials. Therefore, we propose the following strategy as the safest diagnostic approach for LGMD2A. First, a sequence analysis at the transcriptional level on cDNA obtained from the RNA from muscle or blood samples should be performed or, alternatively, on DNA at the genomic level. If a blood sample is used, an additional genomic analysis of exon 15 is compulsory, given that the isoforms expressed in blood do not include this exon. Second, in apparently homozygous patients, or cases in which sequence analysis revealed only 1 disease-causing mutation, we would suggest carrying out an additional MLPA analysis to establish the gene dose to rule out the presence of large genomic rearrangements. Overall, this combined strategy should lead to an increase in the mutation-detection rate and allow for more accurate genetic diagnosis in LGMD2A.

APPENDIX

Sequence variations are described as recommended by the Ad-Hoc Committee for Mutation Nomenclature (AHCMN), with the following suggested additions[19]:

- c.2465G>T: Mutation in the 2465 nucleotide of the coding DNA; substitution of a guanine for a thymine.
- p.(*822Leuext62*): Protein change as a consequence of the mutation; substitution of the natural translation termination codon (*) in the 822 amino acid by a leucine and extension of the protein until a new translation termination codon (*) is created 62 amino acids later.

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