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## Chapter 3

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## **Sarcoglycanopathies and the risk of undetected deletion alleles in diagnosis**

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### Abstract

We have designed Multiplex Amplifiable Probe Hybridization (MAPH) probes for each exon of the sarcoglycan genes *SGCA*, *SGCB*, *SGCG* and *SGCD* (33 in total). The set was used to screen DNA from limb-girdle muscular dystrophy (LGMD) patients for the presence of pathogenic deletion or duplication mutations. An unexpected heterozygous deletion of *SGCG* exon 7 was detected in a patient from a consanguineous family segregating a known c.525delT mutation. The exon 7 deletion was inherited from the father, who was part of the consanguineous c.525delT branch of the family but who screened negative for the c.525delT mutation. The deletion breakpoint was mapped, isolated and sequenced. Identical breakpoints were detected in 2 unrelated LGMD patients from Southern Italy. Haplotype analysis showed identical alleles segregating with the mutation in all three patients, suggesting a common ancestor. Exonic deletions in sarcoglycanopathies appear to be rare events. However, we recommend screening for exonic deletions / duplications in patients where a mutation has not been identified in both alleles, as well as in seemingly homozygous cases where segregation of the mutations can not be confirmed in the parents.

### Introduction

The Limb-Girdle Muscular Dystrophies (LGMD) are a heterogeneous group of neuromuscular disorders, characterized by progressive wasting of the limb-girdle musculature. The proteins involved show a wide range of functions and cellular localization (1). A subset of these are  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  sarcoglycan, which are transmembrane glycoproteins that form part of the dystrophin-associated glycoprotein complex (DAG)(2,3). The absence of any one of these subunits leads to the reduction or loss of the other sarcoglycans, which in turn disrupt the DAG complex.

The sarcoglycanopathies are recessive disorders, however there are often cases where a mutation can be identified in only one allele. In addition, it is not always possible to confirm homozygous mutations through analysis of the parents. As the majority of sarcoglycan mutations described have been point mutations, screening at the DNA level has been almost exclusively performed by direct sequencing. This method however is not quantitative, meaning that deletions or duplications will not be detected.

Although several different methods have been described for detecting copy number changes (4), these have not yet been applied for the sarcoglycanopathies in a routine setting. The most commonly used methods include quantitative Southern blotting (5), quantitative multiplex PCR (6) and fluorescent in situ hybridization (FISH) (7), but these are either time consuming, technically challenging or of insufficient resolution to find small rearrangements. We have used Multiplex Amplifiable Probe Hybridization (MAPH) (8) to look for copy number changes in the sarcoglycan  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  genes in 5 sarcoglycanopathy patients diagnosed as being homozygous for a point mutation, as well as a patient where a mutation in only one allele could be found.

### Methods.

#### Patient details.

All patients were diagnosed by a neurologist as having a neuromuscular disorder, based on their clinical features. Immunohistochemical analysis was performed as previously described (9), and had shown in each case the presence of dystrophin and abnormal levels of the sarcoglycan proteins. Based on these results, sequence analysis of the gene(s) showing the lowest levels of expression was performed as previously described (9). This revealed an apparent homozygous point mutation in one of the sarcoglycan genes in 5 cases (LG 1-5), whereas a mutation was detected in only one allele in patient LG 6 (Table 1).

Patient	Gene Affected	Mutations detected by sequencing	exon(s) of gene
L1	<i>SGCG</i>	[c.525delT]+ [c.525delT]	6
L2	<i>SGCG</i>	[c.525delT]+ [c.525delT]	6
L3	<i>SGCG</i>	[c.525delT]+ [c.525delT]	6
L4	<i>SGCA</i>	[c.229C>T]+ [c.229C>T]	3
L5	<i>SGCB</i>	[c.275C>T]+ [c.275C>T]	3
L6	<i>SGCG</i>	[c.525delT]+ [c.579-4730_702+1721delinsACACTA]	6 and 7

Table 1. The patients tested in this study. The mutations are described based on the following reference sequences; *SGCA*:NM\_000023.1, *SGCB*:NM\_000232.3, *SGCG*:NM\_000231.1. In each case +1 is taken to be the A of the ATG initiation codon.

This was a 5-year-old boy, born of non-consanguineous North African parents. He showed tip-toe walking and progressive lower limb weakness from the age of 2 years. Motor skills such as stair climbing and cycling were progressively lost. Clinical examination at the age of 5 years showed muscular weakness of the upper and lower limb girdle and Gower's sign, with no overt cognitive impairment. The patient has three healthy siblings. Further investigation showed a family history of neuromuscular disorders (Figure 1). The brother of the patient's paternal grandfather has three grandchildren (one boy and two girls) with a DMD-like phenotype: these grandchildren born from consanguineous Moroccan parents were diagnosed as LGMD2C patients, and one has been described in a previous paper (9). The siblings were homozygous for a 1 bp deletion (525delT) in the  $\gamma$ -sarcoglycan gene (*SGCG*), and both parents were demonstrated to be carriers for this mutation.

#### MAPH

To allow screening for whole exon rearrangements MAPH probes for the exons of the  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  sarcoglycan genes were developed (33 in total). Probe preparation and the MAPH protocol have been described (10). Briefly, a DNA sequence covering or in close proximity to each exon was PCR amplified and cloned into the pGEM-T easy vector (Promega). This sequence was reamplified with vector-specific primers. In this way all probes could be co-amplified with only one pair of primers. The probes were combined and hybridized to immobilized genomic DNA. Following stringent washing the remaining probes were recovered from the filter and amplified by PCR. Using a fluorescent primer and capillary electrophoresis the peaks corresponding to each

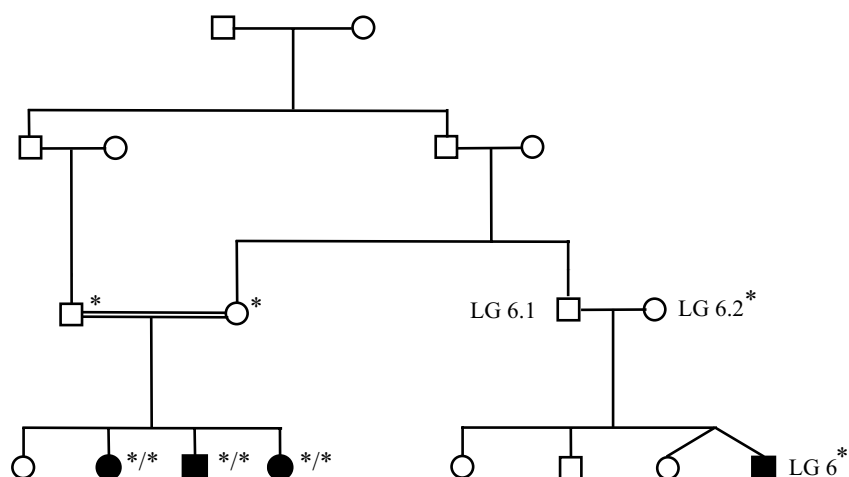


Figure 1. Extended pedigree of patient LG 6. Individuals affected by the sarcoglycanopathy are shown in black. All individuals indicated with a \* are heterozygous for the point mutation c.525delT in the *SGCG* gene, \*\*/\* indicates homozygosity for the same mutation. The mutation is described based on reference sequence NM\_000231.1, with +1 taken to be the A of the ATG initiation codon.

probe were separated and quantified. Probes were normalized against the 4 nearest probes not from the same gene. A deletion was defined as a normalized ratio below 0.75, found in duplicate.

#### Long range PCR

Primers were designed using the Primer 3 program (11), based on sequence derived from the human genome working draft (April 2003). PCR was performed on 200-300 ng genomic DNA using the Expand Long Template PCR system (Roche), with products analyzed on a 0.7% agarose gel. Any samples that were to be sequenced were purified with the Qiagen PCR purification kit (Qiagen). Sequencing was performed at the Leiden Genome Technology Center using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI 3700 Sequencer (Applied Biosystems).

#### Haplotype Analysis

The following polymorphic markers were used; D13S232, D13S292 and D13S115. The sequences used for amplification were obtained from the UNISTS site of the NCBI (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unists>), with the forward primer of each pair being labeled with the fluorescent labels FAM or HEX. After PCR amplification the three marker products were pooled and analyzed on an ABI 3700 Sequencer (Applied Biosystems).

## Results

### MAPH analysis

MAPH analysis was initially performed on genomic DNA from 12 healthy control samples to determine probe variation. In total 28 of the 33 probes gave consistent ratios, and were included in subsequent screening tests. The five probes excluded (*SGCA* exons 5 and 7, *SGCG* exon 3 and *SGCD* exons 2 and 4), did not cover any of the homozygous point mutations to be analyzed. Following MAPH analysis no copy number mutations were detected in any of the 5 patients diagnosed as having a homozygous point mutation. Analysis of DNA from patient LG 6 showed that exon 7 of the *SGCG* gene was heterozygously deleted, i.e. the height of the corresponding peak was ~50% of that in normal controls, whereas the other 27 probes were within normal limits. Testing DNA from the parents showed that the father (LG 6.1) was a carrier for the deletion, whereas the mother (LG 6.2) did not have the deletion. As there were several relatives of the father who also had been diagnosed with  $\gamma$ -sarcoglycanopathy, genomic DNA from these patients was also tested for an exon 7 deletion. No deletion was detected (data not shown).

### Long Range PCR

To confirm the deletion and determine the breakpoints, long range PCR was initially performed on genomic DNA from both LG 6 and LG 6.1. As exon 8 of the *SGCG* gene was known to be present, the reverse primer was located within this sequence. Using the DNA sequence from the Human Genome Working Draft (April 2003) several forward primers were designed in intron 6. These primers were spaced at ~5kb intervals, extending from the 5' end of exon 6, until exon 7. With one particular pair of primers (forward – TGGATGTGGTATATATGCGATCGTG; reverse - GCTTGGGTAAGCACACAGTTTCAG) a 9kb fragment was expected, but a product of ~2.5 kb was amplified from both the patient and his father. Sequence analysis showed that the PCR product spanned the deletion breakpoint, and that the entire exon was deleted. It was not a simple rearrangement, with an insertion of 6 bp between the breakpoints (Figure 2).

Two unrelated patients (N66 and N950) from Italy, previously reported as being homozygous for a deletion of exon 7 of the *SGCG* gene (12), were also tested with the same primer pair. Both DNA samples gave a similar size PCR junction fragment, and sequence analysis revealed the identical deletion breakpoint as seen in LG 6. Analysis of the parents of N950 showed that both were heterozygous carriers of the deletion. As the identical mutation suggested either a recurrent mutation or a common ancestor, haplotype analysis was performed on the 3 patients and 25 CEPH control samples. The haplotype D13S115 (165 bp), D13S232 (112 bp) and D13S292 (205 bp) was found in 5/6 chromosomes in the affected individuals, and in a maximum of 4/50



chromosomes in the control samples. Analysis with Fisher's exact test showed this difference to be significant ( $p < 0.001$ ), strengthening the argument for a common ancestor.

```

22788031 CCAACTGGTGACCTCAGCAGAGAAGGATTTTAATAATCAAGTG 22788073
          |||||-----
          CCAACTGGTGACCTCAGACTAGCTCCGATGTCCTTCACAGA
          -----|||
22794597 GTGTTGCAAGGGATGGGCCAGGCGCTCCGATGTCCTTCACAGA 22794639

```

**Figure 2.** The sequence across the deletion breakpoint (middle row). The six nucleotides underlined indicate the insertion. The numbers indicate the nucleotide position based on the sequence of chromosome 13 from the Human Genome working draft (May 2004).

### Discussion.

We report here the identical single exon deletion in three unrelated individuals diagnosed with  $\gamma$ -sarcoglycanopathy. The extended family of patient LG 6 has been described (9). Several individuals had been diagnosed with  $\gamma$ -sarcoglycanopathy, but were all concluded to be homozygous for the single nucleotide deletion c.525delT. Unexpectedly, these were all on the paternal side, and the index patient inherited the point mutation c.525delT from his mother. We found that the father has an exon 7 deletion in one allele, with no other affected relatives tested having the same deletion.

Exonic deletions in the *SGCG* gene have been rarely described (table 2). There was a single report in the literature of a deletion of exon 7 of *SGCG*, which had been found homozygously in two unrelated individuals in a village in Southern Italy (12).

Comparison of the deletion junction in our patient (of North African heritage) and those of Italian origin showed that they were identical. Analysis of the deletion breakpoint showed an insertion of 6 bp, suggesting that the mechanism involved was non-homologous end-joining. Such rearrangements are associated with scattered breakpoints and insertions at the junction site (13,14), making it less likely that the deletions had occurred independently of each other. Indeed, haplotype analysis showed that the mutation segregated with the same haplotype in all 3 patients, supporting the hypothesis of a common ancestor.

There have been founder mutations described for several LGMD genes, although these have all been small mutations that were detected by sequencing. The c.525delT mutation in *SGCG*, seen

heterozygously in LG 6 and homozygously in LG 1, 2 and 3, is common within the North African population, segregating with the rare 122 bp allele of marker D13S232 (15). The mutation has also been found in Brazilians of African heritage, again segregating with the same allele (16). Another change within the *SGCG* gene, c.87insT was found in patients in Northern Italy (17). This mutation has not been described in other populations, and it was proposed that the mountainous surroundings in that region of Italy contributed to the relative genetic isolation. In contrast, it is likely that villages in Southern Italy had more contact with other regions via sea travel. It is therefore not surprising that a mutation in this region spreads across the Mediterranean.

<i>SGCG</i> deletion	Times Reported	References
-124-?_1500+?del (entire gene)	2	(20,21)
c.506-?_578(702)+?del (exon 6 <sup>*</sup> )	1	(22)
c.579-4730_702+1721delinsACACTA (exon 7)	2	(12), this report

**Table 2.** Deletions of one or more exons described for the *SGCG* gene. The mutations are described based on reference sequence NM\_000231.1, with +1 taken to be the A of the ATG initiation codon. \* In this case it was not determined whether exon 7 was also deleted.

Partial gene deletions or duplications as founder effects have been described, although less commonly than mutations that can be detected by sequencing. Within the Dutch population deletions of one or more exons of the *BRC1* gene occur in ~30% of breast cancer cases (18). There was a report of a partial gene duplication within the *SCL3A* gene, found in several individuals of German descent (19). Although no haplotype analysis was performed, it was a complex rearrangement with identical breakpoints.

The relatively low number of partial gene rearrangements detected as a founder mutation is probably due, at least partly, to the use of sequencing as the predominant method for mutation detection, and it is likely that the number of such cases will increase as more genes are screened with quantitative methods.

It has already been suggested that exonic deletions may be present in  $\gamma$ -sarcoglycanopathy (20), particularly since many cases were reported to be homozygous. If parental DNA is not available it is difficult to distinguish with certainty whether the mutation is truly homozygous or

is a compound heterozygote, with the loss of the second allele masking the true situation. In addition, when parental DNA is unavailable, the fact that one of either parent does not carry the mutation may be erroneously explained as either non-paternity or a de novo occurrence of the mutation. An associated danger in the latter situation is that the de novo change suggested is taken as proof that this change has pathogenic consequences.

As exonic deletions have been shown to occur in at least some sarcoglycanopathy patients it suggests that screening for such mutations should be routinely implemented in a diagnostic setting. Our report shows that such analysis is highly recommended in cases where a mutation can be found in one allele only, or when segregation from the parents can not be confirmed. An accurate diagnosis is not only important for the patient but also for subsequent counseling of family members.

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