

# Genetic epidemiology of muscular dystrophies resulting from sarcoglycan gene mutations

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## Abstract

**Background**—The autosomal recessive limb-girdle muscular dystrophies (LGMDs) are a group of genetically heterogeneous muscle diseases characterised by progressive proximal limb muscle weakness. Six different loci have been mapped and pathogenetic mutations in the genes encoding the sarcoglycan complex components ( $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan) have been documented. LGMD patients affected with primary “sarcoglycanopathies” are classified as LGMD2D, 2E, 2C, and 2F, respectively.

**Methods**—A geographical area in north east Italy (2 319 147 inhabitants) was selected for a genetic epidemiological study on primary sarcoglycanopathies. Within the period 1982 to 1996, all patients living in this region and diagnosed with muscular dystrophy were seen at our centre. Immunohistochemical and immunoblot screening for  $\alpha$ -sarcoglycan protein deficiency was performed on all muscle biopsies from patients with a progressive muscular dystrophy of unknown aetiology and normal dystrophin. Sarcoglycan mutation analyses were conducted on all patient muscle biopsies shown to have complete or partial absence of  $\alpha$ -sarcoglycan immunostaining or a decreased quantity of  $\alpha$ -sarcoglycan protein on immunoblotting.

**Results**—Two hundred and four patient muscle biopsies were screened for  $\alpha$ -sarcoglycan protein deficiency and 18 biopsies showed a deficiency. Pathogenetic mutations involving one gene for sarcoglycan complex components were identified in 13 patients:  $\alpha$ -sarcoglycan in seven,  $\beta$ -sarcoglycan in two,  $\gamma$ -sarcoglycan in four, and none in the  $\delta$ -sarcoglycan gene. The overall prevalence of primary sarcoglycanopathies, as of 31 December 1996, was estimated to be  $5.6 \times 10^{-6}$  inhabitants.

**Conclusion**—The prevalence rate estimated in this study is the first to be obtained after biochemical and molecular genetic screening for sarcoglycan defects.

(J Med Genet 1997;34:973-977)

The autosomal recessive limb-girdle muscular dystrophies (LGMDs) are a clinically heterogeneous group of diseases characterised by progressive muscle weakness of the pelvic and shoulder girdles. Several genes are involved in the aetiology of autosomal recessive LGMDs and a new classification has been proposed<sup>1</sup> according to the molecular basis (table 1): LGMD2A is mapped on chromosome 15q15,<sup>2</sup> LGMD2B on 2p12,<sup>3</sup> LGMD2C on 13q12,<sup>4</sup> LGMD2D on 17q12,<sup>5</sup> LGMD2E on 4q12,<sup>6,7</sup> and LGMD2F on 5q33.<sup>8</sup> Apart from LGMD2B, all other genes have been cloned and their muscle specific protein products identified: calpain-3 is involved in LGMD2A,<sup>9</sup>  $\alpha$ -sarcoglycan (50 kDa dystrophin associated glycoprotein DAG, adhalin) in LGMD2D,<sup>10</sup>  $\beta$ -sarcoglycan (43 kDa DAG) in LGMD2E,<sup>6,7</sup>  $\gamma$ -sarcoglycan (35 kDa DAG) in LGMD2C,<sup>11</sup> and  $\delta$ -sarcoglycan (35 kDa DAG) in LGMD2F.<sup>12</sup>

The  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -sarcoglycan proteins form the sarcoglycan (SG) complex, one component of the dystrophin-glycoprotein complex on the skeletal muscle membrane.<sup>13,14</sup> Pathogenetic mutations in each gene for SG complex components determine a group of diseases called “sarcoglycanopathies”. Because the entire SG complex is disrupted, all sarcoglycanopathies are characterised by biochemical deficiency of all subunits independently of any primary gene defect.<sup>4,7,11,14,16</sup>

After the first description of primary sarcoglycanopathy in a French family,<sup>10</sup> many patients from different countries have been reported<sup>4,7,11,15-30</sup> in whom a variety of gene mutations and heterogeneous clinical phenotypes were found.

Genetic epidemiological studies of muscular dystrophies resulting from SG gene defects have been inhibited by several factors: these diseases are rare, the methodology used to detect small mutations was made available only recently, and, even more important, the collection and screening of all the cases in a defined geographical area is time consuming.

The present study was undertaken to estimate the prevalence of autosomal recessive LGMD resulting from SG genes mutations (LGMD2C, 2D, 2E, 2F) in a restricted area of north east Italy.

## Materials and methods

### METHODS OF ASCERTAINMENT

The present epidemiological survey was done in north east Italy and included three neighbouring administrative districts (Padova,

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Received 7 March 1997  
Revised version accepted for publication 9 July 1997

**Keywords:** autosomal recessive limb-girdle muscular dystrophy; Duchenne-like muscular dystrophy; sarcoglycan complex

Table 1 Classification of autosomal recessive limb-girdle muscular dystrophies (LGMDs)

Nomenclature	Chromosomal localisation	Deficient protein
LGMD2A	15q15.1	Calpain-3 or CANP3
LGMD2B	2p13-p16	Unknown
LGMD2C	13q12	35 kDa DAG or $\gamma$ -sarcoglycan
LGMD2D	17q12-q21.33	50 kDa DAG or $\alpha$ -sarcoglycan
LGMD2E	4q12	43 kDa DAG or $\beta$ -sarcoglycan
LGMD2F	5q33-34	35 kDa DAG or $\delta$ -sarcoglycan

CANP3: calcium activated neutral protease-3. kDa: kiloDaltons. DAG: dystrophin associated glycoprotein.

Treviso, Vicenza). This area is 7341 km with 2 319 147 inhabitants at 31 December 1996. This study was based on the collection of cases affected with muscular dystrophy, diagnosed at the Regional Neuromuscular Centre, University of Padova within the period 1982 to 1996. The diagnostic procedures always included clinical examination, serum creatine kinase (CK) determination, electromyography, and muscle biopsy, obtained after written consent.

The ascertainment of all cases of muscular dystrophy in this area was ensured by the following: (1) the Regional Neuromuscular Centre in Padova is the referral unit for muscle diseases in this area; since 1975 it has regularly collected clinical records and muscle biopsies from muscular dystrophy patients from all the hospitals in this area and from most specialists in the field (neurologists, paediatricians, geneticists); (2) the Regional Centre for Genetic Counselling on neuromuscular disorders has operated in this area regularly from 1973 and represents a referral centre in this region.

#### SELECTION CRITERIA

Of all muscle biopsies processed in our centre in the period 1982-1996, 515 patients received a diagnosis of "progressive muscular dystrophy". They all had a clinical history and muscle weakness compatible with muscular dystrophy, muscle biopsy findings corresponding to dystrophic changes, and increased CK levels.

The first exclusion criterion involved the patients who had been diagnosed on a molecular basis as having Duchenne muscular dystrophy (144), Becker muscular dystrophy (135), merosin deficient congenital muscular dystrophy (11), and symptomatic carriers of dystrophinopathy (12). Patients in whom an autosomal dominant pattern of inheritance was reported were also excluded (9). In the remaining 204 muscular dystrophy patients, in whom the primary genetic defect was unknown and dystrophin was normal, biochemical (immunohistochemical and immunoblotting) screening for  $\alpha$ -SG was carried out.

#### BIOCHEMICAL METHODS

Muscle biopsy cryosections were immunostained for  $\alpha$ -SG by overnight incubation at room temperature with 1:20 diluted monoclonal antibodies (Novocastra, Newcastle, UK). Indirect immunofluorescence visualisation was obtained by the avidin-biotin system, as described elsewhere.<sup>17</sup> Immunoblotting for  $\alpha$ -SG was performed using the same antibody as described elsewhere.<sup>29</sup>

#### MOLECULAR METHODS

Analysis of the genes coding for  $\alpha$ -SG,  $\beta$ -SG,  $\gamma$ -SG, and  $\delta$ -SG was performed by direct sequencing of abnormal SSCP (single strand conformation polymorphism) conformers obtained from reverse transcription (RT-PCR) of muscle biopsy RNA or exon specific PCR amplification of genomic DNA, as described elsewhere.<sup>22 25 28 30</sup>

The size of the PCR products is such that the sensitivity of our SSCP analysis is estimated to be 90 to 95%<sup>31</sup>; in addition we run more than one SSCP procedure and therefore this increases the probability of detecting a mutation.

As we were testing patients with autosomal recessive inheritance, where two gene mutations should be detected, the large majority of mutation positive patients would have been detected as homozygotes or heterozygotes (>99%).

## Results

#### SCREENING FOR PROTEIN AND GENE DEFECTS IN THE SARCOGLYCAN COMPLEX

A biochemical  $\alpha$ -SG defect was found in 18 patients, 13 of whom (72%) showed a pathogenetic mutation in the  $\alpha$ -SG,  $\beta$ -SG, or  $\gamma$ -SG genes. Three mutation positive patients were identified because they are sibs of the index patients undergoing a muscle biopsy (fig 1). A mutation in the  $\alpha$ -SG gene (LGMD2D) was found in seven patients, in the  $\beta$ -SG gene (LGMD2E) in two patients, and in the  $\gamma$ -SG gene (LGMD2C) in four patients (table 2).

Detailed molecular data on patients 1-3 and 8-11 have been reported previously.<sup>7 23 28 29</sup> In the remaining six patients (three pairs of sibs) the molecular data showed the following. Patients 4 and 5 were heterozygous for an  $\alpha$ -SG G→A (nucleotide 739) base change resulting in a valine to methionine amino acid substitution. Patients 6 and 7 were homozygous for an  $\alpha$ -SG C→T (nucleotide 850) base change resulting in an arginine to cysteine amino acid substitution. Patients 12 and 13 were heterozygous for both  $\gamma$ -SG T insertion (nucleotide

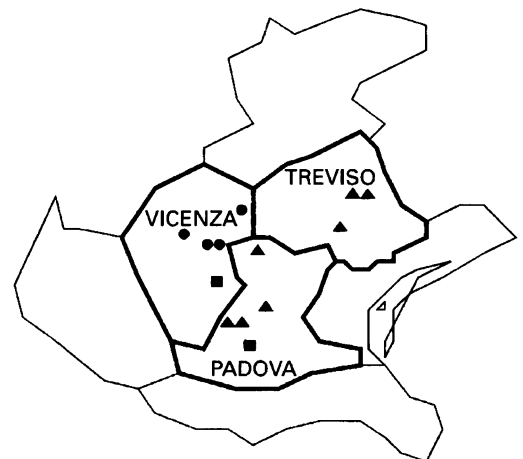


Figure 1 The geographical area selected in this study (thick border) includes three administrative districts (Padova, Vicenza, Treviso) of the Veneto region in north east Italy. The places where the affected patients live is shown by triangles ( $\alpha$ -SG), squares ( $\beta$ -SG), or circles ( $\gamma$ -SG).

Table 2 Familial and clinical data in patients affected with primary sarcoglycanopathy in three districts of Veneto region

Patient No	District	Sex	Affected relatives	Parental consanguinity	Age at diagnosis (y)	Clinical phenotype	SG gene defect	Ref
1	Padova	M	+	-	9	Duchenne-like	$\alpha$ (LGMD2D)	29
2	Padova	F	-	+	44	Limb-girdle	$\alpha$ (LGMD2D)	28†
3	Treviso	M	-	-	16	Limb-girdle	$\alpha$ (LGMD2D)	28†
4 §	Padova	F	+	-	36	Limb-girdle	$\alpha$ (LGMD2D)	
5 §	Padova	M	+	-	31	Limb-girdle	$\alpha$ (LGMD2D)	
6 ¶	Treviso	F	+	-	40	Limb-girdle	$\alpha$ (LGMD2D)	
7 ¶	Treviso	M	+	-	36	Asymptomatic*	$\alpha$ (LGMD2D)	
8	Padova	M	-	-	9	Duchenne-like	$\beta$ (LGMD2E)	28†
9	Vicenza	F	-	-	3	Duchenne-like	$\beta$ (LGMD2E)	7
10	Vicenza	F	-	-	19	Duchenne-like	$\gamma$ (LGMD2C)	23‡
11	Vicenza	M	-	-	23	Limb-girdle	$\gamma$ (LGMD2C)	23‡
12	Vicenza	M	+	-	16	Duchenne-like	$\gamma$ (LGMD2C)	
13	Vicenza	F	+	-	13	Duchenne-like	$\gamma$ (LGMD2C)	

SG: sarcoglycan; LGMD: limb-girdle muscular dystrophy. \*Kyphoscoliosis and high serum creatine kinase level. †Patients 2, 3, and 8 are referred to as patients 33, 25 and 29 in Duggan *et al.*<sup>28</sup> ‡Patients 10 and 11 are referred to as patients 44 and 26 in McNally *et al.*<sup>23</sup> §, ¶, || : sibs.

87-88) and a two base pair (TC) deletion (nucleotide 801).

In all except two patients, mutations on both alleles (either as homozygotes or as compound heterozygotes) were confirmed against parental genomic DNA; the parents were found to carry one of the two identified mutant alleles in a heterozygous state. In the remaining two cases (patients 4 and 5) only one mutant allele was identified by SSCP and confirmed in their mother.

#### PRIMARY $\alpha$ -SARCOGLYCANOPATHY (LGMD2D), $\beta$ -SARCOGLYCANOPATHY (LGMD2E), AND $\gamma$ -SARCOGLYCANOPATHY (LGMD2C)

Seven patients from five different families were shown to suffer from a primary  $\alpha$ -sarcoglycanopathy. The clinical severity of these patients ranged from a severe Duchenne-like muscular dystrophy to a very mild LGMD phenotype.

An autosomal recessive pattern of inheritance was reported in four of the five families: patient 2 was born to unaffected second cousins, while patients 3-7 (including two sets of affected sibs of both genders) were born to healthy, non-consanguineous parents. Patient 1 had an affected cousin who was not available for study. The prevalence of  $\alpha$ -sarcoglycanopathy (LGMD2D) was estimated to be  $3.02 \times 10^{-6}$  (7/2 319 147) inhabitants (table 3).

Two unrelated patients (8 and 9) were shown to have a primary  $\beta$ -sarcoglycanopathy. Both patients had a progressive, severe DMD-like phenotype. Both family histories were negative for neuromuscular disorders and consanguinity. The prevalence of  $\beta$ -sarcoglycanopathy (LGMD2E) was estimated to be  $0.86 \times 10^{-6}$  (2/2 319 147) inhabitants (table 3).

Table 3 Estimate of the prevalence of primary sarcoglycanopathies in three districts of Veneto region

Sarcoglycan gene defect	No of affected cases	Prevalence* ( $\times 10^{-6}$ )
$\alpha$ -sarcoglycan (LGMD2D)	7	3.02
$\beta$ -sarcoglycan (LGMD2E)	2	0.86
$\gamma$ -sarcoglycan (LGMD2C)	4	1.72
Total primary sarcoglycanopathies	13	5.60

\*The prevalence rate was calculated at 31 December 1996 (population 2 319 147). LGMD: limb-girdle muscular dystrophy.

Four patients (two unrelated patients and two sibs) were shown to have a primary  $\gamma$ -sarcoglycanopathy (Nos 10-13). Three of the four patients suffered from a severe DMD-like muscular dystrophy (Nos 10, 12, and 13) and one from milder LGMD (No 11). Consanguinity was not reported in any of these patients. The prevalence of  $\gamma$ -sarcoglycanopathy (LGMD2C) was estimated to be  $1.72 \times 10^{-6}$  (4/2 319 147) inhabitants (table 3).

#### RELATIVE PROPORTION OF PRIMARY SARCOGLYCANOPATHIES AMONG PATIENTS WITH DMD-LIKE AND LGMD PHENOTYPES

The overall prevalence of  $\alpha$ -SG,  $\beta$ -SG,  $\gamma$ -SG, and  $\delta$ -SG gene defects was estimated to be  $5.60 \times 10^{-6}$  (13/2 319 147) subjects (table 3).

In the selected area, 11 patients were diagnosed on their clinical phenotype as DMD-like muscular dystrophy (prevalence  $4.7 \times 10^{-6}$ ) and six of them (54.5%) were affected with primary sarcoglycanopathy. In the same area, 40 patients were diagnosed on their clinical phenotype and biopsy findings as having an LGMD phenotype (prevalence  $17.2 \times 10^{-6}$ ) and seven of them (17.5%) were affected with primary sarcoglycanopathy.

#### Discussion

Primary sarcoglycanopathies have been described in families with a broad range of clinical presentations.<sup>6 7 11 15 17-30</sup> Many cases have been previously described under the name SCARMD (severe childhood autosomal recessive muscular dystrophy) or DMD-like muscular dystrophy. However, the term SCARMD, used to describe the severity of the disease, is not always accurate as a description of the myopathy associated with primary sarcoglycanopathy, because the phenotype may be milder with juvenile or adult onset. Thus, the genetic and clinical heterogeneity of this group of disorders has made the current LGMD nomenclature useful.<sup>1</sup>

Since identification, correct classification, and prevalence estimates of the sarcoglycanopathies cannot be based on clinical criteria, the molecular characterisation of muscular dystrophies with normal dystrophin represented the basis of this study. Our neuromuscular centre is working in an area where full ascertainment

and epidemiological statistics are possible. We believe that all the affected cases came to our notice since muscular dystrophy patients living in this area are referred here to receive either diagnosis or genetic counselling. All the cases suspected to be affected with muscular dystrophy usually undergo a muscle biopsy to obtain clinical and molecular definition. Muscle biopsies from patients shown to have a progressive muscular dystrophy of unknown aetiology (normal dystrophin) have been screened for  $\alpha$ -SG.

Data acquired to date suggest that  $\alpha$ -SG immunostaining can be considered a marker of SG complex integrity, being absent or highly reduced in every primary sarcoglycanopathy.<sup>6 7 11 15 16</sup> Thus, we believe that we have detected all SG deficient patients using biochemical  $\alpha$ -SG studies as the primary screening tool. A possible source of inaccuracy in the prevalence estimate is if we were unable to detect all mutations in the biopsies tested by the SSCP method. Since the rate of mutation detection using SSCP in our study is estimated to be 90-95%, the stated prevalence rates could increase by a maximum of 10%.

In the area selected in this study, primary LGMD2D is twice as frequent as LGMD2C and four times more frequent than LGMD2E; primary LGMD2F was not found. Five patients with  $\alpha$ -SG deficiency did not show mutations of the  $\alpha$ -,  $\beta$ -,  $\gamma$ -, or  $\delta$ -SG genes under investigation, suggesting that the observed  $\alpha$ -SG deficiency was secondary to an as yet unidentified primary protein defect.

The overall prevalence of primary sarcoglycanopathies is estimated to be 5.6 per million, a value that is lower than that estimated for other recessive neuromuscular diseases in the same area: 11 per million for spinal muscular atrophy types II and III<sup>32</sup> and 6.8 per million for congenital muscular dystrophy.<sup>33</sup> Parental consanguinity observed in this and other studies<sup>10 13</sup> confirms that the sarcoglycanopathies are rare recessive disorders.

Comparison between published genetic epidemiological data and those obtained in the present study is difficult for several reasons. First, the estimates currently available for the prevalence of LGMD were based only on the clinical phenotype and precede any of the molecular characterisation of this group of disorders. Furthermore, because of the wide clinical heterogeneity, primary sarcoglycanopathy patients might have been previously included in the estimates of either X linked and autosomal recessive DMD-like muscular dystrophy or LGMD phenotypes.

Most of the prevalence rates reported<sup>34</sup> for LGMD lie between 7 and  $40 \times 10^{-6}$ , while for an autosomal recessive DMD-like muscular dystrophy they are less than  $5 \times 10^{-6}$ . Two studies dealt with the relative proportions of the various defined gene localisations in LGMD. Stec et al<sup>35</sup> estimated that the proportion of patients suffering from autosomal DMD-like muscular dystrophy that could be the result of sarcoglycan genes mutations is 11.8% of all the patients with a DMD phenotype. However, the frequency reported in this study is speculative,

since molecular studies on SG genes were not performed.

Passos-Bueno et al<sup>36</sup> reported that among nine autosomal recessive LGMD families they studied, 22% mapped to chromosome 17q (primary  $\alpha$ -SG). Since this study was performed with families in whom only linkage analysis could be done, this represents a relatively small minority of affected patients and the data cannot be extrapolated from a few families to the whole population.

The only population study on  $\alpha$ -SG deficient patients was reported by Hayashi et al<sup>37</sup> who analysed 243 patients' biopsies showing normal dystrophin and identified five cases with immunohistochemical  $\alpha$ -SG defects. The frequency of  $\alpha$ -SG protein deficiency in Japan was estimated to be  $1.0-2.1 \times 10^{-6}$ . However, no screening for mutations was done on these patients, so not knowing how many of these patients represent primary defects of  $\alpha$ -,  $\beta$ -,  $\gamma$ -, or  $\delta$ -SG makes this number a rough estimate.

In a previous epidemiological study<sup>38</sup> surveying the same geographical area, the prevalence rates for DMD-like muscular dystrophy and autosomal recessive LGMD based on clinical phenotypes were calculated to be  $3.7 \times 10^{-6}$  and  $17.3 \times 10^{-6}$ , respectively. These values are very close to those found in the present study ( $4.3$  and  $17.2 \times 10^{-6}$ , respectively). The reliability of our results is therefore proven by the consistency of the data with those derived from a previous study surveying the same area and having the same clinical and epidemiological ascertainment methods.

We found that the relative proportion of patients with defects in the SG genes among those who received the clinical diagnosis of DMD-like muscular dystrophy was 54.5% (6/11) and 17.5% (7/40) for LGMD cases. In a cumulative series of 556 myopathic patients screened for an  $\alpha$ -SG defect,<sup>28</sup> 22% of DMD-like muscular dystrophy and 6% of LGMD patients were affected with primary sarcoglycanopathies. The difference between the two studies probably represents a difference in patient diagnosis (DMD-like versus LGMD), or the ethnic diversity of the two patient cohorts (a more heterogeneous North American population versus a more homogeneous Italian population). Alternatively, the difference may reflect variation in the frequency of sarcoglycan gene mutations in various world populations. In the study conducted by Hayashi et al,<sup>37</sup> they found approximately 5% of Japanese patients with a DMD/BMD-like diagnosis to have an  $\alpha$ -SG protein deficiency. Although this percentage reflects the biochemical frequency of  $\alpha$ -SG deficiency, the molecular frequencies cannot exceed this number (assuming all mutation positive patients show a biochemical deficiency). Thus, we see that in three different world populations, the frequency of sarcoglycan gene mutations in a particular diagnostic category differs. Therefore, the prevalence rates of the individual sarcoglycanopathies and the frequencies of the sarcoglycanopathies in the various patient populations may need to be determined for all the different world populations.

The possibility that additional SG mutation positive cases may be detected among LGMD or mild myopathic patients showing a nearly normal biochemical  $\alpha$ -SG defect cannot be completely excluded.

The present study has addressed two issues: (1) the estimate of the prevalence rate of each primary sarcoglycanopathy, assessed by both biochemical and molecular screening; and (2) the relative proportion of patients with primary sarcoglycanopathies among those who received the clinical diagnosis of DMD-like or LGMD muscular dystrophy.

Because of the extensive clinical heterogeneity in LGMD patients with normal dystrophin, the correct classification of these patients by molecular analysis should be pursued because of its usefulness in the differentiation between autosomal recessive and dominant and X linked recessive inheritance, an issue that is crucial for genetic counselling.

The financial support of Telethon (grants 695 to MF and C19 to CA) is gratefully acknowledged.

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