Main clinical features of the three mapped autosomal recessive limb-girdle muscular dystrophies and estimated proportion of each form in 13 Brazilian families

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Abstract

Autosomal recessive limb-girdle muscular dystrophies (AR LGMD) represent a group of muscle diseases with a wide spectrum of clinical signs, varying from very severe to mild. Four different loci that when mutated cause the AR LGMD phenotype have been mapped or cloned or both: in two of them the linked families seem to have a relatively mild phenotype (LGMD2a and LGMD2b), in the third one the reported linked families show a more severe clinical course (LGMD2c), while mutations in the fourth locus may cause severe or mild phenotypes (LGMD2d). The relative proportion of each of these genetic forms among the LGMD families and whether there are other genes that when mutated cause this phenotype is unknown. The closest available informative markers for each of the mapped AR LGMD genes have been tested in 13 Brazilian families with at least three affected patients. The findings from the present report confirm non-allelic heterogeneity for LGMD and suggest that in our population about 33% of the LGMD families are caused by mutations in the 15q gene, 33% in the 2p gene, 17% by mutations in the adhalin gene, and less than 10% may be by mutations at the 13q locus. They also suggest that there is at least one other gene responsible for this phenotype. In addition, the main clinical features of the different forms are discussed.

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Key words: autosomal recessive limb-girdle muscular dystrophy; genetic heterogeneity; severe childhood autosomal muscular dystrophy.

Autosomal recessive limb-girdle muscular dystrophies (AR LGMD) represent a group of muscle diseases with a wide spectrum of clinical severity. In the most severe form of AR LGMD, onset occurs usually between 3 and 5 years; the progression is very rapid with loss of ambulation usually before the third decade. Since this form clinically resembles Xp21 Duchenne muscular dystrophy (DMD), it has been called "Duchenne-like muscular dystrophy, DLMD" or "severe childhood autosomal recessive muscular dystrophy, SCARMD".¹⁻⁴ In the milder forms (LGMD), onset may be in the first,

second, or even third decade; the loss of ambulation usually occurs after the third decade and longevity may be reduced or nearly normal. There is significant variability of clinical symptoms in unrelated patients and within families.¹⁵

Despite extensive clinical, epidemiological, and pathological studies, it is still impossible to distinguish the different forms of AR LGMD. It is now generally accepted that the diagnostic controversies involving this group of diseases will only be resolved when the genes responsible are identified and cloned and their specific mutations are determined.

Several genes have been tested as candidates for AR LGMD, such as genes which show homology to dystrophin (the protein product of Xp21 DMD/BMD) as well as the glycoproteins associated with dystrophin. The first to be excluded was the gene on chromosome 6 with homology to dystrophin and responsible for utrophin synthesis.⁶⁻⁹ However, deficiency of one of the dystrophin associated glycoproteins, the 50 kDa protein adhalin, was reported in patients with a SCARMD phenotype.¹⁰

Beckmann *et al*¹¹ mapped the first locus that causes one form of mild AR LGMD to chromosome 15q. Recently, it was shown that this gene codes for calpain.¹² This form of LGMD was classified as LGMD2a.¹³ However, while some LGMD families from Brazil and other populations linked to this 15q locus have been reported,^{14,15} other Brazilian families were unlinked to this region, thus showing genetic heterogeneity for the mild forms of AR LGMD.¹⁵

A second locus responsible for another mild form of AR LGMD has been mapped to chromosome 2p, based on linkage analysis in two large families, one Palestinian and the other Sicilian.¹⁶ Recently, two Brazilian families have also been shown to be linked to this gene on chromosome 2p.¹⁷ This form of AR LGMD has been classified as LGMD2b.¹³

On the other hand, the severe forms of AR LGMD (SCARMD) do not seem to be caused by mutations at 2p or 15q.^{18,19} Othmane *et al*¹⁹ mapped the first locus associated with the more severe phenotype to chromosome 13q. Subsequently, genetic heterogeneity was also observed for this locus and phenotype.^{20,21} Adhalin has been tested in SCARMD patients linked and unlinked to the 13q gene and it was observed that 13q linked patients have a negative

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pattern for this protein; however, among patients belonging to families unlinked to the 13q gene, some were found to be positive for adhalin while others were negative.²⁰⁻²² These results suggested that, besides genetic heterogeneity, adhalin is probably not encoded by the 13q gene and that deficiency of adhalin could represent a secondary effect.²⁰⁻²²

In 1994, Roberds *et al.*²³ mapped and cloned the gene encoding human adhalin to chromosome 17q and identified point mutations at this locus in patients from one family in which muscle biopsy from affected subjects was negative on testing for adhalin.²¹ The muscular dystrophies linked to 13q and 17q have been classified as LGMD2c and LGMD2d, respectively.¹³ Linkage to the adhalin gene has been confirmed in other populations; however, unexpectedly, a proportion of 17q linked patients present a relatively mildly phenotype.¹³

Therefore, up to now, four different genes that when mutated cause the AR LGMD phenotype have been mapped or cloned or both: in two of them the linked families seem to have a relatively mild phenotype (LGMD2a and LGMD2b), in the third one the reported linked families show a more severe clinical course (LGMD2c), while mutations at the fourth locus may cause severe or mild phenotypes (LGMD2d). The relative proportion of each of these genetic forms among the LGMD families and the possible existence of other gene(s) that when mutated cause this condition is unknown.

With these questions in mind, we have tested 13 large families with a relatively mild clinical course for the closest available markers to the four mapped genes, on 15q, 2p, 13q, and 17q.

Methods

FAMILY DATA

Thirteen AR LGMD families (63 patients and 117 unaffected relatives) with a minimum of three affected patients and at least one affected female per family were selected for this study. The families were ascertained at the Centro de Miopatias, Instituto de Biociências, Universidade de São Paulo, SP, Brazil. Diagnosis was based on clinical examination and course of the disease, family history, grossly raised serum creatine kinase levels, and assessment of dystrophin through immunocytochemistry and western blotting in muscle biopsies.²⁴ All the patients were classified as having a mild phenotype according to the following criteria: onset during the second or even first decade but still ambulant at the age of 16.¹⁵ Linkage analyses for 15q markers have previously been reported¹⁵ in six of the 13 families reported here (families 1, 2, 7, 15, 19, and 23 which correspond, respectively, to families 17, 16, 24, 21, 22, and 7 from the present study). Only patients from families linked (or with a high probability of being linked) to a specific candidate gene were included for comparative clinical assessment.

DNA ANALYSIS

DNA was extracted from whole blood, according to the method of Miller *et al.*²⁵ Subjects

were genotyped using the following microsatellites. (1) For the LGMD2a gene markers D15S143 or D15S132, THBS1 or both were used. The LGMD2a locus lies within 1 cM and 1.7 cM of D15S143 and D15S129 respectively²⁶; D15S132 is within this interval (D Love, personal communication) and THBS1 is very close to D15S129.²⁶ (2) For the LGMD2b gene markers D2S282 and D2S286 were used for all the families and AFM205 for some families. The LGMD2b gene maps between D2S379 and D2S286, which are approximately 10 cM apart.¹⁷ D2S282 is very close to D2S379 and AFM205 lies in the 10 cM interval flanked by D2S282 and D2S286. The most probable order of these markers is: D2S379/D2S282-AFM205-LGMD2b-D2S286 (J Weissenbach, personal communication). (3) For the LGMD2c gene D13S115 or D13S120 or both were used. D13S115, the closest available marker, is approximately 3 cM from the disease gene (confidence interval: 0.001–0.09); D13S120 is approximately 16 cM from the LGMD2c gene (confidence interval: 0.08–0.34).¹⁹ (4) For the LGMD2d gene the adhalin intragenic marker D17S1319 was used.²³

LINKAGE ANALYSIS

Two point linkage analysis between the disease gene and each of the markers was performed using the computer program Linkage.²⁷ The gene frequency for the AR LGMD gene was taken to be 0.001. The recombination rate was assumed to be equal in males and females. A homogeneity test was carried out on the two point lod scores (15q and 2p markers) using the HOMOG program.²⁸

Since there is genetic heterogeneity in LGMD families and not all families are large enough to give a lod score higher than 3.0, we considered that a family was likely to be linked to a candidate locus if no recombinants were identified using the closest informative marker for the region tested. In families in which the maximum positive lod score was lower than 2.0, we also verified if recombinants (or negative lod scores) were detected with markers from the other candidate loci.

Results

All the families were first screened for the 15q gene (pedigrees summarised in the figure). Two point lod scores for all of them are given in table 1. A positive maximum lod score at $\theta = 0.0$ was observed in four of these families. In one of them (family 22), a lod score of 6.8 was observed; in two others (families 17 and 47) a lod score of 2.0 was observed and a probability of 98% of linkage to 15q was estimated through the HOMOG test. In family 12, although positive, the lod score was lower owing to lack of informativeness; this family was also tested with another marker closely linked to the LGMD2A gene (D15S132) and a lod score of 2.1 at $\theta = 0.0$ was estimated. Therefore, we considered that these four families have a high probability of being linked to the chromosome 15q gene. Linkage in families 17 and 22 has recently been

Simplified pedigrees of 13 autosomal recessive limb-girdle muscular dystrophy families.

confirmed through the identification of the pathogenic mutations in the calpain gene.¹² Screening for mutations in the two other families is under investigation.

Two of these 15q linked families (17 and 22) are highly inbred while the two others are apparently non-consanguineous (12 and 47). In addition, they are from different ethnic groups: two are white (family 17 and 47) and the other two (12 and 22) are negroids. There is a total of 19 affected and four preclinical cases in these four pedigrees. The mean age of ascertainment was 20.6 (SD 11.8) years, with a mean age of onset of 13.5 (SD 3.8) years. Only three were wheelchair bound before the age of 25 years. Serum creatine kinase (CK) was raised (on average 11-fold) in all affected people including the three who had lost ambulation. The highest values were observed among the youngest and preclinical cases (table

2). Calf hypertrophy and asymmetry of muscle involvement was observed in some patients. However, these features varied within and between the families.

Two point linkage analysis between the disease gene and two markers flanking the LGMD2b locus was performed in the remaining eight non-15q LGMD families (table 3). Besides the two large inbred families previously reported to be linked to this locus (families 21 and 39¹⁵), two additional families (24 and 54) showed a high posterior probability of being linked to this gene (HOMOG (H) analysis with marker D2S282: family 24, $H = 98\%$ and family 54, $H = 99\%$). In addition, negative lod scores were observed with 13q (table 4) and 17q (D17S1319: family 24, $Z = -4.09$ at $\theta = 0.01$ and family 54, $Z = -1.81$ at $\theta = 0.01$) (table 5) markers for these two families. Although these two families seem to be linked to the 2p locus, we considered for future analysis only families 21 and 39 to be linked to the chromosome 2p gene.

These two LGMD2b families (figure) are highly inbred and from different ethnic groups (family 39 is white and family 21 is negroid). There are 10 patients and four preclinical cases in these two pedigrees (table 2). The mean age of ascertainment was 28.9 (SD 15.6) years and the mean age of onset was 22.1 (SD 7.3) years. Only one out of the 14 patients had lost ambulation at 27 years. Serum CK was raised (on average 34-fold above normal) in all affected subjects including the one who is wheelchair bound. There was also extreme variability of clinical symptoms within and between families. For example, in family 39, with the exception of one affected patient who was wheelchair

Table 1 Two point lod scores between marker D15S143 and the LGMD2 gene

Family	0	0.01	0.05	0.1	0.2	0.3
7	-0.0	-3.61	-1.67	-0.96	-0.41	-0.18
11	-0.0	-1.09	-0.45	-0.23	-0.06	-0.02
12*	0.87	0.86	0.80	0.71	0.52	0.30
16	-0.0	-1.38	-0.72	-0.45	-0.21	-0.09
17	2.21	2.15	1.94	1.67	1.14	0.65
21	-0.0	-3.79	-1.79	-1.02	-0.38	-0.12
22	6.89	6.73	6.10	5.31	3.70	2.11
24	-0.0	-0.96	-0.34	-0.14	-0.01	0.01
39	-0.0	-3.59	-1.63	-0.89	-0.31	-0.09
42*	0.03	0.03	0.03	0.02	0.02	0.02
47	2.18	2.14	1.98	1.76	1.30	0.78
54	-0.0	-1.53	-0.83	-0.52	-0.23	-0.09
61	-0.0	-2.51	-1.18	-0.67	-0.25	-0.09

* These families have also been tested with the marker D15S132 or THBS1.

Family 12 (marker D15S132): $Z_{\max} = 2.1$ at $\theta = 0.0$; family 42 (marker THBS1): $Z = -1.50$ at $\theta = 0.01$.

Table 2 Main clinical findings in LGMD patients caused by mutations at different loci

Family	Subject	Sex	Ascertainment	Onset	PGW*	SGW†	Wheelchair	CK (x fold)‡
(A) 15q linked (LGMD2a)								
17	III-1	F	32	16	Yes	Yes	No	6
17	III-5	M	26	13	Yes	Yes	No	5
17	III-7	F	32	?	Yes	No	No	2.5
17	III-8	F	27	16	Yes	Yes	21	16.5
17	II-2	F	28	?	Yes	Yes	No	2.7
12	II-1	F	25	14	Yes	Yes	No	4
12	II-5	M	15	13	Yes	Yes	No	5
12	II-9	F	10	—	No	No	No	28
47	II-1	M	37	16	Yes	Yes	No	4
47	II-2	F	36	16	Yes	Yes	No	5
47	II-3	F	34	23	Yes	Yes	No	3.2
47	II-7	M	38	17	Yes	Yes	No	Not tested
22	II-5	F	21	13	Yes	Yes	No	64
22	II-6	M	24	12	Yes	Yes	No	2.7
22	II-10	F	13	8	Yes	No	No	8
22	II-13	M	24	11	Yes	Yes	18 y	4
22	II-14	M	19	10	Yes	Yes	18 y	11
22	II-17	M	10	10	Yes	No	No	26
22	II-21	M	6	§	No	No	No	13
22	II-23	F	3	§	No	No	No	12
22	III-1	F	8	8	Yes	No	No	12
22	III-3	M	4	§	No	No	No	12
22	III-4	F	2	§	No	No	No	4
(B) 2p linked (LGMD2b)								
21	II-1	F	28	20	Yes	Yes	No	22
21	II-2	F	31	16	Yes	Yes	No	15
21	II-7	F	19	13	Yes	Yes	No	28
21	II-8	M	15	§	No	No	No	67
21	II-9	M	13	§	No	No	No	43
21	II-10	F	11	?	Yes	No	No	100
21	II-11	M	10	§	No	No	No	10
39	II-2	F	52	35	Yes	Yes	No	7
39	II-3	F	49	27	Yes	Yes	No	6
39	II-4	M	59	24	Yes	Yes	27 y	5
39	II-7	M	38	20	Yes	Yes	No	26
39	II-8	F	29	?	Yes	No	No	65
39	II-9	M	26	§	No	No	No	32
39	II-10	F	25	?	Yes	No	No	52
(C) 13q linked (LGMD2c)								
42	II-2	F	18	?	Yes	No	No	17
42	II-4	M	16	10	Yes	No	No	12
42	II-7	M	10	§	Yes	Yes	No	40
(D) 17q linked (LGMD2d)								
7	II-2	M	24	16	Yes	No	No	17
7	II-3	F	22	12	Yes	No	No	6
7	II-4	M	21	12	Yes	Yes	No	19
7	II-5	F	17	12	Yes	No	No	40
16	II-3	M	—	—	—	—	16	—
16	II-8	F	18	6	Yes	Yes	Walking with support	16
16	II-11	F	11	1	Yes	Yes	Walking only with support	18
16	III-1	F	7	?	Yes	No	No	175
16	III-2	F	5	?	No	No	No	180

* PGW = pelvic girdle weakness; † SGW = shoulder girdle weakness; ‡ x fold = increased x fold above normal level. CK is measured in Sigma units and normal levels are considered to be up to 10 SU for adults and up to 20 SU for children.

§ Asymptomatic; ? = the affected patient does not know when the weakness started or does not consider himself affected.

bound at the age of 27 (III-9), the other six have a mild phenotype. One subject (IV-3) was asymptomatic at the age of 26, but has a raised serum CK (32-fold) and his diagnosis was confirmed through muscle biopsy.

Although the gene mapped on chromosome 13 had been previously reported in pedigrees with a more severe DLMD phenotype, we tested all the non-15q families for the 13q markers (table 4). Negative lod scores were observed in all the families, except for family 42. In this non-consanguineous family a maximum lod score of 1.7 at $\theta = 0.0$ was observed (testing three affected patients, four normal sibs, and their parents); two other markers from this region were also tested in this family, but no improvement of the lod scores was obtained (D13S120, $Z_{\max} = 0.5$ at $\theta = 0$, and D13S143, not informative). The age of onset in the proband (II-4) was around 10 years old with complaints of difficulties in running and climbing stairs; at ascertainment, he was 16 years old and still walking with no difficulties. Serum CK activities were increased 12-fold in the

proband, 17-fold in his 18 year old sister (II-2), and 40-fold in his 10 year old younger brother (II-7). Careful clinical evaluation showed that the only clinical sign in II-2 is difficulty in standing up from the floor, while II-7 has enlargement of the calves but without any apparent weakness.

The remaining four families (families 7, 11, 16, and 61), excluded for these three loci at $\theta = 0.01$, were tested with intragenic adhalin markers. Intragenic recombinants in the adhalin gene were not expected to be found owing to its small size.^{23,29} Therefore, we considered that only families 7 and 16 were linked to the adhalin gene. This was further supported by the recent finding of pathogenic mutations in this gene in the patients from these two 17q linked genealogies.³⁰

The main clinical features in nine patients from the two 17q linked families are summarised in table 2. The clinical progression in patients from family 7 is milder than in those from family 16. The youngest of family 7 (II-5) is currently 27 years old and able to perform

Table 3 Two point linkage analysis between chromosome 2p markers and the disease locus for the informative families

Family	0	0.01	0.05	0.1	0.2	0.3
(A) Marker D2S282						
7	-0.0	-3.60	-1.63	-0.89	-0.31	-0.10
16	-0.0	-4.95	-2.3	-1.28	-0.45	-0.13
21	3.78	3.71	3.44	3.08	2.33	1.55
24	1.33	1.30	1.18	1.03	0.71	0.39
42	0.80	0.79	0.72	0.64	0.45	0.26
54	1.70	1.66	1.49	1.27	0.82	0.41
(B) Marker D2S286						
7	-0.0	-6.65	-3.29	-1.97	-0.82	-0.31
11	-0.0	-2.69	-1.35	-0.83	-0.37	-0.15
16	-0.0	-4.49	-1.88	-0.92	-0.2	0.001
21	-0.0	1.48	1.87	1.78	1.29	0.71
24	0.93	0.91	0.82	0.70	0.47	0.25
39	4.62	4.53	4.13	3.62	2.59	1.54
42	-0.0	-3.84	-1.83	-1.05	-0.4	-0.14
54	1.10	1.06	0.89	0.68	0.31	0.08
61*	-0.0	-0.70	-0.10	0.7	0.12	0.08

* This family was also tested with marker AFM205: $Z = -2.13$ at $\theta = 0.10$.

Table 4 Two point linkage analysis between marker D13S115 and the LGMD gene

Family	0	0.01	0.05	0.1	0.2	0.3
7	-0.0	-0.79	-0.16	0.05	0.14	0.11
11*	0.22	0.22	0.215	0.19	0.13	0.06
16	-0.0	-4.20	-2.16	-1.33	-0.58	-0.23
21	-0.0	-5.19	-2.69	-1.05	-0.64	-0.24
24	-0.0	-2.68	-1.32	-0.78	-0.31	-0.11
39	-0.0	-2.96	-1.05	-0.37	-0.08	0.13
42	1.7	1.67	1.53	1.34	0.9	0.53
54	-0.0	-2.03	-0.76	-0.32	-0.04	0.02
61	-0.0	-4.48	-1.87	-0.91	-0.19	0.001

* This family has also been tested with marker D13S120: $Z = -1.11$ at $\theta = 0.01$.

Table 5 Two point linkage analysis between D17S1319 and the disease locus

Family	0	0.01	0.05	0.1	0.2	0.3
7	1.53	1.50	1.38	1.23	0.91	0.56
11*	-0.0	-0.57	0.03	0.21	0.25	0.16
16	2.68	2.62	2.35	2.01	1.31	0.64
61*	-0.0	-0.50	0.68	0.99	0.98	0.67

* Muscle from patients belonging to these families was also tested for adhalin and a positive pattern was observed (unpublished results).

all functions. In family 16, one patient (II-3) lost ambulation at the age of 16 and died aged 27 (according to his parents). His two sisters, currently aged 26 and 19 (II-8 and II-11 respectively) have severe weakness in both upper and lower limbs, but are still ambulant. In this family two girls aged 7 and 5 years old were identified because of extremely high levels of serum CK (III-1 and III-2 respectively), but both were completely asymptomatic at this age.

Discussion

The present report confirms that there are Brazilian LGMD families with a mild phenotype linked to the loci on chromosomes 15q, 2p, 17q, and one possibly linked to the 13q locus. In addition, one family with six affected patients (LG61) was excluded from the four known genes suggesting that there is at least a fifth locus responsible for mild LGMD.

All the LGMD patients included in this study have a relatively mild phenotype, which illustrates the difficulty in classifying the subforms of this muscular dystrophy based only on clinical symptoms and complementary

laboratory examinations. Highly raised serum CK levels were observed only among affected and preclinical cases suggesting that a grossly raised serum CK in young people from families with LGMD patients may be considered as a preclinical diagnosis.

Preliminary clinical analysis suggests that LGMD2b may be the mildest of these four forms of LGMD. The average age of onset in LGMD2b patients was significantly later than in the LGMD2a form ($t = 3.04$, $p < 0.05$) and none of the 2p linked patients lost ambulation before 25 years of age (table 2).

In relation to the 13q linkage analysis, although the positive lod scores were not high enough to confirm linkage in family 42 owing to its small size, it is important to observe that negative values were estimated for all the other loci, including 17q (D17S1319, $Z = -0.75$, at $\theta = 0.01$). As soon as this gene is cloned, it will be extremely important to verify if the disease in these apparently 13q linked patients is the result of pathogenic mutations in this locus. If confirmed, these observations will indicate that the 13q gene may not necessarily lead to a severe phenotype, as previously reported,¹⁹ since patients from the present family have a mild course.

Adhalin deficiency, as originally reported, should cause a severe phenotype.¹⁰ However, all the patients from the two adhalin linked families have a relatively mild clinical course comparable to that observed in LGMD2a and LGMD2b patients. Therefore, these data suggest that mutations in this gene may also lead to mild phenotypes.

A tentative clinical classification would be important before testing the patients for specific mutations that may cause the LGMD phenotype, in particular among the isolated cases. Only four of 57 patients from the present report were confined to a wheelchair (one aged 16, two aged 18, and one aged 27). Therefore, as in the Xp21 Duchenne and Becker muscular dystrophies, we suggest the inclusion of the age at loss of ambulation (before or after the age of 16) as an additional criterion to classify patients as severe or mild LGMD.

In conclusion, the present study confirms non-allelic heterogeneity for LGMD. If we consider only the nine families with strong (or proven) evidence of linkage to one of the known candidate loci, we would estimate that in our population about 44% of the mild LGMD families are caused by mutations at the 15q gene, 22% by the 2p gene, 22% by mutations in the adhalin gene, and 11% by mutations in other loci. However, if families 24 and 54 are included in the analysis, then the proportion of LGMD families caused by mutations in the 15q and 2p loci is similar, that is 33%. The finding of specific mutations in these families will be very important to confirm these data.

In order to determine the relative frequency of each of the LGMD genes (15q, 2p, 13q, and 17q) among families from different ethnic origins, as well as to identify clinical differences among these forms, it is important to test and report all available informative ARLGMD pedigrees. This approach will facilitate efforts to

map and clone all of the AR LGMD loci, thereby improving the classification and understanding of this group of conditions.

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