Limb girdle muscular dystrophy due to mutations in POMT2

Limb girdle muscular dystrophy due to mutations in *POMT2*

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Abstract

Background

Mutations in the gene coding for protein O-mannosyl-transferase 2 (*POMT2*) are known to cause severe congenital muscular dystrophy, and recently, mutations in *POMT2* have also been linked to a milder limb-girdle muscular dystrophy (LGMD) phenotype, named LGMD type 2N. Only four cases have been reported so far.

Methods

We report 12 new cases of LGMD2N, aged 18-63 years. Muscle involvement was assessed by MRI, muscle strength testing and muscle biopsy analysis. Other clinical features were also recorded.

Results

Presenting symptoms were difficulties in walking, muscle pain during exercise, delayed motor milestones and learning disabilities at school. All had some degree of cognitive impairment. Brain MRIs were abnormal in three of ten patients, showing ventricular enlargement in one, periventricular hyperintensities in another, and frontal atrophy of the left hemisphere in a third patient. Most affected muscle groups were hip and knee flexors and extensors on strength testing. On MRI, most affected muscles were hamstrings followed by paraspinal and gluteal muscles. The 12 patients in our cohort carried 11 alleles with known mutations, while 11 novel mutations accounted for the remaining 13 alleles.

Conclusion

We describe the first cohort of LGMD2N patients and show that unlike other LGMD types, all patients had cognitive impairment. Primary muscle involvement was found in hamstring, paraspinal and gluteal muscles on MRI, which correlated well with reduced muscle strength in hip and knee

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flexors and extensors. The study expands the mutational spectrum for LGMD2N, with the description of 11 novel *POMT2* mutations in the association with LGMD2N.

Introduction

Recessive mutations in the gene coding for protein O-mannosyl-transferase 2 (*POMT2*) are known to cause severe congenital muscular dystrophies (CMD), such as Walker-Warburg syndrome and muscle-eye-brain disease. These conditions are, characterized by structural brain and muscle involvement at birth and a low chance of survival past childhood. In 2007, mutations in *POMT2* were linked to a milder limb-girdle muscular dystrophy (LGMD) phenotype, named LGMD type 2N (LGMD2N).[1] LGMD is a group of heterogeneous diseases characterized by wasting and weakness of the muscles of the shoulder and hip region.[2]

The POMT2 protein forms a complex with protein O-mannosyl-transferase 1 (POMT1), and catalyzes the first step in the synthesis of O-mannosyl glycan, located on the extra-cellular protein, α-dystroglycan. Alpha-dystroglycan (α-DG) is part of the dystrophin-associated glycoprotein complex at the sarcolemma. Here, it forms an essential link between the sub-sarcolemmal cytoskeleton and the extracellular matrix of the muscle cells, and plays an important role in membrane integrity and force transmission.[3]

Only four young patients (age 4-18 years) with a LGMD2N phenotype have been reported as separate cases so far.[1,4-6] Thus, detailed knowledge about the disease characteristics is lacking for LGMD2N. Through an international collaboration between seven clinical centers, and facilitated by the use of next-generation sequencing (NGS) in unclassified myopathies with limb girdle weakness, we have identified a group of 12 patients affected by LGMD2N. We report on the specific clinical features of this rare form of LGMD, extend the mutational spectrum, and describe findings on MRI of brain and muscle as well as immunohistochemical analyzes on muscle biopsies.

Methods

The study was approved by the Danish National Committee on Health Research Ethics (H-3-2012-163 with amendment #41665, #43449 and #50556), as well as the local Ethical Review Boards of the participating centers. All patients consented to participate.

Subjects

Patients affected by LGMD2N were identified primarily by NGS in cases with undiagnosed limb girdle and in a few cases by direct Sanger sequencing. Twelve patients with genetically verified LGMD2N were included in the study (Table 1). Two of the patients (cases 1 and 2) were siblings of consanguineous parents, and cases 9 and 10 were siblings of non-consanguineous parents. All other patients were unrelated.

	Case 1[a]	Case 2[a]	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9[b]	Case 10[b]	Case 11	Case 12
Gender/age, years	Female/25	Male/20	Female/51	Male/52	Male/54	Male/41	Female/18	Female/18	Female/33	Male/29	Female/63	Female/32
Phenotype	LGMD	LGMD	LGMD	LGMD	CMD/LGMD	LGMD	CMD/LGMD	CMD/LGMD	LGMD	LGMD	LGMD	LGMD
Disease onset, years	2	5	4	8	From birth	2.5	From birth	From birth	4	2	55	25
Presenting symptoms	Difficulties walking and running	Difficulties walking and running	Learning difficulties, oriented to special school	Difficulties in learning math and science in school	Delay in cognitive development**	Walked at age 2,5 and increasing weakness	Congenital, delay in development	Congenital, delay in development	Difficulties walking and running	Difficulties walking and running	Weakness of lower legs	Difficulty getting off the floor and climbing stairs
Age at muscle MRI, years	25	20	49	49	54	38	17	18	22	18	60	28
Ethnicity	Arab (DK)	Arab (DK)	CA (FR)	CA (FR)	CA (DK)	CA (AZ)	CA (SP)	CA (SP)	CA(BE)	CA(BE)	CA(UK)	Fars(IR)
Brain MRI	Central atrophy, ventricular enlargement	Normal	Normal	Periventricular hyperintensities	Frontal atrophy in left hemisphere	ND	Normal	Normal	Normal	Normal	Normal	ND
MMSE/ Age at exam, years	16/30 25	19/30 20	26/30 51	25/30 52	10/30 54	ND	28/30 18	20/30 18	27/30 33	28/30 29	29/30 63	26/30 32
FVC	ND*	ND*	76%	51%	ND*	76%	71.7%	44.2%	72%	ND	83%	56%
ECG	Single premature ventricular contraction	Sinus bradycardia with right bundle branch block	Normal	Normal	Normal	Normal	Normal	Normal	Nonspecific repolarization abnormalities	ND	Normal	Normal
Cardiac echo	ND	Normal	Normal LVEF = 67%	Normal LVEF = 65%	LVEF = 50%	Normal	Dilated cardiomyopathy, LVEF = 45-50%	Normal	Normal	ND	LVEF = 65% Mild tricuspid insufficiency	Normal
10 m walk test	10.98 sec	6.26 sec	16 sec	16 sec	70.67 sec	Can't walk	<6 sec	<6 sec	Can't walk without support	7.41	ND	ND
Walking aids	None	None	Two canes	One cane	Walking frame with wheels	Wheelchair	None	None	Wheelchair	None	None	One crutch

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CK (U/L)	1590	5410	4201	398	585	2762 – 4000	2640	2000-4000	7646	5086	3000	3120

Table 1: Baseline characteristics of 12 patients with LGMD2N

ND = not done. CA = Caucasian. DK = Danish. Fr = French. Arab = Arabic. AZ = Azerbaijan. SP= Spanish. BE = Belgian. UK = British. IR = Iranian. MMSE = minimal mental status examination. FVC= forced vital capacity. CK = Creatine Kinase. a and b = siblings. * = Could not cooperate during the test. ** = Hypoxic at birth.

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Clinical evaluation

A thorough medical history was taken and focused on presenting symptom, age at disease onset, muscle cramps, and myalgia. Sometimes disease onset and presenting symptom had to be obtained from parents or medical records. Walking capability was examined by the 10-meter walk test, and by recording the use of walking aids. Limb muscle strength was evaluated at participants' present age by manual muscle testing (MRC scale). Respiratory function was assessed by a spirometry (Forced Vital Capacity, FVC) and cardiac function by ECG and echocardiography. Cognitive function was screened by using the Minimal Mental Status Examination (MMSE).

MRI

Whole-body muscle MRI was performed in all patients and brain MRI in 10 of the 12 patients. The MRI-scanners differed among the participating clinical centers, but only axial T1-weighted images were assessed. Four cross-sectional slices at the level of calves, thighs, L4 and pelvis were chosen for evaluation of muscle involvement (Figure 1). Replacement of muscle by fat was graded according to the Mercuri scale.[7]

Muscle biopsies

Muscle biopsies were obtained from the tibial anterior, gastrocnemius or deltoid muscles and stained with H&E for general histopathological evaluation. For immunohistochemical evaluation of α-DG glycosylation, muscle sections were stained with the antibodies VIA4-1 and IIH6C (Merck-Millipore, Temecula, CA) and goat anti-mouse Alexa Fluor 594 antibodies (ThermoFisher, Waltham, MA) using standard protocols.[8] Biopsies for immunohistochemistry were only available for cases 1, 5, 9 and 10.

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Molecular findings

Blood samples were drawn for determination of plasma creatine kinase (CK) concentration and for extraction of DNA from leucocytes, according to standard procedures. Mutations in *POMT2* were identified by whole exome sequencing of leucocyte DNA at the Broad Institute's Genomics Platform, using Illumina exome capture, 38 Mb baited target, and the Broad's in-solution hybrid selection process. The mutations were, confirmed by Sanger sequencing. In two cases (3 and 4), mutations were found directly by Sanger sequencing. Mutation frequencies were estimated using Exome Aggregation Consortium (ExAC) with 60,706 unrelated individuals as control population (Table 2).

Table 2: Mutations identified with estimated frequency in control population.

	Case 1[a]	Case 2[a]	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9[b]	Case 10[b]	Case 11	Case 12
Mutation (Frequency in control population)	Homozygote c.713G>T (0)	Homozygote c.713G>T (0)	c.796G>A (1:63,090) c.1997A>G (1:16,620)	c.1031C>T (Unknown) c.1762C>T (1:70,270)	c.1238G>C (1:30,000) c.1496A>G (0)	c.1261C>T (1:62,000) c.1030A>C (0)	c.1997A>G (1:24,000) c.406T>C (0)	c.306C>A (0) c.816+1G>A (0)	c.1462C>T (1:62,000) c.1792_1793insG (0)	c.1462C>T (1:62,000) c.1792_1793insG (0)	c.557G>A (0) c.1637A>C (1:122,000)	c.1654-5T> (0) c.878T>A (0)
Protein product	p.Gly238Val	p.Gly238Val	p.Gly266Arg p.Tyr666Cys	p.Thr344Ile p.Arg588*	p.Arg413Pro p.Glu499Arg	p.Arg421Trp p.Thr344Pro	p.Tyr666Cys p.Tyr136His	p.Phe102Leu	p.Arg421Trp p.L577AfsX7	p.Arg421Trp p.L577AfsX7	p.Cys186Tyr p.His546Pro	p.Leu293H

a and b = siblings.

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Results

Clinical evaluation

Disease onset varied from birth to 55 years (Table 1). Three participants had disease onset at birth, and were classified as CMD/LGMD, because they had a typical LGMD phenotype as adults. Two with onset at birth, still walked unassisted at age 18 years and one was still ambulatory with assistance at age 54 years. Similar reasoning to classify patients as LGMD2N, despite disease onset at birth, was also applied in two of the four previously reported young cases of LGMD2N. [1,5]. Presenting symptoms were in most cases related to ambulatory function, showing either as a delay in the ability to walk, or troubles in walking, climbing stairs or running. Ten patients were ambulatory while one was wheelchair bound, and one could only walk when assisted. Some patients presented with learning difficulties in school, especially case 3, who attended a special school. The MMSE score was decreased relative to normal in most patients (Table 1), and all were described by their physicians as having some degree of cognitive impairment, although no formal neuropsychological tests were performed.

Two cases had reduced left ventricular ejection fraction (LVEF) (Table 1). Case 7 was diagnosed with dilated cardiomyopathy at age 18 years, and treated with an ACE inhibitor. The echocardiography of case 5 revealed a mild reduction of LVEF without any cardiac symptoms. Case 3 had hypertension and was treated with an antihypertensive drug. FVC was measured in 8 patients and was reduced in all with an average FVC of 66% of the predicted value. Cases 1, 2 and 5 could not cooperate during pulmonary function tests, because of poor intellectual capacity, and case 10 was unavailable for FVC measurements, because he was lost to follow-up.

Muscle strength examination showed reduced force in all patients (Figure 2), especially in hip and knee flexors and extensors. Knee flexor muscles were weaker than the extensors and the opposite

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pattern was seen across the hip. As apparent from Figure 2, muscle weakness was mostly symmetric, except in case 6, who had a much weaker left relative to right leg. Muscle strength of forearm and finger muscles showed normal force in all patients.

Proximal muscle atrophy was observed in most patients, and atrophy of the shoulder girdle was found in cases 9, 10 and 12, who had significant scapular winging (Figure 3). CK levels were highly elevated in all participants, except two cases (#4 and 5) in whom it was slightly elevated (Table 1).

MRI

Brain MRIs were abnormal in three of the 10 patients in whom brain imaging was carried out. Mild ventricular enlargement due to central and cortical atrophy was found in case 1, periventricular hyperintensities in case 4 and frontal atrophy of the left hemisphere in case 5 (Figure 4).

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Muscle MRI revealed a pattern of selective muscle involvement, most strikingly affecting the hamstring, paraspinal and gluteal muscles (Figure 1). Consistent with the evaluation of muscle strength, the hamstring muscles were more severely affected (average 3.8 on the Mercuri scale) than the anterior thigh muscle group (average 3.3 for the quadriceps).

In the muscles of the lower leg, both degree of fatty infiltration and muscles involved differed among the participants (Figure 1), but there was a predilection for selective involvement of the muscles of the posterior compartment; on average the gastrocnemius muscles (average 3.2 on Mercuri) were more severely affected compared to the tibialis anterior muscle (average 1.7 on Mercuri).

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Muscle biopsy

A dystrophic pattern with fiber size variation and central nuclei was present in muscle biopsies from 6 cases. Immunofluorescent staining of α-DG glycosylation demonstrated a clear signal reduction

(Figure 5). Glycosylation was also absent or severely reduced in muscle biopsies of cases 1, 9 and 10 (data not shown).

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Molecular findings

The patients carried 11 novel mutations in *POMT2* (Table 2). Case 3 carried the known mutation, c. 1997A>G, which previously has been linked to a CMD phenotype.[6,9]

Discussion

We describe the first cohort of patients affected by LGMD2N due to mutations in the *POMT2* gene, which so far has only been described in a few single-case reports. The major new findings of the study are: 1) patients with LGMD2N, unlike other recessively inherited LGMDs, are cognitively

impaired, 2) the disease primarily affects hamstring, paraspinal and gluteal muscles, and 3) the mutational spectrum of LGMD2N is expanded by the addition of 11 new mutations in *POMT2*, adding to the 5 known mutations causing LGMD2N and 4) patients with LGMD2N seem to have a wide range of disease onset.

The muscle involvement and clinical presentation of patients with autosomal recessive LGMD differ highly among the various subtypes. In this study, the degree of muscle involvement also varied among the 12 patients. One patient had an asymmetric appearance of muscle involvement, although generally the pattern on strength testing and MRI showed consistent involvement, preferentially of the hamstring, paraspinal and gluteal muscles in a symmetrical pattern. This pattern of muscle involvement was similar to that found in the worldwide most prevalent form of recessive LGMD, LGMD2A, which is caused by mutations in the *CAPN3* gene. Besides the pattern of leg involvement, prominent scapular winging, as seen in three of our LGMD2N patients, and asymmetry are also occasionally encountered in LGMD2A.[10] The same pattern of muscle involvement is also seen in another glycosylation defect of α-DG, LGMD2I, caused by mutations in *FKRP* (fukutin-related protein gene). Patients with LGMD2I present with a similar, preferential involvement of the posterior thigh. When calf muscles are involved in LGMD2I, the medial gastrocnemius and soleus muscles are affected first, which was also seen in our LGMD2N patients together with a rather hypertrophic appearance of the calf muscles.[11] Assessment of calf muscle hypertrophy was based on the clinical examination performed by an experienced myologist at each center; cross-sectional area measurements of different muscle groups could aid in reliably judging muscle bulk. The combination of different MRI features can, however, yield valuable clues, as e.g. LGMD2I is typically associated with muscle hypertrophy, contrasting with the atrophic phenotype of LGMD2A. If imaging is performed in relatively early disease stages, a similar pattern seems to be observed in different dystroglycanopathies.

The other way around, MRI imaging can aid in the interpretation of candidate sequence variants obtained by NGS techniques.

Dystroglycanopathies comprise more than 18 separate disorders and more remain to be discovered.[12] The disorders affect glycosylation of α-DG, and in the majority of cases result in CMD associated with brain abnormalities. POMT2 deficiency was first reported to cause CMD,[13] and only later was the defect associated with a rare phenotype compatible with LGMD.[1,4-6] Only in rare cases have patients with a LGMD phenotype been systematically associated with brain involvement. First, LGMD2I has been proposed to have occasional involvement of the frontal and posterior parietal lobes of the brain, as suggested by brain MRI scans from ten LGMD2I patients.[14] Patients were reported to have mild problems in graphic element integration, but this cognitive impairment was not related to the MRI findings.[14] Thus, although the general clinical experience is that patients with LGMD2I have normal cognitive function, there is evidence to suggest a mild impairment.[14,15] Patients with LGMD2M, caused by mutations in the *FKTN* gene, have been reported to have normal cognitive function and brain structure.[16,17]. A recent study of two siblings with POMT1 deficiency and a LGMD phenotype (LGMD2K) were reported to have intellectual disability and focal cortical dysplasia on brain MRI.[18] The same pattern of cognitive and structural brain involvement has been found in a cohort of five patients with LGMD2K.[19] POMT1 and POMT2 dimerize to attach the initial O-linked mannose onto α-DG. It can be argued that the mutations in POMT1 and POMT2 generally cause more severe phenotypes due to the requirement of this initial mannose for extracellular matrix anchoring. However, most of the enzymes responsible for the transfer of sugar-moieties to the initial mannose, and subsequent expanding glycan, fukutin, FKRP, POMTGNT1, LARGE, may result in CMD, muscle-eye-brain disease or Walker-Warburg Syndrome when the genes are mutated.[16,20,21] The absence of

phenotypes without some brain involvement in patients with POMT2 deficiency, suggests that minor changes to the structure of POMT2, which has nine transmembrane helices and requires N-glycosylation at multiple sites, are likely to affect function or binding to POMT1 significantly.[22,23] Cognitive function was not quantitatively assessed in our patients, due to practical difficulties in doing so across 7 centers in 6 countries. However, the cognitive impairment was evident from MMSE and the patients' inability to succeed in school. Cognitive function in our patients was not quantitatively assessed using a neuropsychological examination, due to practical difficulties in coordinating this effort across 7 centers in 6 countries. The use of the MMSE score is not validated for this cohort, but was used as an instrument to gain access to an index of cognitive function, which together with information on performance at school allowed a general judgement of the intellectual capabilities. Three of our patients had abnormal brain MRI, but with an inconsistent pattern of brain MRI involvement. The patients with MRI abnormalities were the ones with the lowest MMSE scores, suggesting a link with cognitive impairment. However, cognitive impairment was also present in patients with normal brain MRI (Table 1). Abnormal brain MRI findings could have other causes than LGMD2N. As shown in Figure 4, case 5 has frontal atrophy of the left hemisphere, which may relate to the patient's hypoxia at birth. Periventricular hyperintensities found in case 4 are often linked to cerebrovascular diseases, and this patient also had hypertension, but learning difficulties had been present since age 13 in school, which suggests that the periventricular lesions likely played no role in the cognitive function. The finding of diffuse central and cortical atrophy in case 1, a 25-year-old woman with no history of other organic diseases, is likely related directly to the LGMD2N.. Although our study is the first to suggest consistent cognitive dysfunction in a LGMD subtype, case reports of LGMD patients affected by glycosylation defects of α-DG suggest that brain abnormalities may be present in some LGMD

subtypes, especially POMT1 and POMT2. Attention to cognitive aspects should therefore be exercised when diagnosing patients with dystroglycanopathies and an LGMD phenotype.

α-DG is also glycosylated in cardiac muscle cells, which might account for the frequent occurrence of cardiac affection in CMD and LGMD caused by glycosylation defects of α-DG. Dilated cardiomyopathy has been reported in patients with mutations in the *fukutin* gene,[24,25] and a third of patients with LGMD2I develop cardiomyopathy.[26-28] Two patients with *POMT1* mutations and a LGMD phenotype have also been reported with ventricular dilatation of the heart.[29] In the present study, two cases had reduced LVEF (cases 5 and 7), suggesting that patients with LGMD2N are at risk of developing pump failure. In accordance with this, Martinez et al. recently reported three siblings with a CMD phenotype and a homozygous c.1997A>G mutation in *POMT2*, which was also present in case 3 in our study. The three siblings had reduced LVEF, dilatation of the aortic root and/or left ventricular wall motion abnormalities.[30] These and our findings suggest that regular cardiac investigations should be carried out in patients with LGMD2N.

Our study disclosed 11 new mutations in POMT2, which were either inherently pathogenic, because they were frame-shifting mutations, or predicted by various in-silico prediction tools (PolyPhen, Mutation Taster and FATHMM) to be pathogenic. No prediction was given for the mutation c.1654-5T>G in case 12, as it was predicted to be located at an extended splice site. These new mutations were all absent or extremely rare in the background population. As mutations affect wide range of sites in the *POMT2* gene, including the transmembrane helices, N-glycosylation sites and variable effects on hydrophobic/hydrophilic changes, there appears to be little resilience in the POMT2 structure before mutations become pathogenic (Table 2).

In conclusion, we demonstrate the clinical features in the first cohort of patients with LGMD2N, showing a pattern of muscle affection similar to other LGMD's, most notably LGMD2A and 2I, and a consistent cognitive affection, which has not been described as a signature feature of other LGMD types. Our study quadruples the number of cases of LGMD2N reported in the world, and therefore suggests that the condition may be more prevalent than hitherto considered.

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Legends

Table 1: Baseline characteristics of 12 patients with LGMD2N

Footnotes: ND = not done. CA = Caucasian. DK = Danish. Fr = French. Arab = Arabic. AZ = Azerbaijan. SP= Spanish. BE = Belgian. UK = British. IR = Iranian. MMSE = minimal mental status examination. FVC= forced vital capacity. CK = Creatine Kinase. a and b = siblings. * = Could not cooperate during the test. ** = Hypoxic at birth.

Moved down [1]: Mutation frequencies were estimated using Exome Aggregation Consortium (ExAC browser) with 60,706 unrelated individuals as control population.

Table 2: Mutations identified with estimated frequency in control population. Mutation frequencies were estimated using Exome Aggregation Consortium (ExAC browser) with 60,706 unrelated individuals as control population.

Moved (insertion) [1]

Footnotes: a and b = siblings

Figure 1: T1-weighted, cross-sectional MR images of muscles in six cases. Images were acquired at L4 at spine level, at pelvic level, the middle of the thighs and at the thickest part of the calves.

Commented [WDR4]: The meaning of the abbreviations is obvious, but still these are abbreviations… shouldn't we specify flx = flexion ext = extension, L = left, R = right?

Figure 2: Muscle strength evaluation by using the Medical Research Council (MRC) scale. Values 0-5, including plus and minus for 4 and 5 (4+ equals 4.33 and 5- equals 4.66). Boxplots showing the distribution of MRC-scores for each motion, including a median line.

Figure 3: Picture of case 10, showing prominent scapular winging.

Figure 4: T1-weighted brain MRI with sagittal and transverse slices showing central and cortical atrophy and mild ventricular enlargement in case 1 and frontal atrophy of left hemisphere in case 5.

Figure 5: Muscle biopsy from a healthy control and case 5, displaying myopathic features (increased internalized nuclei and increased variation of muscle fiber diameter). Staining, using α-dystroglycan glycosylation specific antibodies IIH6C and VIA4-1, demonstrates loss of glycosylation. Bar is 50µm.