VALIDITY OF HOSPITAL DISCHARGE DATA FOR IDENTIFYING CASES OF AMYOTROPHIC LATERAL SCLEROSIS

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ABSTRACT: Inpatient hospital encounters and emergency department visits were examined to identify cases of amyotrophic lateral sclerosis (ALS). The ninth edition of the International Classification of Disease, clinical modification (ICD-9-CM) for ALS was confirmed for ALS was confirmed in 93% of inpatient discharges and in 91% of emergency department visits by the diagnostic standard (chart review). Yearly prevalence rates ranged from 2.94 to 3.28 per 100,000 residents. The low calculated prevalence rates suggest that this method of case identification is inadequate and must be combined with other data sets to maximize confirmation of the clinical diagnosis.

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The ALS Registry Act, signed into law in October 2008, requires the establishment of a national registry to collect data on amyotrophic lateral sclerosis (ALS) and other motor neuron diseases (Public Law 110-373). The creation of a registry in the USA faces significant logistical challenges over those faced by well-established European registries, and it is complicated by the absence of a universal healthcare system.

Given the medical complications and interventions that occur with the progression of ALS, the hospital patient discharge uniform billing (UB) data source is important for case identification. We examined UB data for the state of South Carolina to determine the prevalence of the ALS code (335.20) according to the ninth edition of the International Classification of Disease, clinical modification (ICD-9-CM), and to give recommendations regarding the use of this data source in future registries.

METHODS

South Carolina state law mandates that all general, short-term acute care, and specialized hospitals transmit inpatient and emergency visit discharge data to the Office of Research and Statistics, South Carolina Budget and Control Board (ORS). Data adhere to uniform billing standards and meet completeness and accuracy requirements as required by the ORS.[1] To "link across" data sources the ORS has developed a series of algorithms using personal identifiers to create a global, unique identifier that enables staff to analyze the data while protecting confidentiality. This study was approved by the South Carolina Data Oversight Council and the institutional review board of the Medical University of South Carolina.

Patients with a primary or secondary motor neuron disease (MND) discharge diagnosis code (ICD-9-CM codes 335.2–335.29) were identified from all 63 South Carolina acute care hospitals during a 5-year period (2001–2005). Subjects who did not reside in South Carolina or were <18 years of age at the time of the first MND encounter were excluded from the study ($n = 17$).

Data to verify MND codes were obtained from records by trained abstractors. Inpatient records with a neurology specialty code noted in the UB data file were selected first for review, followed by the most recent admission. Emergency department records were used when inpatient records were unavailable. Abstractors collected information using standardized forms. The South Carolina Department of Health and Environmental Control, using its legislative authority to review medical records, conducted this phase of the study on behalf of the ORS. A chart diagnosis of ALS required the presence of the diagnosis of ALS in the provider notes available for review.

Annual prevalence ratios of the ICD-9-CM code for ALS in inpatient and emergency department services were calculated for South Carolina using yearly population estimates.[2]

RESULTS

The 5-year study period included over 2.2 million and 5.3 million inpatient discharges and emergency department visits, respectively. The inpatient discharges included 791 encounters with the MND codes (465 unique patients). Five hundred sixty-

Abbreviations: ALS, amyotrophic lateral sclerosis; ED, emergency department; ICD-9-CM, International Classification of Diseases, 9th revision, clinical modification; MND, motor neuron disease; ORS, Office of Research and Statistics; PPV, positive predictive value; UB, uniform billing

Key words: amyotrophic lateral sclerosis, case identification, epidemiology, hospital discharge data, prevalence, registry

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Table 1. Annual prevalence ratios based on ALS code (335.20) found in UB data source*

Study year	Number of unique cases with ALS code	SC population (18 and older)	Prevalence ratio (per 100,000 residents)
2001	94	3,046,170	3.09
2002	97	3,084,193	3.15
2003	101	3,125,625	3.23
2004	104	3,172,939	3.28
2005	95	3,227,881	2.94

*Excluded non—South Carolina residents and patients <18 years of age at time of first motor neuron disease encounter. Occurrences of a primary or secondary diagnosis code for ALS were unduplicated by study year and patient's unique identifier. Annual prevalence ratio = (number of unique cases with ALS code per year / South Carolina population 18 and older) × 100,000.

seven encounters included the ALS code (335.20) (309 unique patients). The emergency department encounters consisted of 238 visits with an MND code present in 160 unique patients, 200 of which included the ALS code (133 unique patients). After combining two data sources and removing multiple encounters, there were 369 unduplicated patients with the ALS code. Of the 369 unique patients coded for ALS, records with the ALS diagnosis code were available for abstraction for 336 (91%) patients.

Of the 283 inpatient records reviewed, a chart diagnosis of ALS was noted for 262 patients (93%). Of the 21 cases in which the medical record did not agree with the coded diagnosis, 5 were obvious miscodes. The other records included a variety of neurological conditions, such as multiple sclerosis, neuropathy, progressive supranuclear palsy, spinal stenosis, and spinal tumor.

An additional 53 ED records were reviewed in cases where an inpatient record was not available. Medical record review returned a chart diagnosis of ALS for 48 patients (91%).

Of the 101 inpatient hospital and emergency department visit records with the ALS code cited as the primary diagnosis in UB data files, record review supported a chart diagnosis of ALS in 98 (97%) reviews. Medical record review supported a chart diagnosis of ALS in 210 of the 235 (89%) reviews when the ALS code was listed as a secondary diagnosis.

Annual prevalence ratios for code 335.20 calculated for each year of the study ranged from 2.94 to 3.28 per 100,000 residents of South Carolina (Table 1).

DISCUSSION

The multiple medical complications in patients with ALS and the high frequency of hospitalizations and emergency department admissions suggest that discharge data could be a valuable source for case identification.[3]

Prior evaluations of the accuracy of ICD-9 codes from hospital discharge data in identifying ALS cases have had variable conclusions. In an evaluation from Piemonte, Italy, a positive predictive value (PPV) of 39% was determined when both primary and secondary diagnoses were considered.[4] In the Lombardy region of Italy, a PPV of 70% was found when only primary diagnoses from the discharge data were considered.[5]

The proportion of people assigned ICD-9-CM code 335.20 in the hospital billing dataset who had ALS was higher in our study compared with previous evaluations. This likely resulted from the use of the five-digit code that resulted in more accurate case identification and the methods used to validate cases. By using ICD-9-CM codes as a mechanism to screen patients, the National Registry of Veterans with ALS also showed a higher rate of case verification when a five-digit (335.20) code was used.[6] We found that the clinical data in the hospital records were limited, and case confirmation using the revised El Escorial criteria was possible in <20% of the charts reviewed, necessitating the use of the chart diagnosis to confirm cases.[7]

The calculated prevalence ratios are lower when compared with another North American study from the 1990s and two European studies of ALS.[8–10] The lower values are likely secondary to the incomplete nature of case identification and underrepresentation of early cases of ALS using the single UB data source, raising concerns about the validity of this single source to identify cases. As a result, our prevalence ratios should be interpreted as minimum prevalence ratios, because we would not have captured non-admitted patients.

Although these results illustrate that UB data represent a potentially valuable source for public health surveillance for ALS, this study also demonstrates the limitations of the use of hospital discharge data as a single source to identify individuals with ALS. Thirty-nine and 25 states actively collect all-payer, encounter-level UB discharge data for the databases of the Healthcare Cost and Utilization Project State Inpatient and Emergency Department, respectively, and our methods could not immediately be applied at the national level.[11] We have demonstrated that an ICD-9 code from UB data has a high probability of identifying an ALS patient, but we also found that patients can be missed. Therefore, algorithms for case identification must combine UB data with other data sources to maximize case finding and diagnosis confirmation in future ALS registries in the USA. The delays in the release of data sets limit our ability to capture cases early in the disease course so that

the primary utility of the data will be for epidemiological studies.

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MUSCLE METABOLIC ALTERATIONS ASSESSED BY 31-PHOSPHORUS MAGNETIC RESONANCE SPECTROSCOPY IN MILD BECKER MUSCULAR DYSTROPHY

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ABSTRACT: Although the molecular defect causing Becker muscular dystrophy (BMD) has been identified, the biochemical mechanisms that lead to muscle necrosis remain unclear. Exercise-related muscle metabolism in 9 mildly affected BMD patients was assessed by muscle 31-phosphorus magnetic resonance spectroscopy (^{31}P MRS) during an incremental workload. Compared with normal controls, BMD patients showed deregulation of resting pH and intramuscular membrane breakdown. We also observed increased reliance upon anaerobic metabolism during sustained submaximal contraction and maintenance of oxidative function during recovery.

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Despite current knowledge regarding the molecular defect causing Duchenne and Becker muscle muscular dystrophy (DMD, BMD), the cellular mechanism in which the lack of dystrophin triggers skeletal muscle dysfunction and ultimately leads to muscle necrosis is still unclear, and available therapies are consequently inadequate.

A better knowledge of the muscle metabolic changes that accompany exercise in DMD/BMD can be useful in understanding the role energy utilization plays in contractile insufficiency and pathogenetic mechanisms and the underlying dystrophinopathies.

31-Phosphorus magnetic resonance spectroscopy (^{31}P MRS) is a non-invasive tool to investigate skeletal muscle metabolism in different physiological conditions (rest, exercise, and recovery) by measuring high-energy phosphate metabolites such as adenosine triphosphate (ATP), phosphocreatine (PCr), inorganic phosphate (Pi), phosphodiesters (PDE), phosphomonoesters (PME), and cytosolic pH.[1,2]

^{31}P MRS has been used in studies of DMD/BMD patients. It has been mainly used to study relative intracellular alkalosis at rest, similar to other muscular dystrophies.[3–9] It has also shown an increased Pi/PCr ratio and, in some cases, increased [PDE], possibly due to sarcolemmal breakdown.[5,8] During exercise, both a premature drop in PCr and reduced acidosis have been described in BMD.[4,6]

Abbreviations: ^{31}P MRS, 31-phosphorus magnetic resonance spectroscopy; ADP, adenosine diphosphate; ATP, adenosine triphosphate; BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophies; E, proton efflux rate; MRC, Medical Research Council; MVC, maximal voluntary contraction; PCr, phosphocreatine; $PCr_{end\text{-}ex}$, the concentration of PCr at the end of exercise; $PCr_{end\text{-}ex}$ (%), the residual PCr in percentage at the end of exercise, relative to its value at rest; PDE phosphodiesters; PME phosphomonoesters; $pH_{end\text{-}ex}$, the pH at the end of the exercise; pH_{min}, the minimum pH observed during recovery; Pi, inorganic phosphate

Key words: 31-phosphorus magnetic resonance spectroscopy, Becker muscular dystrophy, exercising muscle, muscular energy metabolism, phosphocreatine

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The present studies were performed in clinically heterogeneous patients who exhibited mild to advanced stages of the disease. Our aim was to analyze mildly affected BMD patients with a ^{31}P MRS exercise protocol in order to investigate metabolic abnormalities detectable in the early stages of disease, when fiber loss and fibrosis are still limited.

METHODS

We selected 9 patients (median age 30 years, range 24–44 years) diagnosed with BMD based on clinical, muscle biopsy, and genetic findings. They were ambulant and mildly impaired (grades I–II on the Vignos scale[10]). None had a history of exercise-related myoglobinuria or muscle cramps. ^{31}P MRS data of the patients were compared with those obtained in 10 age-matched, healthy male volunteers (median age 30 years, range 24–35 years).

All subjects performed an incremental workload exercise test consisting of isometric intermittent plantar flexions of the dominant leg through an MR-compatible ergometer.[11] Test normalization was obtained with reference to individual isometric maximal voluntary contraction (MVC), a valuable force indicator in contracting muscles.[12–14] Near MVC supramaximality was assured during enrollment sessions by using the superimposed twitch elicitation technique. The in-magnet exercise protocol consisted of an incremental workload starting from 20% of the mean MVC (re-measured at rest 1 hour before the examination) and progressively increased by 10% MVC every 30 seconds until the subject's exhaustion.

^{31}P spectra were acquired using a 1.5-T MR system (Signa LX; GE Healthcare) through an 8-cm transmit/receive spectroscopy ^{31}P surface coil wrapped around the calf muscle with a short-echo-time (TE = 2.5 ms), slice-selective spin–echo sequence.[15] Signals were treated using jMRUI[16] and quantified in the time domain by AMARES.[17] Metabolite concentrations were referenced to an adenosine triphosphate (ATP) signal assumed to be constant and equal to 8.2 mmol/L of tissue water. Intracellular pH, free adenosine diphosphate (ADP) concentration at rest, proton efflux rate (E) at the start of recovery,[18] and initial PCr recovery rate (V) were also calculated.[1,2,19]

Statistical analysis was performed by independent-sample *t*-test between BMD and normal groups. The relationships between variables were studied using a linear-regression analysis and expressed by the Pearson correlation coefficient.

RESULTS

At rest, BMD patients had a significantly ($P = 0.008$) higher pH compared with controls and no significant difference in any phosphate metabolites except for a slight ($P = 0.061$) increase in phosphodiesters (PDE).

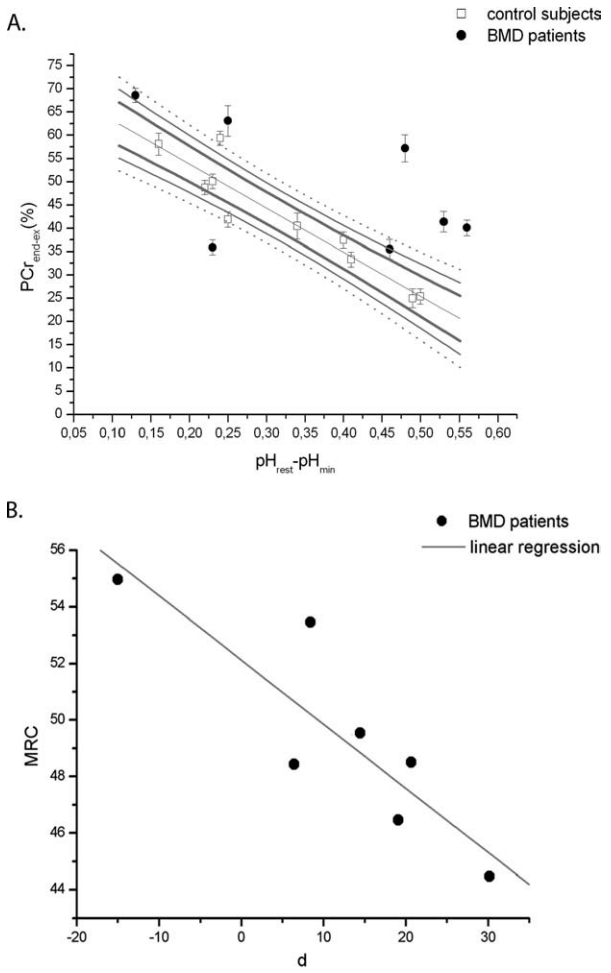

FIGURE 1. (**A**) Residual PCr at the end of exercise in percentage with respect to that at rest, $PCr_{end\text{-}ex}$ (%), versus the acidification index, ΔpH, defined as the difference between the basal pH (pH_{rest}) and the minimum pH (pH_{min}). All patients (filled circles) were outside the prediction interval characteristic of healthy subjects (open squares) calculated at 68% and corresponding to 1 standard deviation (thick gray line). The thin gray line and dotted line correspond to a prediction interval calculated at 1.5 and 2 standard deviations, respectively. (**B**) Linear correlation between the MRC score and the exercise efficiency index, *d*, obtained as the Euclidean distance between each point representing the patient metabolic status at the end of exercise and the best-fit line of the healthy control data of (**A**) ($R = -0.875$, $P = 0.010$). Arbitrary positive or negative values have been assigned to each exercise efficiency index *d* according to whether it was lying above or below the best-fit line in (**A**).

Two of the 9 patients were not able to complete the exercise, as they tired too quickly and were excluded. The remaining 7 patients completed the exercise with a mean workload of 64% MVC (controls 66%), indicating an exercise duration comparable to that of controls (159 ± 11 vs. 178 ± 9 seconds). No significant differences were found in mean values of metabolic variables at the end of exercise, including $PCr_{end\text{-}ex}$ [mM], its percentage residual relative to rest value $PCr_{end\text{-}ex}$ (%), $pH_{end\text{-}ex}$, and the minimum value pH_{min},

recorded in the first minute of the recovery after exercise as a more stable parameter when detected in relaxing muscle. The acidification index, ΔpH ($\Delta pH = pH_{rest} - pH_{min}$), that is, the difference between basal pH and the minimum attained pH, although not statistically different, when plotted with $PCr_{end\text{-}ex}$ (%), showed a different metabolic profile compared with controls (Fig. 1A). All patients lay far outside the 68% (corresponding to 1 SD) predicted interval characteristic of controls. Two of them were between 87% and 95% (1.5 and 2 SDs, respectively), whereas all the others exceeded the 95th percentile. All but 1 patient exhibited lower PCr consumption for a normal acidification index. Only 1 patient, who had a 45–51 deletion and very mild features (grade I on Vignos scale, MRC 55/55, MVC in gastrocnemius muscle = 326 N), showed a lower index of acidification and a higher PCr consumption.

To express metabolic exercise efficiency an "exercise efficiency index," *d*, was determined as the point-by-point Euclidean distance between single-patient exercise metabolic status at the end of exercise, as defined previously, and the best-fit line of controls. A significant linear correlation ($R = -0.875$, $P = 0.010$) was found between Medical Research Council (MRC) score and index *d* (Fig. 1B). A negative *d* value was exhibited again in the case described earlier.

No difference in mean values of initial rate of PCr recovery (V) was found in controls, whereas the rate of proton efflux, E, at the start of recovery was slightly reduced in BMD patients ($P = 0.064$).

DISCUSSION

Similar to findings from previous investigations of exercise-related metabolic profiles in DMD or more severe BMD patients,[4–6] we found an intracellular pH higher than in controls at rest. The meaning of this cytosolic alkalosis is not clear, but factors linked to alterations in sarcolemmal proton efflux mechanisms, also under rest conditions, may play a role.[20]

In contrast to the high intracellular Pi level reported at rest with PCr loss,[5,7,8] which is interpreted as ensuing from secondary mitochondrial dysfunction in muscle fibers undergoing necrosis, our study revealed normal Pi and PCr levels. This is consistent with the mild degree of muscle involvement in our patients.

Moreover, as in other muscular dystrophies,[9] we found a slight increase in the PDE signal, which results from different precursor compounds or degradation products of membrane phospholipids. It could represent a precocious catabolic marker of membrane damage.

At the end of the exercise all but 1 patient showed a reduced level of PCr consumption for the degree of acidification as compared with controls, and only 1 exhibited, as has been cited in the literature,[6] an inverse profile. A possible explanation for this metabolic pattern in our BMD patients could be the presence of defective consumption of PCr in concomitance with excessive activation of lactate production during incremental exercise. A number of mechanisms that interfere with intracellular H^+ handling in exercising muscles can explain this result, ranging from the sarcolemmal Na^+/H^+ exchange system and proton homeostasis impairment[21] to altered nitric oxide–mediated blood terminal flow regulation and anticipated shift to anaerobic ATP synthesis during incremental exercise.[22–24] The slight reduction of proton efflux rate at the start of recovery could confirm this latter hypothesis. Relative preservation of contractile performance of skeletal muscles in mild BMD patients could then match with a greater reliance upon anaerobic metabolism in order to maintain some metabolic exercise efficiency (see "index *d*"). This mechanism differs from what happens in the advanced state of disease, when fiber necrosis and interstitial fibrosis dramatically reduce muscle force generation.

No difference in PCr recovery was noted between patients and controls, which confirms that aerobic metabolism is not impaired in this phase. This finding has already been reported in DMD carriers[3] and sarcoglycanopathies.[9]

In conclusion, ^{31}P MRS muscle abnormalities of mildly affected BMD patients appear to set off a number of events mainly related to impairment of sarcolemmal function, rather than to insufficiency of the contractile machinery. It remains to be elucidated the extent to which these events are linked to pathogenic alterations of the disease—that is, the alteration of the dystrophin–sarcoglycan subsarcolemmal complex—or indicate an adaptive response of the muscle.

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FOUR NOVEL POINT MUTATIONS IN THE PMP22 GENE WITH PHENOTYPES OF HNPP AND DEJERINE–SOTTAS NEUROPATHY

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ABSTRACT: We report four novel point mutations in the *PMP22* gene with two different phenotypes: mutation p.Ser79Thr arose de novo in a patient with the Dejerine–Sottas neuropathy (DSN) phenotype; and mutations c.78+5 G>A, c.320-1 G>C, and p.Trp140Stop segregated with HNPP in 5 families. Our findings show that point mutations in *PMP22* may be more likely in HNPP patients than in CMT1 patients after exclusion of CMT1A/HNPP.

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Abbreviations: CMT, Charcot–Marie–Tooth neuropathy; DSS, Dejerine–Sottas neuropathy; HNPP, hereditary neuropathy with liability to pressure palsies; PCR, polymerase chain reaction
Key words: Charcot–Marie–Tooth neuropathy, CMT1A, Dejerine–Sottas neuropathy, hereditary neuropathy with liability to pressure palsies, PMP22 gene
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The most frequent type of hereditary neuropathy, Charcot–Marie–Tooth (CMT) disease type 1A (CMT1A), is caused by a duplication containing the *PMP22* gene on chromosome 17.[1] Hereditary neuropathy with liability to pressure palsies (HNPP) is caused by a deletion of the same region.[2] HNPP is the mildest form of hereditary neuropathy, where patients typically suffer from recurrent peripheral paresis after minor pressure trauma to the peripheral nerves, most frequently in the upper limbs. The most affected are the nerves at points of anatomical narrowing.[3] Some HNPP deletion carriers may remain clinically asymptomatic, but nerve conduction studies usually show typical abnormalities.

Table 1. Summary of four novel *PMP22* mutations.

Family/patient	Nucleotide change	Amino acid change/ protein domain	Position	Phenotype
1	c.235 T>A	p.Ser79Thr/transmembrane 2	Exon 3	DSN
2	c.78+5 G>A	5′ splice-site	Intron 1	HNPP
3	c.78+5 G>A	5′ splice-site	Intron 1	HNPP
4	c.320-1 G>C	3′ splice-site	Intron 4	HNPP
5	c.320-1 G>C	3′ splice-site	Intron 4	HNPP
6	c.419 G>A	p.Trp140Stop/transmembrane 4	Exon 4	HNPP

In 84% of HNPP patients, a deletion of the region on chromosome 17p is found.[4] Point mutations in the *PMP22* gene are another rare cause of HNPP, CMT1A, or Dejerine–Sottas neuropathy (DSN).[5,6] DSN is a severe form of demyelinating neuropathy with onset in infancy, frequently delayed early motor milestones, and severely reduced nerve conduction velocities.[5]

We report four novel point mutations in the *PMP22* gene and its associated phenotypes. Three of the mutations were found in HNPP families and predict loss of one functional allele, and one mutation arose de novo in a patient with DSN.

METHODS

The new mutations were detected during routine DNA diagnostic testing in our laboratory. The CMT1A duplication/HNPP deletion was previously excluded in all patients using a set of 17 microsatellite markers.[7] All patients signed informed consent for analysis of hereditary neuropathy–related genes.

When a novel mutation was detected, all available family members were tested for segregation of the mutation with the phenotype.

All four coding exons of the gene *PMP22* were amplified with polymerase chain reaction (PCR) using a set of four pairs of primers (sequences available upon request). Direct sequencing was performed with a sequencing kit (BigDye Terminator v3.1) and analyzed on a genetic analyzer (ABI 3130; Applied Biosystems). HGVS nomenclature and reference sequence NM_153322.1 were used for assigning the mutations. Mutations were analyzed in silico to support their pathogenicity. The intron mutations were tested with NetGene2[8] and ExonScan,[9–11] and the exon mutations were tested with MutationTaster[12] (the *P*-value is the probability of the prediction, a value close to 1 indicates a high "security" of the prediction). A set of 17 microsatellite markers were used to test the parentity of the de novo mutation.

RESULTS

The summary of four novel *PMP22* mutations is shown in Table 1. Three mutations were found in 5 families with the HNPP phenotype, which were compatible with autosomal dominant inheritance. The one de novo mutation caused Dejerine–Sottas-type neuropathy.

Mutation p.Ser79Thr was found in patient 1, a boy who was the product of in vitro fertilization in which the pregnancy and delivery were normal. Early motor milestones were delayed from birth; he did not walk independently until the age of 25 months. The patient was examined at the age of 3 years and had general hypotonia and absent tendon reflexes. His walking was insecure, and he had mild bilateral pes planovalgus. Nerve conduction velocities in the upper limbs were severely reduced to 3 m/s (see Supplementary Material, Table S1). The patient's fraternal twin brother is healthy. A mutation was not found in the patient's parents or in his twin brother and was thus compatible with a de novo origin.

Mutation c.78+5 G>A was found in 2 families (families 2 and 3). Patient 2 presented with right footdrop diagnosed as fibular nerve palsy at the age of 14 years. At age 15 he had recurrent episodes of numbness in an ulnar nerve distribution on the left and radial nerve distribution on the right. The patient's mother (2a) and aunt suffered from recurrent episodes of weakness with numbness of the upper limbs after exertion. Examination of the patient's sister (2b) also showed evidence of demyelinating neuropathy, but clinically she did not report any motor or sensory problems at the age of 19 years and had never experienced any episodes of weakness or numbness.

Patient 3 was examined at 51 years of age. He first reported problems at age 18 with transient weakness of the right wrist. Since then he suffered several further episodes of transient weakness of the upper extremities. His daughters (3a and 3b) have signs of HNPP on nerve conduction studies, but clinically neither reported any episodes of weakness or numbness at age 20 and 22 years.

Another splice-site mutation, c.320-1 G>C, was found in families 4 and 5.

Patient 4 had only one episode of transient peripheral mononeuropathy, with paresthesias affecting both wrists at 54 years of age. Her daughter (4a), 28 years old, has the same mutation, and her electrophysiological examination supports HNPP neuropathy, but she has not yet shown clinical evidence of mononeuropathy.

Patient 5 had transient weakness and numbness of the upper limbs at 18 years of age. His daughter

(5a), age 24 years, suffers from paresthesias of the upper limbs after exertion. His son (5b) suffered bilateral fibular nerve palsy after surgery at 13 years of age. Now, at 26 years of age, he reports paresthesias of the lower and upper limbs after prolonged pressure stimulus.

Mutation p.Trp140Stop was found in patient 6 who has suffered from recurrent peripheral paresthesias since childhood but without weakness. The parents are, by history, clinically healthy, but they were not available for testing.

Nerve conduction studies in patients with the HNPP phenotype show primary evidence for demyelinating motor and sensory peripheral neuropathy with the most affected sensory nerves in the upper limbs and signs of predominant slowing over compression sites (see Table S1).

In silico analysis of all mutations supports their pathogenic character. The p.Ser79Thr mutation revealed high probability ($P = 0.989$) for being disease-causing. For p.Trp140Stop there is a high probability ($P = 0.999$) of truncation of the PMP22 protein (−21 amino acids) according to MutationTaster.[12] The intron mutations abolish the donor (intron 1) and acceptor (intron 4) sites for the splicing compared with the wild-type sequence, where the donor and acceptor sites are recognized with high confidence by NetGene2[8] and ExonScan.[9–11]

DISCUSSION

We have reported three mutations that predict a loss of one functional copy of *PMP22* and are very likely causal for the HNPP phenotype in 5 families, and one mutation that predicts an amino acid substitution p.Ser79Thr, which arose de novo and is responsible for the DSN phenotype.

We suggest that the splice-site mutations c.78+5 G>A and c.320-1 G>C alter the normal splicing of *PMP22* by exon skipping, intron retention, or by use of the alternative or cryptic splice site. Abnormal splicing will lead to a non-functional protein. The causality of both these mutations is supported by the segregation of each mutation with the HNPP phenotype in 2 families. Families 2 and 3 have the same mutation, as do families 4 and 5. They probably have a common ancestor, but we were not able to shed further light on this possibility.

Only three splice-site mutations with the HNPP phenotype have been reported to date. mRNA analysis of leukocytes from patients with the c.78+1 G>T mutation suggests that exon 1 is skipped, and the mutant allele is functionally a null allele.[13] The second is the c.179-1 G>C mutation, which probably leads to skipping of exon 3, or alters the splicing by use of a cryptic splice site.[14] The third c.179+1 G>C mutation affects the splicing by partial retention of intron 2 and produces the abnormal mRNA.[15]

The nonsense mutation, p.Trp140Stop, was found only in the proband, but the character of the mutation (a stop mutation) and in silico analysis support its pathogenicity and causality for HNPP. The premature stop codon will lead to a truncated protein that is probably degraded.

Two different mutations at p.Ser79 were previously reported.[16,17] The p.Ser79Pro mutation was found in a girl with delayed walking and nerve conduction velocities <10 m/s in median and tibial nerves. Mutation p.Ser79Cys was reported in a family with the CMT1A phenotype, where the first clinical symptoms appeared at 10 years of age. These findings support the pathological character of the p.Ser79Thr mutation. Codon 79 is clearly a mutation hotspot in the *PMP22* gene.

Point mutations in the *PMP22* gene are generally rare in CMT1 patients, but our experience shows that it is more likely that they will be detected in suspected HNPP patients than in CMT1 patients after exclusion of CMT1A/HNPP. From 108 unrelated patients sequenced for mutations in *PMP22* in our laboratory, only one mutation was found in a group of 51 patients with suspected CMT1, whereas five mutations were found among 57 patients suspected to have HNPP. Therefore, *PMP22* gene sequencing may be more useful in HNPP patients without the HNPP deletion than in CMT1 patients without the CMT1A duplication, where mutations in other genes, including *GJB1*, *MPZ*, or *SH3TC2*, are the more likely cause of CMT1.

Our findings further highlight the importance of splice-site changes and intronic mutations in DNA diagnostic of HNPP patients.

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BECKER MUSCULAR DYSTROPHY DUE TO AN INVERSION OF EXONS 23 AND 24 OF THE DMD GENE

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ABSTRACT: The use of hybridization-based methods for Duchenne muscular dystrophy (DMD) mutation analysis is increasingly common. We report a case of Becker muscular dystrophy in which discrepant results between a polymerase chain reaction (PCR)-based single-condition amplification/internal primer (SCAIP) and a comparative genomic hybridization assay incompletely characterized the mutation (an inversion of exons 23 and 24). These results demonstrate the limits of sensitivity and specificity of both tests, and highlight the need for more detailed analysis when intronic deletions are detected by comparative genome hybridization methods.

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Becker muscular dystrophy (BMD) and the more severe Duchenne muscular dystrophy (DMD) are both caused by mutations in the *DMD* gene that can be detected in genomic DNA samples in 93–96% of cases.[1,2] Approximately 65% of *DMD* mutations are deletions of one or more exons, and another 5% are exonic duplications that require methods for detecting exon dose. Most non-deletion/non-duplication mutations are point mutations, including nonsense mutations, subexonic frameshifting insertions and deletions, and rarely missense mutations.[3,4]

For many years, the standard diagnostic test utilized multiplex polymerase chain reaction (PCR) of a limited number of exons within deletion "hotspots," where 98% of deletions occur.[5] Relatively inexpensive and reliable for detection of deletions in hemizygous males, the multiplex PCR test has been largely replaced by methods that interrogate all exons and also readily allow dose measurements in female carriers. These include multiplex ligation-dependent probe amplification (MLPA) that detects exon copynumber,[6] and comparative genomic hybridization (CGH) arrays that may also detect copynumber changes in non-exonic regions.[7–9] Meanwhile, detection of point mutations has become feasible through the routine use of direct sequencing methods from genomic template DNA[3] or chip-based resequencing. Sequencing of cDNA derived from muscle mRNA remains useful for detecting all mutation classes and for validating the implications of certain mutations detected at the genome level (such as putative splice site mutations). In addition, cDNA sequencing is necessary for detecting the small percentage of mutations, such as deep intronic point mutations, that are not readily detectable with current genomic screening methods but result in inclusion of the intronic sequence as pseudoexons within the mRNA transcript.[10]

Herein we report the characterization of an unusual *DMD* mutation that led to discrepant results between PCR-based and comparative genomic hybridization (CGH) array–based diagnostic methods. Both methods were performed accurately, and their combined results gave a clue to the presence

Abbreviations: BMD, Becker muscular dystrophy; CGH, comparative genomic hybridization; DMD, Duchenne muscular dystrophy; MLPA, multiplex ligation-dependent probe amplification; NAHR, non-allelic homologous recombination; NHEJ, non-homologous end-joining; PCR, polymerase chain reaction; SCAIP, single-condition amplification/internal primer; UDP, United Dystrophinopathy Project

Key words: Becker muscular dystrophy, CGH, inversion mutation, MLPA, SCAIP

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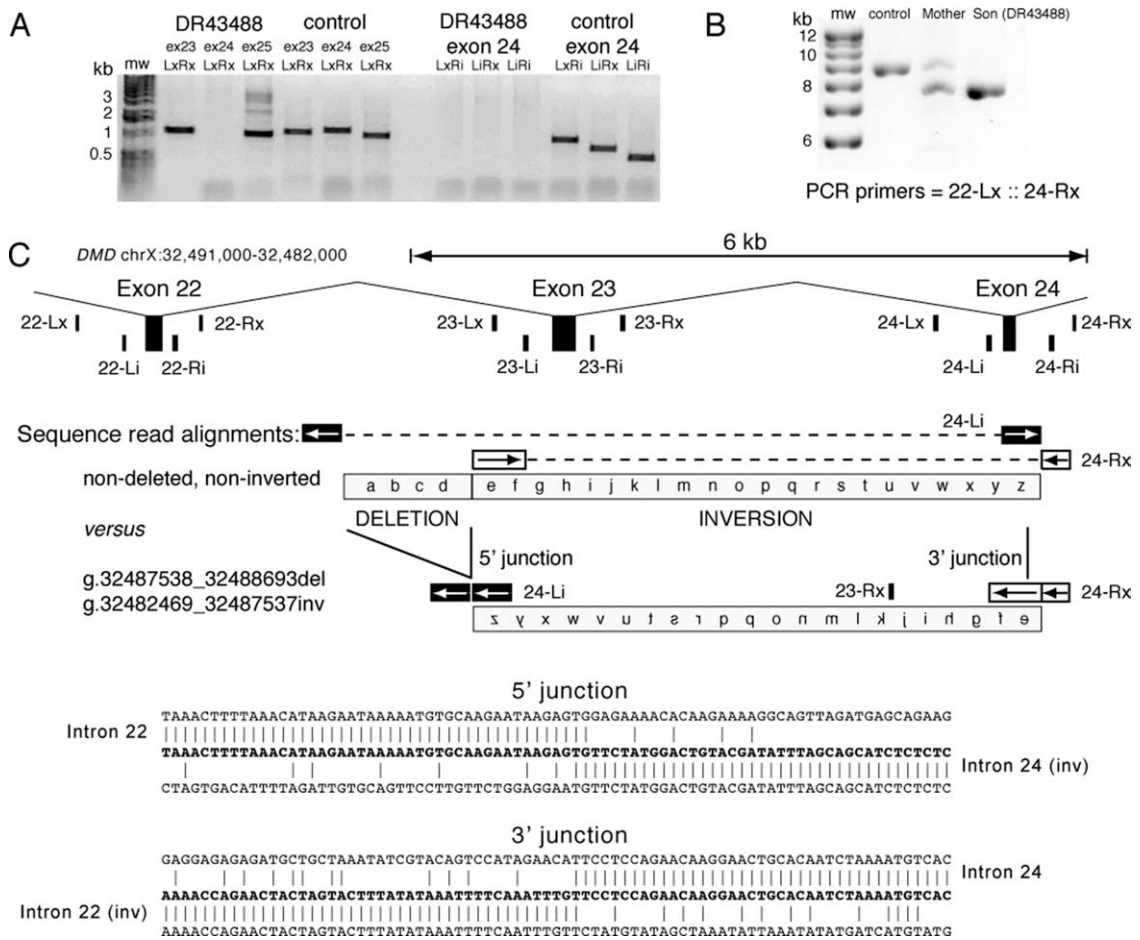

FIGURE 1. PCR and sequencing confirmation of the *DMD* deletion/inversion mutation. (**A**) PCR amplicons from SCAIP deletion analysis of exons 23, 24, and 25 in the proband (DR43488) and a normal control. (**B**) PCR amplicons using primers 22-Lx and exon 24-Rx for a normal control, the mother of DR43488, and patient DR43488. (**C**) Locations of PCR/sequencing primers in a 9-kb interval of the *DMD* gene (coordinates chrX: 32,491,000–32,482,000), including exons 22, 23, and 24. Diagrammatic alignment of sequence reads generated from primers 24-Li and 24-Rx on normal versus inverted exon 23 to exon 24 segments. The breakpoint sequence from the 22-Lx::24-Rx amplicon from patient DR43488 defines the 5′ junction (sequence from 24-Li primer) and 3′ junction (sequence from 24-Rx primer).

of the actual mutation, an inversion of exons 23 and 24 within the gene.

CASE REPORT

The patient was initially found to have an incidental elevation of serum transaminases that led to a gastroenterological evaluation around 18 months of age. By 5 years of age, he was found to have an elevated serum creatine kinase (1035 IU/L by report; exact upper limit for the lab used is unknown). At age 12 (his most recent examination), he demonstrated myalgias with exertion and decreased stamina compared with peers, but only minimal limb-girdle weakness (modified Medical Research Council grade 5⁻/5 in hip flexors and deltoids, with strength otherwise 5/5).

At age 5 years he underwent a clinical diagnostic *DMD* mutation analysis that included multiplex PCR and Southern blot analysis using eight cDNA probes covering the *DMD* coding region. The clinical report noted that: (1) Southern blot with probe 30-2 (covering exons 20–30) revealed a band shift of the HindIII fragment that normally contains exons 22–25 to a position that corresponds with a slightly lower molecular weight; and (2) exons 22–25 were not included in the multiplex PCR reaction.

Based on the clinical features and this result, the patient (DR43488) was eligible for enrollment in the United Dystrophinopathy Project (UDP; http://dystrophy.genetics.utah.edu) database. Under an institutional review board–approved protocol and following informed consent, his initial testing in our laboratory was performed under the UDP protocol using the SCAIP (single-condition amplification/internal primer) method, as described elsewhere.[3] Amplification

of all exons (as the first step in SCAIP analysis[3]) from a genomic DNA template revealed the absence of an amplification product for exon 24 alone. As per standard laboratory protocol, amplification was repeated using alternate internal primer sets flanking exon 24 in order to exclude the possibility of polymorphisms at the primer hybridization site. No product was seen by agarose gel electrophoresis using the standard (LxRx) or three alternate exon 24 primer configurations (Fig. 1A).

In order to reconfirm the results, and to ascertain the mother's carrier status, genomic DNA samples from the boy and his mother were tested at a clinical laboratory using a *DMD* oligonucleotide CGH array.[9] This revealed a normal gene dose signal for all exons, including exon 24, in both the mother and the patient. However, it revealed a deletion in intron 22 in a heterozygous fashion in the mother, and in a hemizygous fashion in the patient. This 1.1-kb deletion included nucleotides 32,487,572–32,488,656 of the genomic sequence (chrX reference sequence from GRCh37 assembly); this intron 22 segment is found approximately 1.6 kb from the 3′ end of exon 22, and 0.7 kb from the 5′ end of exon 23.

We presumed that both results were performed in a technically correct fashion and postulated that a complex mutation comprised of an inversion of exons 23–24 and an intron 22 deletion could account for both results. Repeat blood samples were obtained, and another round of diagnostic testing was therefore undertaken in our laboratory. MLPA analysis[11] revealed the presence of both exons 23 and 24 in the patient and his mother, with a normal dose for all *DMD* exons. PCR amplification with primers 23-Rx and 24-Rx resulted in a 1.6-kb amplification product that would be expected only in the setting of an inversion (data not shown). We then undertook long-range PCR (Roche) using the manufacturer's recommended conditions with primers flanking exon 22 (22-Lx) and exon 24 (24-Rx), and successfully amplified a band of approximately 8 kb from DNA from both the boy and his mother, and a band of approximately 9 kb from a wild-type control and the boy's mother (see Fig. 1B).

Sequencing of this ∽8-kb PCR product from the patient revealed an inversion of exon 23 through exon 24 associated with the intron 22 deletion breakpoint (Fig. 1C and D). The 5′ inversion breakpoint is at the site of the 1.16-kb deletion first detected by CGH array within intron 22. The 3′ inversion breakpoint is located within intron 24 between the splice donor site of exon 24 and the location of the internal PCR primer site (24-Ri). The sequence generated from the 24-Li primer aligns with intron 23, exon 24, and intron 24 (196 nt to nucleotide 32,482,469) before aligning with intron 22 at the 5′ junction of the deletion (nucleotide 32,488,694). The sequence generated from the 24-Rx primer begins in intron 24, aligning through to nucleotide 32,482,468 and then continues aligning in intron 22 beginning at nucleotide 32,487,537. These alignments are consistent with a complex deletion/inversion: intron 22 deletion (g.32487538_32488693del, 1.16 kb) plus an exon 23–24 inversion (g.32482469_32487537inv, 5.07 kb).

DISCUSSION

These results show limitations inherent in CGH, MLPA, and SCAIP methods, which are in widespread clinical use. The parsimonious explanation for the discrepant results from CGH and SCAIP testing was an inversion, and an amplification using predicted primer pairs resulted in the confirmation of that explanation and characterization of the breakpoints. The reported absence of exon 24 by SCAIP is explainable by the location of the 3′ breakpoint between exon 24 and the flanking PCR primer site (24-Ri).

The 5′ deletion/inversion junction occurs in unique sequence, whereas the 3′ inversion junction occurs within a 235 nt DNA transposon-like medium reiterated frequency repeat element (MER20) located in intron 24. The nucleotides encompassing the 1.16-kb deletion are derived from intron 22, with no additional insertion of nucleotides at either the 5′ or 3′ inversion junction. These breakpoint sequence features suggest that the mechanism underlying this rearrangement was non-homologous end-joining (NHEJ) and not a non-allelic homologous recombination (NAHR). *DMD* deletions account for ∽65% of mutations, and studies involving sequencing of dozens of deletion breakpoints have shown that NHEJ appears to be the major mechanism, often with the breakpoint junction exhibiting a few nucleotides of homology between 5′ and 3′ flanking sequences or the addition of a few extraneous bases.[12,13] A sequenced non-recurrent deletion breakpoint involving the 17p11.2 Smith–Magenis syndrome region involved a MER5B element, and it was suggested that double-strand break substrates within low-density MER elements may not be able to be repaired by homologous recombination in a timely manner, thus leading to error-prone NHEJ.[14] The *DMD* exon 23–24 inversion/deletion observed herein may be another example of a complex rearrangement involving the NHEJ pathway.

This case—only the fifth intragenic *DMD* inversion in the literature[15–18]—provides a cautionary note with regard to the interpretation of any individual gene dose test. Both MLPA and CGH revealed a normal exon dose. In our standard diagnostic algorithm, the absence of a detectable mutation by gene dose and SCAIP methods leads us to recommend muscle biopsy to determine mRNA structure. In this case, a biopsy had not been performed as part of the diagnostic work-up; furthermore, even after recontacting the patient and his family, we were unable to obtain tissue for mRNA or protein analysis, as they declined any tissue sampling whatsoever. This refusal included skin biopsy, from which fibroblast cultures could have been established for MyoD-forced myogenesis and subsequent DMD expression; this option is less invasive than muscle biopsy yet still allows reliable characterization of *DMD* mRNA structure. Nevertheless, we would predict that the mRNA contains an in-frame deletion of exons 23 and 24, which would be consistent with the patient's relatively mild clinical course. Such a mild course suggests that these exons encode protein regions of limited significance, and that exon skipping of small mutations in this region may prove beneficial. As in all of the previously reported cases, the inversion mutation was flanked by a deletion, in this case the smallest (1.16 kb) yet reported; this pattern suggests that the deletion event is intrinsic to the formation of the inversion. We emphasize that the report of a novel intronic deletion of any size on a diagnostic CGH test should lead to more detailed analysis by additional genomic or mRNA studies. Neuromuscular clinicians should have a high index of suspicion for the presence of an inversion mutation when interpreting CGH results.

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DILATED CARDIOMYOPATHY WITH CONDUCTION DEFECTS IN A PATIENT WITH PARTIAL MEROSIN DEFICIENCY DUE TO MUTATIONS IN THE LAMININ-α2-CHAIN GENE: A CHANCE ASSOCIATION OR A NOVEL PHENOTYPE?

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ABSTRACT: Patients with a partial reduction of merosin due to mutations in the laminin-α2 chain gene usually present with a mild form of congenital muscular dystrophy or a limb-girdle–like muscular dystrophy. To our knowledge, cardiac impairment has never been reported in such patients. A longitudinal study of a patient with partial laminin-α2 deficiency secondary to mutations in the *LAMA2* gene revealed dilated cardiomyopathy with ventricular arrhythmias. Is this a chance association or a novel phenotype?

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Merosin-deficient congenital muscular dystrophy type 1A (MDC1A) is the most common form of congenital muscular dystrophy (CMD).[1,2] It is characterized by the complete absence of laminin-α-chain expression in skeletal muscles. A more rare allelic form of muscular dystrophy is characterized by partially reduced expression of the laminin-α2 chain. Patients with this less severe form of CMD frequently present with a limb-girdle syndrome[3,4]; to date, there are no reports of cardiac compromise in these patients.

CASE REPORT

A previous report[5] described the case of a man who had white-matter alterations, proximal myopathy, and demyelinating polyneuropathy. His family history was negative for muscular dystrophy or cardiac disorders, including conduction defects or dilated cardiomyopathy. Histological examination of the muscle biopsy specimen revealed changes resembling inclusion-body myositis (IBM), and immunohistochemistry showed partial reduction of laminin-α-chain expression. Analysis of the *LAMA2* gene revealed two different dominant mutations: a missense mutation in exon 29 (c.4405 T→C, p.Cys1469Arg) and a nonsense mutation in exon 31 (c.4645 C→T, p.Arg1549Stop).

Abbreviations: CK, creatine kinase; CMD, congenital muscular dystrophy; ECG, electrocardiogram; EF, ejection fraction; HLA, human leukocyte antigen; IBM, inclusion-body myositis; MDC1A, merosin-deficient congenital muscular dystrophy type 1A; MHC, major histocompatibility complex; MRC, Medical Research Council

Key words: dilated cardiomyopathy with conduction defects, partial merosin dficiency, *LAMA2* gene mutations

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The patient is now 42 years of age, and over the last 10 years his cardiac and neurological conditions have been closely monitored in our neuromuscular unit. Long-term follow-up included neurological assessment, cardiac examination, echocardiography, and Holter electrocardiography (ECG). Muscle biopsy was repeated when he was 41 years of age. Common histological staining was performed, as was immunohistrochemistry for dystrophin, sarcoglycans, emerin, desmin, caveolin-3, major histocompatibility complex (MHC) class 1 human leukocyte antigen (HLA), and lamin A/C. Sequencing of the lamin A/C gene was also performed.

Recent neurological examination, including assessment of muscle power and evaluation of contractures, showed a waddling gait with lordosis and bilateral steppage, a generalized decrease of muscle tone, pseudohypertrophy of the calves and toe extensors, and atrophy of the proximal muscles of the legs.

The Medical Research Council (MRC) scale for testing muscle strength yielded a selective pattern of muscle impairment. Evaluation of shoulder muscles showed that muscle strength against resistance was not full (MRC grade 4). A variable reduction in power was observed for the muscles of the pelvic girdle with hip flexors and abductors at MRC grade 4, and hip extensors and adductors at MRC grade 3. Performance of trunk flexors and extensors, knee extensors, and dorsiflexors was also reduced (MRC grade 3). Sensory examination revealed hypesthesia of the lower limbs. Moreover, the patient had dysphagia to liquids. Table 1 summarizes the cardiac condition of the patient at the ages of 30, 37, and 40 years.

The patient was 30 years of age when he first complained of palpitations and precordial pain. He also recalled having a single fainting episode. Clinical evaluation did not show any signs of cardiomyopathy. He was in sinus rhythm, but sporadic ventricular ectopic beats were detected by 24-hour Holter ECG monitoring. Echocardiography showed mild left ventricular dilation and a reduced

Table 1. Instrumental investigation of cardiac conditions in a patient carrying the c.4405 T→C, p.Cys1469Arg, and c.4645 C→T, p.Arg1549Stop LAMA2 gene mutations.

Age	Standard ECG	Echocardiography	24-hour Holter monitoring
30 years	Sinus rhythm; HR 64 bpm	Mild dilation of left ventricle; EF 39%	Sinus rhythm; rare asymptomatic ventricular ectopic beats
37 years	Sinus rhythm; HR 66 bpm	Mild dilation of left ventricle; EF 35%	Synus rhythm; several asymptomatic ventricular ectopic beats; rare asymptomatic supraventricular ectopic beats
40 years	Sinus rhythm; HR 65 bpm	Left atrial dilation; left ventricular dilation; diffuse left ventricular hypokinesia; EF 33%	Sinus rhythm; several asymptomatic ventricular ectopic beats; rare asymptomatic supraventricular ectopic beats

ECG, electrocardiography; HR, heart rate; bpm, beats per minute; EF, ejection fraction.

ejection fraction (EF). Angiocardioscintigraphy performed at the same age confirmed diffuse hypokinesia with an EF of approximately 39%. Repeated echocardiographic assessment and Holter monitoring over the next 5 years did not show any significant variations in cardiac status. Clinical conditions and instrumental parameters remained stable until age 40, when the patient had an episode of syncopal ventricular tachycardia. There was evidence of a progressive decrease in ventricular function (EF 33%), which required implantation of an intracardiac defibrillator.

Atypical histological findings on muscle biopsy included a few inflammatory infiltrates, rimmed vacuoles devoid of any glycogen content, moderate variation in fiber size and shape, split fibers, increased endomysial and perimysial connective tissue and adipose tissue, and a few degenerating fibers. Increased acid phosphatase activity was related to degenerating fibers. Immunohistochemistry confirmed a decrease in the expression of laminin-α2-chain antibodies against both the 80- and 300-kDa fragments; immunolabeling of all the other proteins examined was normal. HLA expression was seen only on blood vessels.

Sequencing of the *LMNA* gene did not indicate any pathogenic alterations. A recently obtained creatine kinase (CK) was 250 IU (normal range 10–120 IU).

DISCUSSION

The long-term clinical and instrumental evaluation of our patient led to a diagnosis of dilated cardiomyopathy with life-threatening ventricular arrhythmias.

Dilated cardiomyopathy with ventricular arrhythmias is described as a complication of a peculiar phenotype of *LAMA2*-related gene mutations, which were previously characterized by proximal myopathy, demyelinating polyneuropathy, and white-matter alterations.

Immunohistochemistry excluded underlying inflammatory processes and the more common causes of dilated cardiomyopathy related to neuromuscular conditions. *LMNA* gene sequencing ruled out the presence of underlying mutations in the lamin A/C gene.

Previous reports have indicated that cardiac impairment may occur in individuals with MDC1A who have cardiac failure or left ventricular hypokinesia[6]; recently, it has been reported that up to one third of individuals with MDC1A develop left ventricular dysfunction.[7] Also, it was shown that the majority of cardiac manifestations in these patients are not clinically relevant,[8] although the case of a young patient with congestive cardiomyopathy has been described. Conversely, to our knowledge, there have been no prior reports of cardiac alterations in patients with partial merosin deficiency due to *LAMA2* gene mutations.

The patient described herein did not have any clinical or laboratory manifestations of viral myocarditis at follow-up. No other mechanisms of myocardial injury that could reduce myocardial contractility were noted in the follow-up period.

This is the first report describing such clinical and histological findings in a patient carrying *LAMA2* gene mutations. Although the possibility of a novel phenotype related to *LAMA2* gene alterations is intriguing, it is difficult to prove. In particular, severe compromise of cardiovascular function along with histological findings has not been described among subjects with partial merosin deficiency due to *LAMA2* gene alterations; therefore, one should be cautious in considering this complex clinical entity as being caused exclusively by a decrease in laminin-α2-chain expression.

Another limitation relates to the fact that we cannot rule out the possibility that this complex phenotype may be caused by a digenic disorder or that an environmental factor may be responsible for the deterioration of the heart.

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