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Charcot-Marie-Tooth disease type 2G redefined by a novel mutation in *LRSAM1*

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Abstract

Objective: To identify the unknown genetic cause in a large pedigree previously classified with a distinct form of axonal Charcot-Marie-Tooth disease type 2G and to explore its transcriptional consequences.

Methods: Clinical re-evaluation of the pedigree was performed, followed by linkage analysis with the redefined disease statuses, whole genome and exome sequencing. The impact of the mutation was investigated by immunoblotting and transcriptome sequencing.

Results: Thirteen affected individuals over three generations displayed mild and quiescent lower-limb axonal sensorimotor neuropathy. MRI of lower-limb musculature systematically showed fatty atrophy in clinical and subclinical mutation carriers. We redefined the disease-linked region to chr9q31.3-q34.2 and subsequently identified a novel missense variant in the E3 ubiquitin-protein ligase LRSAM1 (p.Cys694Tyr). Unlike previous reports, we demonstrated in patients' lymphoblasts that the mutation does not influence overall protein levels of LRSAM1, neither of its ubiquitylation target TSG101. The mutation is associated with several transcriptional changes, including a significant up-regulation of another E3 ubiquitin-protein ligase, NEDD4L, and of TNFRSF21, a key regulator of axonal degeneration.

Interpretation: Our findings demonstrate that the isolated genetic entity CMT2G is caused by the first missense mutation in LRSAM1 and should be reclassified as CMT2P. MRI of lower limb musculature can be used to detect minimal signs of the disease. Transcriptome analysis of patients' cells highlights novel molecular players associated with LRSAM1 dysfunction, and reveals pathways and therapeutic targets shared with amyotrophic lateral sclerosis and Alzheimer's disease.

Introduction

Charcot-Marie-Tooth disease (CMT) is the most common inherited neuromuscular disorder affecting peripheral nerves¹, characterized by progressive weakness and wasting of distal muscles, motor impairment, sensory loss and skeletal deformities.² CMT was initially classified according to mode of transmission and electrophysiological or nerve biopsy features. Characteristically, motor conduction velocities (MCV) in median nerve are <38 m/s for the demyelinating form (CMT1) and >38 m/s for the axonal form (CMT2).³ CMT is clinically and genetically extremely heterogeneous, with causal mutations in >70 genes implicated in diverse cellular pathways.⁴

CMT2G was attributed to a single large Spanish family presenting with a mild, slowly progressive axonal CMT.⁵ Initial clinical examinations were performed from 1977-1985, ages of patients ranged between 9-76 years (mean 38). Almost all patients had developed symptoms during the second decade. Two cases were asymptomatic but showed signs of disease. The main presenting symptoms were foot deformity and walking difficulty; no hand disturbances were noted. Patients were at most only moderately disabled and symptoms showed little or no progression. Slight NCV slowing was observed in 36% of nerves and 3:10 affected individuals had entirely normal NCVs. The amplitude of the sensory action potentials of median and peroneal nerves was almost uniformly reduced. Twelve years ago, the disease locus was assigned to chr12q12-q13.3 and designated as CMT2G.⁶

Here, we describe novel clinico-electrophysiological and magnetic resonance imaging (MRI) findings in this challenging pedigree, prompting renewed linkage analysis. Ultimately, we used next-generation sequencing technologies to elucidate the genetic defect underlying the CMT2G category. This study pinpoints disease-specific pathways triggered by the *LRSAM1* mutation.

Methods

Patients and evaluation

This CMT2 pedigree was first described in 1986⁵ and has been regularly updated since. Currently reported investigations were performed in the last five years.

Standard protocol approvals, registrations and patient consents

Written informed consent was obtained from all participants. This study was approved by the institutional review boards of the Santander University Hospital and the University of Antwerp.

Electrophysiological investigation

Serial nerve conduction studies were done as reported⁷ according to CMT neuropathy score⁸ (CMTNS) requirements. Needle electromyography (EMG) was performed, analyzing motor unit potential duration and morphology, presence of spontaneous activity, and pattern at maximum voluntary effort.

MRI protocol

Lower-limb musculature MRI (1.5T clinical scanner, Signa, General Electric Medical Systems, Milwaukee, Wis.) was performed as reported.⁹

Array comparative genomic hybridization

A high-density 4x180k microarray was developed using eArray (Agilent Technologies, Santa Clara, CA) containing 96,775 tiling probes (60bp each, ± 20 bp overlap) targeting the CMT2G locus on chr12q12-q13.3.⁶ Array hybridization was executed as reported.¹⁰ Results were visualized in arrayCGHbase.¹¹

Linkage studies

After genotyping a custom panel of 422 short tandem repeat markers, genome-wide parametric linkage analysis was performed with SuperLink in EasyLinkage.¹²

Whole genome/exome sequencing

Whole genome sequencing was outsourced to Complete Genomics (Mountain View, CA). Whole exome sequencing (WES) of IL2 and III.1 was performed by Axeq Technologies (Macrogen Corp., Rockville, MD) using the SureSelect V4 capture kit (Agilent Technologies Inc., Santa Clara, CA) and sequenced on the HiSeq2000 platform (Illumina, San Diego, CA). WES of IV.11 was done by the Beijing Genomics Institute (BGI, Shenzhen, China) using the SureSelect V5+UTR capture kit (Agilent Technologies Inc.) on the HiSeq2000 platform (Illumina). Mapping and variant calling were performed with CLC Genomics Workbench (Qiagen Bioinformatics, Redwood City, CA), annotation and filtering was done with GenomeComb.¹³

Mutation analysis of LRSAM1

Mutation analysis was done by Sanger sequencing as described.¹⁴ Nucleotide and amino acid numbering followed mRNA (NM_001005373.3) and protein (NP_001005373.1) LRSAM1 sequences available at NCBI and mutation description was according to HGVS nomenclature.

Immunoblot analysis

Immunoblot was executed as reported.¹⁴ Anti-LRSAM1 (#ab73113, Abcam, Cambridge, UK), anti-TSG101 (#14497-1-AP, Proteintech, Rosemont, IL) and anti-GAPDH (#GTX100118, Genetex, Irvine, CA) antibodies were used. Results were visualized on the ImageQuant™ LAS4000 (GE Healthcare Life Sciences).

RNA isolation and transcriptome sequencing

RNA was extracted as described.¹⁴ Transcriptome sequencing was outsourced to KULeuven Genomics Core (Belgium). Libraries (TruSeq RNA Library Prep Kit, Illumina) were paired-end sequenced (HiSeq2000, Illumina) with 100bp insert size and 50-100x10⁶ reads/sample. Reads were mapped and annotated using TopHat.¹⁵ Differential expression was assessed with DESeq2 and edgeR (Bioconductor).¹⁶

RT-qPCR analysis

RT-qPCR was performed as reported.¹⁴ Statistical data analysis was executed with qBase+ (Biogazelle, Zwijnaarde, Belgium) taking into account primer amplification efficiencies.

Results

Gene hunting within the original CMT2G locus on chr12

To find the CMT2G-causing gene, we initially focused on the chr12q12-q13.3 disease locus.⁶ The genomes of three CMT2G haplotype carriers (IV.2, IV.9, IV.11) were analyzed by whole genome sequencing (WGS). However, no novel, disease co-segregating coding variations were found, even after covering the unsequenced regions by Sanger sequencing. To exclude false negative calls, IV.11 was additionally submitted to whole exome sequencing (WES). Three more coding variations were identified within the locus, but none were co-segregating with disease. Moreover, no copy number variations were found in the locus when comparing four CMT2G haplotype carriers (III.1, IV.2, IV.9, IV.11) to four unrelated controls with array comparative genomic hybridization. To correlate the non-coding variations in the locus to putative changes in expression levels or alternative splicing events, the transcriptome of lymphoblast lines from three haplotype carriers (III.10, IV.8, IV.11) were compared to three non-carriers (III.9, IV.5, IV.10). The only mis-regulated transcript in the locus was excluded due to the lack of genomic alterations in the gene region and inconsistent expression levels in additional samples. Cryptic splice site activation was eliminated by corroborating *in silico* prediction and RNAseq results. Altogether, despite exhaustive genetic investigations, no disease-causing mutation could be found within the chr12 locus, prompting us to reconsider the original linkage results and reassess the clinical data.

Clinical findings

The pedigree was updated and re-evaluated (Fig 1, Table 1). Four patients (II.1, II.2, III.1, III.4) died from causes unrelated to CMT; none of them had exhibited progression of lower-limb

weakness. Both re-examined patients of the third generation (III.8, III.10) showed progressive walking difficulties, requiring no ankle foot orthosis or support. CMTNS was mild. All affected members of the fourth generation have remained asymptomatic with mild CMTNS. Two patients (IV.4, IV.11) showed pes cavus, stocking hypoesthesia, and lower-limb areflexia. Non-symptomatic progression of lower-limb amyotrophy was observed in one patient (IV.4; Fig 2A to D). Figure 2E to I shows patient IV.11 exhibiting the minimal clinical phenotype consisting of forefoot pes cavus, toe clawing, stocking hypoesthesia and ankle areflexia. The remaining patients of the fourth generation exhibited just isolated pes cavus or no sign of disease.

Our longitudinal clinical evaluations revealed differences of some affection statuses compared to previous reports.^{5,6} III.12 is a 57-year-old man displaying pes cavus and minimal stocking hypoesthesia. At age 26 the only positive electrophysiological finding was reduced amplitude of sensory nerve action potential (SNAP) of peroneal nerve ($0.5\mu\text{V}$; normal ≥ 1.5).⁵ He was then considered affected. Serial examinations (latest 2014) have revealed no signs of disease except for minimal pes cavus. Furthermore, a control electrophysiological study was normal. Therefore, we reclassified him as unaffected. III.14 is a 53-year-old man initially evaluated at age 13, when there was just mild pes cavus and minimal stocking hypoesthesia with preserved ankle reflexes and muscle power of foot dorsiflexors/evertors. In our report from 2004, he was considered affected.⁶ Nerve conduction studies were normal. Serial examinations over 20 years revealed no changes. He was thus reassigned as unaffected. At the initial evaluation, members at risk from the fourth generation were between 4-15 years of age and only IV.2 was considered affected. Repeated clinical examination of IV.8 and IV.9 has shown minimal pes cavus with no other signs of peroneal muscular atrophy (PMA). Electrophysiology was normal, furthermore MRI of lower-limb musculature in IV.8 showed no changes (see below). These two patients, considered affected before⁶, were reclassified as unaffected.

Electrophysiological findings

Electrophysiological studies were updated for eight affected individuals (Table 2). Besides isolated SNAP amplitude reduction of median nerve, potentially associated with an incidental carpal tunnel syndrome, two patients (IV.1, IV.12) had normal nerve conduction studies; furthermore, needle electromyography (tibialis anterior and EDB in IV.1; EDB in IV.12) revealed no changes. In the remaining six patients, the most common finding was variable SNAP amplitude reduction of median, ulnar or sural nerves. CMAPs of ulnar and median nerves were systematically preserved, whereas those of tibial and/or peroneal nerve were attenuated in four cases. MCVs and SCVs were normal or slowed in the axonal range; likewise, DML were normal or slightly prolonged. Electromyography of EDB systematically revealed a pattern of chronic denervation.

MRI findings

MRI of lower-limb musculature was performed in eight individuals: two symptomatic (III.8, III.10) and six at risk (IV.1, IV.4, IV.8, IV.10, IV.11, IV.12). Only in IV.8 (not carrying the disease-causing mutation, see below), lower-leg and foot musculature was preserved (Fig 3A to C). Yet, in the other seven (all mutation carriers), MRI revealed changes, more evident with disease progression (Fig 3D to I). Calf musculature showed fatty atrophy, which was bilateral, at most moderate, predominantly distal, and mainly involving superficial posterior compartments. Calf muscle edema (not shown) was exceptional. Contrast enhancement, investigated only in III.8 and III.10, was not observed. Compared to lower-leg muscles, intrinsic foot musculature systematically exhibited more advanced fatty atrophy (Fig 3F and I). Thigh musculature was preserved.

Linkage studies

We recruited additional family members (IV.1, IV.5, IV.10, IV.12, IV.13) and considered the updated affection status of seven individuals (see above). Owing to the minimal clinical phenotype in the fourth generation, the affection status of four asymptomatic individuals (IV.5, IV.8, IV.9, IV.12) was deemed “unknown”. The renewed genome-wide linkage analysis demonstrated that the CMT2G region on chr12q12-q13.3⁶ was no longer linked to the disease phenotype; however,

a new conclusive linkage region appeared on chr9q31.3-q34.2 ($Z_{\max} = 3.186$ at $\theta = 0$; Fig 1B). Genotype analysis confirmed a common haplotype shared by all affected individuals (Fig 1A). Key recombination events (in II.1, IV.4, IV.10, IV.11, IV.12) delineated the disease haplotype to a 23.6 Mb region between markers D9S2026-D9S164.

Mutation analysis

We reinvestigated the WGS data to find novel, coding variants in the chr9 locus shared between the two affected individuals (IV.2, IV.11) and absent from the unaffected individual (IV.9). Data filtering revealed no such variations; therefore, to exclude false negative calls, we additionally performed WES of three affected individuals (II.2, III.1, IV.11). WES analysis revealed a shared, novel missense variation (c.2081G>A, p.Cys694Tyr) in the gene encoding leucine-rich repeat- and sterile alpha motif-containing 1 (*LRSAM1*), which has already been associated with CMT2P^{17,18}. This particular genomic position was not covered in the WGS data of the two affected individuals. The *LRSAM1* variation was present in all disease haplotype carriers and absent from 164 Spanish control individuals. It targets a conserved nucleotide (GERP-score 5.26) and amino acid residue (PolyPhen2-score 0.998) within the catalytic RING-type zinc finger domain of the *LRSAM1* protein.

Protein studies

Reported *LRSAM1* mutations affect the length^{18–20} or abundance¹⁷ of the protein. Therefore, we compared *LRSAM1* protein levels in lymphoblasts from five mutation carriers and four related non-carriers, yet observed no difference in expression (Fig 4A). Additionally, a reported *LRSAM1* mutation affecting the RING-domain disturbs its ubiquitin ligase activity, resulting in increased abundance of its ubiquitylation target TSG101.^{18,21} We tested a potential effect of the C694Y *LRSAM1* mutation on TSG101, but observed comparable TSG101 expression in lymphoblasts from patients and controls (Fig 4A).

Transcriptome analysis

Differential expression analysis of the lymphoblast transcriptome of three *LRSAM1* mutation carriers (III.10, IV.10, IV.11) compared to three non-carriers (III.9, IV.5, IV.8) revealed several mis-

regulated transcripts (Fig 4B, sup. table). With RT-qPCR, we validated 35 protein-coding genes that showed a significant log-transformed fold-change $\geq \pm 0.5$ with both DESeq2 and EdgeR, and that had ≥ 100 raw read counts in all samples of at least one group (sup. table). To expand the relevance of our findings, we added samples of two more mutation carriers (III.8, IV.12) and one non-carrier (IV.9). We identified six significantly up-regulated and two down-regulated transcripts in patients' lymphoblasts (Fig 4C), including *NEDD4L*, another ubiquitin E3 ligase, and *TNFRSF21*, a key regulator of axonal degeneration.^{22,23}

Discussion

Here, we describe a longitudinal study of a large CMT2 pedigree, essentially characterized by very quiescent lower-limb sensorimotor axonal neuropathy and pes cavus. Previous detailed histological investigations in this pedigree led us to conclude that the disease represents a model of primary neuronopathy affecting lumbo-sacral motor and sensory neurons with length-dependent sensorimotor axonopathy.⁵ Updated clinical and genetic data allowed us to finally discover the genetic cause underlying CMT2G. The single family within this category carries the first missense mutation in *LRSAM1* and should thus be reclassified as CMT2P.

At initial evaluation (1977-1985), PMA was relatively easy to diagnose in the individuals of the first two generations, then aged between 25-76 years. By 1985, just one out of nine individuals at risk in the fourth generation showed subclinical signs of disease. Since then all six *LRSAM1* mutation carriers of this generation, now aged 35-47 years, have remained asymptomatic with minimal or no signs of PMA. The presence of isolated pes cavus in the context of a familial disorder²⁴ prompted us to establish a presumptive diagnosis of PMA in several individuals, which was ultimately not confirmed. Contrastingly, serial evaluations over three decades in IV.4 demonstrated the appearance of overt foot and calf amyotrophy that remained entirely asymptomatic, supporting the need for detailed neuromuscular examination to detect the CMT2 semiology.²⁵ Furthermore, even after serial evaluation four *LRSAM1* mutation carriers remained

entirely subclinical. Updated electrophysiological studies confirm that the most common finding is amplitude reduction of SNAP and CMAP in lower limb nerves, while NCVs are preserved or slightly slowed.⁵ It is worth noting that two subclinical *LRSAM1* mutation carriers showed normal electrophysiology. In short, serial evaluations in the current pedigree highlight the difficulties in establishing a clinical diagnosis in mildly affected patients, which can be an issue in many other forms of CMT.

Mutations in *LRSAM1* were recently found to cause autosomal dominant^{18–20} and recessive¹⁷ CMT2P. The dominant mutations described so far are associated with a relatively mild, very slowly progressive sensorimotor axonal neuropathy initially affecting lower limbs. Disease onset usually ranges between the second and fourth decade, but, notably, in a family with a dominant *LRSAM1* splice-site mutation six out of ten patients were asymptomatic.¹⁹ Additionally, three aged patients carrying a *LRSAM1* frameshift mutation developed Parkinson's disease, suggesting a potential role for mutated *LRSAM1* in substantia nigra degeneration.²⁶

Our study broadens the current knowledge regarding the phenotypic and genetic spectrum associated with *LRSAM1*. Reduced penetrance is generally rarely observed in CMT subtypes²⁷, yet it appears to be a common phenomenon in the context of *LRSAM1*-related neuropathies, as demonstrated by the current and previous investigations.¹⁹ The fact that some mutation carriers display only mild or minimal clinical and electrophysiological abnormalities should be carefully considered. Diagnosis should be based on detailed electrophysiological investigations in combination with clinical and genetic data.

Moreover, our findings highlight the importance of MRI to detect and characterize pathological conditions of the skeletal muscle. In keeping with previous observations for CMT1A and CMT2A^{9,28,29}, the current MRI showed moderate to marked fatty atrophy of foot musculature in all seven subclinical *LRSAM1* mutation carriers. Fatty infiltration of leg muscles was present to a lesser degree, increasing distally throughout the long axis of the muscle belly. As in other dominant axonal CMT phenotypes, fatty atrophy was greater in superficial posterior

compartment muscles.²⁹ The observed predominant fatty infiltration in foot musculature and proximal-to-distal gradient in calf muscles correlates with the dying-back phenomenon found in our previous histological study of the sciatic nerve and its branches in patients III.1 and III.4.⁵ Notably, MRI of a non-mutation carrier showed normal appearance of calf and foot muscles. Our findings establish that patients with a minimal clinical phenotype display abnormalities of lower-limb musculature perceptible by MRI, suggesting that muscle MRI could be a useful tool for diagnosing *LRSAM1*-related neuropathies.

This report illustrates several potential pitfalls of genetic investigations. First, thorough examination of an established disease locus (CMT2G on chr12) with state-of-the-art “omics” technologies did not procure the disease-causing mutation. The ultimate gene identification was facilitated by longitudinal clinical follow-up, reassessment of affection statuses and renewed linkage analysis. Second, the c.2081G *LRSAM1* position was not covered in the WGS data of the two investigated patients, so the mutation could only be retrieved after applying an orthogonal sequencing technology (WES). The relatively high raw base-calling error-rate and variable performance of NGS platforms is well recognized.³⁰ Our study highlights the added value of a dual platform approach employing complementary NGS protocols³¹ to improve variant yield and reduce false negative calls.

LRSAM1 encodes a multidomain RING-type E3 ubiquitin ligase that covalently ubiquitylates target proteins through its catalytic C-terminal zinc finger domain. Post-translational ubiquitylation directs cellular proteins to various fates and functions, including proteasomal degradation, lysosomal targeting, modulation of protein-protein interactions, transcriptional regulation and cell signaling.³² The only known ubiquitylation target of *LRSAM1* is the tumor susceptibility 101 protein (TSG101), involved in lysosomal sorting of ubiquitylated cargo.²¹ Knockdown of the *LRSAM1* homolog in zebrafish results in abnormal motor neuron development¹⁸ and knockout of *Lrsam1* in mice leads to mild peripheral neuropathy and increased sensitivity to a neurotoxic agent.³³ The four reported *LRSAM1* mutations truncate, disrupt or abolish its catalytic RING zinc finger domain.^{17–20} Here, we report the first missense mutation in

LRSAM1, which also targets the RING zinc finger. Synthetic missense mutations in this domain abolish the ubiquitylation function.²¹ Likewise, a CMT2P-causing LRSAM1 insertion results in increased TSG101 levels in a cellular overexpression model, suggesting that it is not ubiquitylated and degraded.¹⁸ We tested if the C694Y *LRSAM1* mutation had a similar effect *in vivo*, but did not detect increased TSG101 levels in patients' lymphoblasts. This implies that either the mutation does not abolish LRSAM1 ubiquitylation function, or the loss of function is compensated by alternative cellular mechanisms. It has been suggested that other ubiquitin ligases also target excess TSG101 for proteasomal degradation to prevent detrimental cellular consequences.³⁴ Interestingly, our transcriptomics analysis appears to support this hypothesis. The results in patients' cells show increased transcription of *NEDD4L*, a gene encoding another E3 ubiquitin ligase known to ubiquitylate TSG101.³⁵ So, NEDD4L might (partially) compensate for loss of LRSAM1 function, which may explain the mild phenotype in LRSAM1 patients. Double knock-out studies³⁶ have revealed that mutations in a functionally redundant gene often lead to a minor or no phenotype, whereas targeting the compensatory gene pair together substantially increases severity, e.g. dystrophin and utrophin A in a mouse model of Duchenne muscular dystrophy.³⁷

Additionally, C694Y mutation carriers have increased transcription of *TNFRSF21*, a key regulator of axonal degeneration.^{22,23} Likewise, TNFRSF21 levels are elevated in the spinal cord of patients with amyotrophic lateral sclerosis (ALS)³⁸ and in the brain of Alzheimer's disease (AD) patients³⁹, promoting neuronal death through activation of apoptotic caspase signaling pathways.³⁸ Blocking TNFRSF21 function with an antagonist antibody results in increased motor neuron survival and motor function improvement in ALS mice³⁸ and protection against A β 42-induced neurotoxicity in cultured neocortical neurons.³⁹ It would be interesting to investigate if this promising therapeutic approach could have a similar neuroprotective effect on CMT2P models and, ultimately, patients. Another up-regulated transcript in CMT2P patients, *PLA2G4C*, was recently found to be a putative causal gene for ALS, harboring a *de novo* missense mutation.⁴⁰ *PLA2G4C* encodes a membrane-bound phospholipase that hydrolyses glycerophospholipids into various signaling molecule precursors⁴¹ but its potential role in neurodegeneration has yet to be explored. Among the other

mis-regulated transcripts are genes involved in apoptotic protease activation (*CTSC*), the mitochondrial ubiquinone respiratory chain (*NDUFA4L2*), or with largely unexplored functions (*CLLU10S*, *MB21D2*, *GRAMD1B*). In summary, our transcriptomic analysis of CMT2P patients offers new insights into cellular pathways associated with LRSAM1 dysfunction and provides novel targets for further studies.

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Author contributions

Study design: K.P., P.P., J.Be., A.J. Data acquisition and analysis: K.P., P.P., A.L.P.-N., A.G., E.G., R.G.-B., L.M., J.Ba., B.M., E.D.V., P.D.J., V.T., J.I., J.Be., A.J. Drafting the manuscript: K.P., P.P., A.L.P.-N., A.G., E.G., L.M., J.Be., A.J.

Conflicts of interest

Nothing to report.

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Figure legends

Figure 1. Genetic investigations. (A) Updated pedigree and haplotype analysis of the Spanish CMT2G family. Square symbols represent males and circles females. Black symbols indicate affected individuals. Red underlined labels specify the individuals whose affection status was altered after clinical re-evaluation. Individuals that were subjected to whole genome, whole exome or RNA sequencing are indicated with G, E and R, respectively. Underlined labels indicate family members whose lymphoblast transcriptome was sequenced. The disease-segregating haplotype on chr9 carrying the *LRSAM1* c.2081G>A mutation is depicted below each analyzed family member. Key recombination events (in II.1, IV.4, IV.10, IV.11, IV.12) delineate the disease locus to a 23.6 Mb region between markers D9S2026-D9S164.

(B) Graph representing the results of the genome-wide linkage analysis. Note that the initial linkage peak corresponding to the CMT2G locus on chr12 disappeared, whereas a new conclusive linkage region appeared on chr9q31.3-q34.2 ($Z_{\max} = 3.186$ at $\theta = 0$). (C) Electropherograms around the *LRSAM1* c.2081 genomic position demonstrating a heterozygous G>A transition in a patient compared to a control.

Figure 2. Clinical phenotype. Serial pictures of case IV.4 (A-D) and pictures of case IV.11 at age 32 (E-I). (A) At age 25, lower limbs appear normal; particularly note the absence of peroneal muscular atrophy and toe clawing. (B) Close-up picture of the sole of the feet showing midfoot hollowing and callosity over transverse arcus plantaris and external foot borders. (C) At age 43, lower-leg amyotrophy is notable mainly involving peroneal musculature; in spite of this there is no evidence of weakness of foot extensors/evertors or difficulty in heel walking. (D) Close-up picture of the left foot showing wasting of extensor digitorum brevis muscle (arrows). (E-G) There is no evidence of lower-leg amyotrophy; note that even under load pes cavus and clawing of toes are visible. (H-I) Close-up pictures of the feet showing marked pes cavus-varus deformity and toe clawing.

Figure 3. MR imaging according to the last examinations performed between 2011 and 2015. T-1 weighted MR images of lower legs in the coronal plane thorough posterior aspect (*upper row*) and the axial plane at mid calves (*middle row*), and of feet thorough metatarsal bones (*lower row*). **(A-C)** In case IV.8, showing normal examination and no *LRSAM1* mutation, note preservation of calf and foot musculature. **(D-F)** In patient III.10 note predominantly distal fatty infiltration of all four muscle compartments mainly involving soleus (So) and gastrocnemius medialis (GM) muscles; there is massive fatty atrophy of intrinsic foot musculature, right interossei (asterisks) and flexus hallucis brevis (FHB) being indicated. **(G-I)** In patient IV.1 note mild and predominantly distal fatty infiltration of soleus (So) and gastrocnemius medialis (GM) muscles; there is more advanced atrophy of intrinsic foot muscles, FHB being indicated.

Figure 4. Protein and transcriptional analyses. **(A)** Immunoblot analysis of LRSAM1 and its interactor TSG101 in lysates of lymphoblasts from five patients and four controls. GAPDH was used to demonstrate equal loading. **(B)** Heat map generated from the transcriptome sequencing data reflecting the expression level of the significantly mis-regulated genes retrieved by DESeq2 (measured in regularized log-transformed read counts) across patient and control samples. **(C)** RT-qPCR analysis showing relative expression levels (normalized to GAPDH, TBP and SDHA) of eight significantly mis-regulated transcripts in lymphoblasts obtained from five patients compared to four controls. ** = $p \leq 0.01$; * = $p \leq 0.05$

Table 1. Clinical features.

Patient		II.1	II.2	III.1	III.8	III.10	IV.1	IV.2	IV.4	IV.10	IV.11	IV.12
Age (years)		84*	85*	58*	64	59	47	41	44	40	38	35
Age at onset (decade)		5	2	2	2	2	A	A	A	A	A	A
Distal weakness (LL)		+	+	+	+	+	-	-	-	-	-	-
Areflexia	UL	-	-	+	-	+	-	-	-	-	-	-
	KJ	-	+	+	-	+	-	-	+	-	-	-
	AJ	+	+	+	+	+	-	-	+	-	+	-
Sensory changes	UL	-	-	+	+	+	-	-	-	-	-	-
	LL	+	+	+	+	+	-	-	+	-	+	-
Pes cavus		+	+	+	+	+	+	+	+	+	+	-
CMTNS		6**	9**	11**	7	9	3	0	5	3	3	0

LL = lower limbs; UL = upper limbs; KJ = knee jerk; AJ = ankle jerk; * = age at death; ** = CMTNS estimated from⁵; + = presence of abnormality; - = absence of abnormality; A = asymptomatic.

Table 2. Electrophysiological features according to the last examinations performed between 2011 and 2015.

Patient		III.8	III.10	IV.1	IV.2	IV.4	IV.10	IV.11	IV.12	Normal
Median nerve	DML (ms)	4.0	3.8	3.1	3.4	2.9	<u>4.6</u>	3.6	2.5	≤ 4.4
	MCV (m/s)	56.2	49.0	58.6	55.4	59.1	52.9	53.0	62.5	≥ 49.0
	CMAP (mV)	6.3	5.6	9.7	4.7	6.9	4.8	7.6	10.1	≥ 4.0
	F wave (ms)	30.4	<u>33.2</u>	28.0	25.7	24.3	30.6	30.8	25.3	≤ 31.0
	SCV (m/s)	48.5	<u>42.3</u>	50.0	48.4	50.8	<u>37.9</u>	52.0	57.1	≥ 45.0
	SNAP (μV)	<u>1.9</u>	<u>2.1</u>	<u>2.4</u>	9.8	<u>3.9</u>	<u>0.3</u>	<u>2.9</u>	8.4	≥ 7.0
Ulnar nerve	DML (ms)	ND	2.6	2.4	ND	2.4	2.9	2.4	2.1	≤ 3.3
	MCV (m/s)	ND	<u>42.3</u>	53.8	ND	61.7	54.3	57.1	63.9	≥ 49.0
	CMAP (mV)	ND	<u>3.7</u>	7.0	ND	7.8	11.0	10.9	7.7	≥ 6.0
	F wave (ms)	ND	<u>36.1</u>	31.4	ND	26.5	27.6	29.1	26.0	≤ 32.0
	SCV (m/s)	ND	48.1	53.2	ND	53.1	47.7	56.0	54.2	≥ 47.0
	SNAP (μV)	ND	<u>0.5</u>	3.0	ND	<u>1.6</u>	3.9	5.5	3.0	≥ 3.0
Peroneal nerve	DML (ms)	<u>6.2</u>	5.1	5.5	<u>5.6</u>	5.5	<u>5.6</u>	4.2	3.9	≤ 5.5
	MCV (m/s)	<u>39.9</u>	<u>33.5</u>	46.4	51.2	<u>41.1</u>	46.1	44.3	51.9	≥ 44.0
	EDB CMAP (mV)	4.7	<u>0.7</u>	3.3	2.6	<u>1.8</u>	<u>1.3</u>	3.0	6.6	≥ 2.0
	TA CMAP (mV)	ND	<u>3.3</u>	7.3	ND	7.6	5.1	6.2	5.4	≥ 5.0
	F wave (ms)	55.4	A	53.8	49.0	A	51.7	48	46.7	≤ 56.0
Tibial nerve	DML (ms)	<u>5.9</u>	A	5.8	5.1	4.8	4.0	<u>6.1</u>	ND	≤ 5.8
	MCV (m/s)	43.0	A	43.7	48.1	<u>36.3</u>	49.2	41.1	ND	≥ 41.0
	CMAP (mV)	<u>2.9</u>	A	5.5	12.5	4.4	9.1	4.8	ND	≥ 4.0
	F wave (ms)	<u>57.6</u>	A	52.6	ND	53.5	51.4	<u>57.4</u>	ND	≤ 56.0
Sural nerve	SCV (m/s)	47.3	A	49.1	<u>36.0</u>	44.8	52.9	46.7	53.1	≥ 40.0
	SNAP (μV)	<u>4.1</u>	A	6.3	<u>0.6</u>	7.0	<u>2.9</u>	<u>1.3</u>	8.1	≥ 6.0

EDB = *extensor digitorum brevis* muscle; TA = *tibialis anterior* muscle; A = absent; ND = not done. Abnormal values are indicated in bold and underlined.