Loss-of-function mutations in the SIGMAR1 gene cause distal hereditary motor neuropathy by impairing ER-mitochondria tethering and Ca\textsuperscript{2+} signalling

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Abstract

Distal hereditary motor neuropathies (dHMNs) are clinically and genetically heterogeneous neurological conditions characterized by degeneration of the lower motor neurons. So far, 18 dHMN genes have been identified, however, about 80% of dHMN cases remain without a molecular diagnosis. By a combination of autozygosity mapping, identity-by-descent segment detection and whole-exome sequencing approaches, we identified two novel homozygous mutations in the SIGMAR1 gene (p.E138Q and p.E150K) in two distinct Italian families affected by an autosomal recessive form of HMN. Functional analyses in several neuronal cell lines strongly support the pathogenicity of the mutations and provide insights into the underlying pathomechanisms involving the regulation of ER-mitochondria tethering, Ca\textsuperscript{2+} homeostasis and autophagy. Indeed, in vitro, both mutations reduce cell viability, the formation of abnormal protein aggregates preventing the correct targeting of sigma-1R protein to the mitochondria-associated ER membrane (MAM) and thus impinging on the global Ca\textsuperscript{2+} signalling. Our data definitively demonstrate the involvement of SIGMAR1 in motor neuron maintenance and survival by correlating, for the first time in the Caucasian population, mutations in this gene to distal motor dysfunction and highlight the chaperone activity of sigma-1R at the MAM as a critical aspect in dHMN pathology.
Introduction

Distal hereditary motor neuropathies (dHMNs), also called distal spinal muscular atrophy (distal SMA), represent a group of progressive neurological diseases caused by degeneration of lower motor neurons with secondary distal muscle weakness and atrophy. Unlike hereditary motor and sensory neuropathy (HMSN), also known as Charcot-Marie-Tooth disease (CMT), sensory abnormalities are absent in dHMN or represent a minor component of the disease (1).

Genetically, dHMN is a heterogeneous condition inherited as an autosomal dominant, autosomal recessive or X-linked trait. Over the last 10 years, mutations in 18 genes (HSPB1, HSPB8, BSC1L, IGHMBP2, SETX, GARS, DYN1H1, DCTN1, ATP7A, TRPV4, SLC5A7, HSPB3, REEP1, HSJ1, BICD2, MYH14, AARS and FBXO38) have been associated to dHMN (2,3). These genes encode proteins implicated in different cellular processes such as protein folding and quality control, RNA processing, axonal trafficking and ion channel function supporting that multiple pathogenic mechanisms may underlie the degeneration of motor neurons in dHMN. Despite these advances in gene discovery, further heterogeneity is expected in dHMN as mutations in the known genes account for only about 20% of dHMN patients (4).

Furthermore, five additional chromosomal loci without identified disease genes have already been reported both for dominant (4q34-q35, 7q34-q36 and 2q14) and recessive dHMN forms (9p21.1-p12 and 11q13) (4). Recently, a mutation in the SIGMAR1 gene has been reported in a consanguineous Chinese family with an autosomal recessive form of dHMN (5).

In this study, we combined whole-genome mapping and exome sequencing approaches to identify the gene responsible for a recessive form of dHMN clinically characterized in two Italian families.

Results

Clinical findings

Family 1 came from a small village of Southern Italy and pedigree reconstruction highlighted a consanguineous relationship between the parents of patient III-4 (first cousins), thus corroborating the recessive inheritance of the disease. On the contrary, no documented relationship was identified for parents of III-1 although all their ancestors came from the same village over four generations (Fig. 1A).

The proband (III-1), a 27-year-old female, displayed difficulties of gait since school age presenting recurrent sprains of the ankles and falls while running. At age 19 years, examination disclosed: bilateral pes cavus and claw hands; bilateral wasting of peroneal and sural muscles and of first interosseus, thenar and hypothenar muscles; stepping gait, impossible on heels and tips. Distal moderate-to-severe weakness was symmetrically present in the four limbs: according to the Medical Research Council (MRC) scale, strength was as follows: toe and ankle dorsiflexion 0/5, plantar flexion 2/5, intrinsic hand muscles 3/5, the strength was normal in the remaining muscular districts. All modalities of sensation were preserved. Cranial nerves, bulbar and cerebellar functions were normal. Cognitive deficit was not detected. Deep tendon reflexes were absent in the distal lower limbs and brisk in the proximal lower and upper limbs; superficial reflexes were normal. Electromyography (EMG) disclosed diffuse signs of chronic denervation in the distal muscles of the four limbs. In nerve conduction studies, compound muscle action potential (CMAP) was unequivocal in the lower limbs and markedly reduced (with conserved motor nerve conduction velocities) in the upper limbs; sensory nerve conduction studies were normal in lower (sural) and upper limbs (median and ulnar) nerves. Brain and spine magnetic resonance imaging (MRI), brainstem auditory, somatosensory and visual evoked potentials were normal. No clinical signs of dHMN were observed in the parents and in the two proband’s siblings. The second affected subject (III-4) was referred to have clinical features similar to the proband, unfortunately she and her family refused to participate in the study.

The proband of the second family (II-2), aged 27 years at the first visit, reported ambulatory difficulties from the infancy and poor performance in physical activities during the school. The symptoms progressed very slowly in the following years, with walking impairment due to ankle distortions and difficulty in fine hand movements. Neurological examination revealed stepping gait and moderate muscle weakness and wasting in lower and upper limbs, more distal than proximal, with the following MRC strength scores: toe and ankle dorsiflexion = 2/5, plantar flexion = 2/5, intrinsic hand muscles = 2/5, wrist extension/flexion 3/5, weakness was absent at other levels. There were bilateral pes cavus, Babinski sign and hyperactive deep tendon reflexes without spasticity signs. Sensory, cerebellar, cranial nerves and bulbar functions were normal as well as respiratory and cognitive functions. Upper and lower limbs ENG revealed diffuse and marked reduction in CMAP amplitude with slightly reduced conduction velocities and preserved sensory conduction. Motor nerve conduction blocks were absent. Upper and lower limbs EMG disclosed diffuse signs of chronic denervation in the hand, forearm and leg muscles with normal findings in proximal muscles. Brain and spine MRI, as well as brainstem auditory, somatosensory and visual evoked potentials were normal.

Molecular genetic studies

In order to identify the disease gene, a genome-wide homozygosity mapping was performed on the proband, her parents and the two healthy siblings of family 1. The analysis of over 160,000 high-quality SNP genotypes identified 11 candidate homozygous regions greater than 1Mb inherited by the proband and not shared by her healthy siblings III-2 and III-3. A genome-wide IBD segment detection analysis was then carried out on the apparently unrelated parents II-1 and II-2 in order to quantify their relatedness and to identify DNA segments inherited from a common ancestor. IBD analysis revealed a single shared region on chromosome 9p with a probability of being inherited from a distant ancestor close to 1 (Fig. 1B). The same IBD interval overlaps with one of the homozygous regions previously identified in the proband and haplotype reconstruction confirmed the co-segregation with the disease (Fig. 1A). Despite the small size of the family, the combination of homozygosity mapping and IBD analysis allowed to identify a single candidate locus of about 7.9Mb (rs17775810; rs1022770) on chromosome 9p21.1-p13.2.
Whole-exome sequencing (mean coverage depth: 159X) was performed in the proband III-1 and no putative disease-causing variants were detected in any of the known genes associated with dHMN or with other related neuropathies. The analysis of WES variants in chr9p21.1-p13.2 identified a homozygous missense substitution (c.412G>C, p.E138Q, coverage depth: 149X, reads G = 0, C = 149) in the \textit{SIGMAR1} gene (NM_005866). Sanger sequencing confirmed the presence of the homozygous variant exclusively in the patient III-1, whereas her parents (II-1 and II-2) and the unaffected sister III-2 were heterozygous (Fig. 1A). No other putative disease-causing variants were identified in other genes in this region neither in other candidate genes in the remaining 10 homozygous regions. In order to confirm the involvement of \textit{SIGMAR1} in the dHMN pathogenesis, a PCR-based mutation screening was performed in other 12 unrelated index patients diagnosed as having dHMN with a putative recessive inheritance.

In all these cases, mutations in the HSPB1, HSPB8, BSC12, IGHMBP2, GARS, HSPB3, HSJ1 genes, commonly involved in dHMN, were previously excluded by sanger sequencing. A second homozygous missense variant (c.448G>A, p.E150K) was identified in one index case belonging to a small family with two affected brothers (Fig. 1C). The analysis of the other available family members confirmed the co-segregation of the homozygous \textit{SIGMAR1} c.448G>A substitution with the disease.

Both c.412G>C and c.448G>A substitutions are neither reported in NHLBI Exome Variant Server, nor in other mutation databases including Leiden Open Variation Database, Human Gene Mutation Database and ClinVar (6–8). Only the c.448G>A is present in the ExAC database as it has been identified in heterozygosis in 1 out of 60315 subjects (allele frequency 0.000008). Moreover, they were not found in 200 control chromosomes from individuals of the same geographic area. Interestingly, \textit{SIGMAR1} shows very high inter- and intra-species conservation; only 5 SNPs (MAF >1%) are reported in its coding sequence according to dbSNP146. In addition, both the identified mutations occur in highly conserved nucleotides (PhyloP scores >5) and were predicted to be deleterious by several in silico analysis algorithms (Supplementary Material, Table S1).
SIGMAR1 encodes for the sigma non-opioid intracellular receptor 1 (sigma-1R), an integral membrane protein of the endoplasmic reticulum (ER) with chaperone activity implicated in many aspects of cellular homeostasis in the nervous system, including regulation of ion channels, calcium signalling (9), neurite outgrowth and autophagy (10).

The two identified amino acid changes occur in the C-terminus sigma-1R domain containing the ligand binding pocket (11) and with a putative chaperone function (12). Both mutations cause the replacement of a negatively charged residue with an uncharged (E138Q) or positively charged (E150K) residue.

**Sigma-1R E138Q and E150K mutations affect neuronal cell survival**

In order to identify the pathophysiological role of the two sigma-1R substitutions found in patients, we utilized three different neuronal cell systems, two human neuroblastoma cell lines, SH-SY5Y and SK-N-BE, and the murine motor neuron-like NSC-34 line. Given the relatively low level of endogenous sigma-1R protein in these cells, we overexpressed the two FLAG-tagged sigma-1R E138Q and E150K variants and compared the phenotype of transfected cells with that of cells overexpressing the WT sigma-1R.

We observed a protective effect of WT sigma-1R expression on cell survival after treatment of transfected cells with different ER stress inducers, namely H2O2 (1 mM) (13), MG132 (2.5 μM) and Thapsigargin (10 μM) (14). Interestingly, the expression of E138Q and E150K sigma-1R variants did not show this protective effect and, on the contrary, it further exacerbated the toxic effect of the treatments (Fig. 2A). To note, we observed a significant induction of cell death in cells expressing the sigma-1R E138Q and E150K mutations compared to cells expressing the WT protein, already in basal conditions (Fig. 2B).

**E138Q and E150K mutations induce sigma-1R mislocalization out of the MAM**

Sigma-1R protein localizes at specialized ER membrane regions in close contact to mitochondria, called mitochondria-associated ER membrane (MAM) (10) and actively participates to the Ca2+ transfer from ER to mitochondria during agonist-induced Ca2+ release in a variety of cellular models (9,14–16). This strategic localization ensures sigma-1R function as chaperone to IP3R channels at the ER-mitochondria interface and guarantees receptor activity and stability after ligand binding.

The immunofluorescence analysis in neuronal cells showed a cytoplasmic distribution of exogenous FLAG-tagged WT sigma-1R while both the E138Q and E150K mutants displayed an abnormal subcellular localization in both murine and human neuronal cells already in basal condition (Fig. 2C and Supplementary Material, Fig. S1A), which became even more striking in differentiated cells (Supplementary Material, Fig. S1B).

We then investigated the precise subcellular localization of overexpressed sigma-1R proteins confirming the ER membrane localization of WT sigma-1R, thus nicely reproducing that of the endogenous protein (10), as revealed by the colocalization with the ER marker calreticulin (Fig. 3A). Differently, both E138Q and E150K sigma-1R variants showed a dramatically reduced colocalization with calreticulin (Fig. 3A and B). This was also confirmed by the colocalization with another ER chaperone, GRP78/Bip (Supplementary Material, Fig. S2), which has been described to associate to sigma-1R and regulate its chaperone activity (10). We then analysed the localization of sigma-1R proteins at MAM, by staining transfected neuroblastoma cells with the mitochondrial marker TOM20. Here again, sigma-1R E138Q and E150K variants showed a significant reduction of colocalization with TOM20 compared to WT sigma-1R (Fig. 4). The same result was obtained by the analysis of colocalization between sigma-1R and the mitochondrial protein Mitofusin1 (not shown). This clearly points to a displacement of sigma-1R mutant proteins away from the MAM.

**Impaired cellular Ca2+ handling in sigma-1R E138Q and E150K expressing cells**

Considering the role of sigma-1R protein in assisting IP3Rs function during ER Ca2+ release in response to IP3-generating stimuli, and the mislocalization of the two sigma-1R mutants out of the MAM, we wondered whether they cause alteration in global Ca2+ homeostasis in neuronal cells. We then measured cytosolic and mitochondrial Ca2+ levels in resting condition and after Bradykinin (50 nM) stimulation in cells transfected with WT, E138Q, and E150K sigma-1R using the ratiometric Fura-2 Ca2+ indicator or cotransfecting with the genetically encoded mitochondrial Ca2+ probe 4mt-GCaMP6f. As shown in Figure 5, both cytosolic Ca2+ increase and mitochondrial Ca2+ uptake were significantly reduced in cells expressing the two mutants compared to those expressing WT sigma-1R, despite both the cytosolic and mitochondrial resting Ca2+ were unaffected. We hypothesize that this is likely due to a loss of sigma-1R chaperone function to the IP3R channels rather than to a defect in the capacity of the ER to store Ca2+, as suggested also in other cellular systems where the sigma-1R C-terminus domain is mutated (17).

**Sigma-1R E138Q and E150K mutations induce p62 and LC3 aggregation**

Considering: i) the position of the two identified mutations within the chaperone domain of sigma-1R; ii) the aggregate-like distribution (Fig. 2C and Supplementary Material, Fig. S1A and B) and iii) the loss of cytoprotection (Fig. 2A and B) of the mutants, we investigated their effect on the cellular pathways involved in protein clearance and autophagy. To test this hypothesis, we analysed the expression and distribution of the two classical markers of autophagy in our cell models: p62 and LC3.

P62 lays at the crossroad of the two major proteolytic systems of the cell: the autophagy pathway and the proteasome-dependent protein degradation. Interestingly, we found that the expression of the mutated sigma-1R proteins induced a robust and significant increase of p62 reactive dots compared to WT sigma-1R in both human SH-SY5Y neuroblastoma and mouse NSC-34 cells (Fig. 6A and Supplementary Material, Fig. S3A).

LC3 is lipiddated and redistributes to autophagosomes during autophagy induction appearing as fluorescent dots with punctate distribution inside the cell (18). The expression of both E138Q and E150K sigma-1R mutants correlated with a significant increase of LC3 positive autophagosomes (Fig. 6B). However, no significant colocalization between sigma-1R and p62 or LC3 was observed. We also investigated the distribution of another stress-activated chaperone involved in protein aggregate clearance, the heat shock protein HSP70 (19,20). HSP70 is found diffused in the cytoplasm in control mouse NSC-34 cells and in WT sigma-1R transfected cells and it did not
Figure 2. The expression of mutated sigma-1R proteins induces aggregate-like structures and increased cell death in neuronal cell cultures. (A) Measurements of cell viability by MTS assay of human SH-SY5Y (left) and SK-N-BE (right) neuroblastoma cells transfected with empty vector (mock) or plasmids coding for WT, E138Q and E150K FLAG-tagged sigma-1R and treated with H2O2 (1 mM), MG132 (2.5 µM) and Thapsigargin (10 µM) for 24 hours or not treated (Basal), as indicated. Cell viability is expressed as percentage of the untreated empty vector transfected cells. Data are presented as mean ± standard error from three independent experiments and a total of 30 replicates each condition. **P < 0.01; ***P < 0.001. (B) The nuclear morphology of SH-SY5Y neuroblastoma cells transfected with FLAG-tagged WT, E138Q and E150K sigma-1R plasmids and immunostained with anti-FLAG antibodies, was assessed by Hoechst staining. The graph on the right represents the quantification of apoptotic cells, expressed as the percentage of transfected cells displaying pyknotic condensed nuclei over the total number of FLAG-positive cells. *P < 0.05; ***P < 0.001. (C) Representative images of mouse NSC-34 motoneuronal cells transfected and treated as in (B). The scale bar represents 10 µm.
colocalize with sigma-1R (Supplementary Material, Fig. S3B). Notably, the expression of sigma-1R patient mutations induced an overall increase of HSP70 positivity and an accumulation of HSP70 signal in punctate structures that were positive for sigma-1R. Unfortunately, the small percentage of transfected cells we recovered (Supplementary Material, Fig. S1C), prevented us to perform quantitative analysis of the HSP70 amount in the whole cell population extract.

Taken together, our observations indicate an induction of autophagosome formation following the expression of the two sigma-1R mutations. However, the mutated proteins seem not to be targeted for autophagic degradation, thus suggesting that their accumulation in aggregate-like structures precede recognition by the autophagy machinery or, alternatively, that they have already overwhelmed this degradation pathway.

**Discussion**

By using a combination of homozygosity mapping, IBD analysis and whole-exome sequencing approaches, we identified two homozygous missense SIGMAR1 mutations in two Italian families affected by a distal form of autosomal recessive hereditary motor neuropathy (dHMN).
In 2011, a SIGMAR1 homozygous mutation has been identified in a consanguineous Saudi Arabian family affected by a form of juvenile amyotrophic lateral sclerosis (21). This form has been classified as jALS16, however, no other mutations have been identified in extensive molecular screenings of ALS and frontotemporal lobar degeneration patients (22,23). More recently, a 3′-UTR SIGMAR1 nucleotide variation (c.672′31A > G) has been reported as candidate in two Pakistani brothers with ALS (24), however, from recent polymorphism databases, the mutated allele seems to be very common in several populations (SNP rs4879809, G-allele frequency >0.95). A SIGMAR1 splice site mutation has been also identified in a single Chinese consanguineous family with a form of dHMN (5) but the small family size may leave uncertainty about the association with the phenotype.

Despite these reports, the association of SIGMAR1 with human diseases remains still controversial as SIGMAR1 mutations have been identified only in small and isolated non-Caucasian families, with limited genetic and functional supporting data and never confirmed in other patients.

In this context, our study identifies, for the first time, SIGMAR1 mutations in Caucasian families with dHMN and definitively demonstrates the role of SIGMAR1 in the pathogenesis of a heterogeneous group of motor neuropathies mainly characterized by distal motor dysfunction (dHMN). This gene should be thus screened in patients with such phenotype worldwide.
as mutations have been now documented in different ethnic populations. Phenotypically, the spectrum of SIGMAR1 mutations manifestations is wide and includes ALS and distal spinal muscle atrophy with pyramidal signs, as reported in the present work and by Li et al. (5) (Table 1).

To note, SIGMAR1 maps within the known recessive Jerash dHMN locus, identified in consanguineous families from the Jerash region of Jordan and still orphan of the disease-causing gene (OMIM: 605726) (25). The phenotype similarity of our families, the Chinese family and the Jordan ones, allows speculation on the involvement of SIGMAR1 in the Jerash dHMN form (Table 1).

The genetic overlap between dHMN and ALS is not unusual; for instance, SETX mutations have been listed as a cause of both distal motor neuropathy with pyramidal features and the jALS-type 4 (26,27).

SIGMAR1 encodes for the sigma non-opioid intracellular receptor 1 (sigma-1R), a ubiquitously expressed integral membrane protein of the ER, mainly associated with the so-called MAM (mitochondria-associated ER membrane), exerting regulatory actions on the UPR, calcium homeostasis through interaction with the IP3 receptors, apoptosis and neuroprotection (28). Sigma-1R has been demonstrated to regulate dendritic spine formation and dendrite arborization (29) and to play a cytoprotective role after tissue injury as infarction and oxidative stress (13). Changes in SIGMAR1 expression or function have been observed in several psychiatric and neurological disorders including depression, schizophrenia, stroke, drug addiction, age-related cognitive impairments and ALS (30,14).

This multifaceted but yet poorly understood functions of sigma-1R strongly complicate the dissection of the mechanisms through which its mutations lead to pathogenesis in dHMN. However, the results of our functional studies obtained from overexpression experiments, clearly showed that both mutations affect cell viability in human neuroblastoma cells. More interestingly, while the expression of WT sigma-1R is per se protective against oxidative and ER stress (Fig. 2A and 13), the E138Q and E150K sigma-1R mutants lack this cytoprotective function.

In addition to that, our immunofluorescence studies evinced a significant mislocalization of mutated sigma-1R proteins out of MAM (Fig. 4) and a reduction of the total MAM number in cells overexpressing the E138Q and E150K sigma-1R (not shown). Despite further studies are needed to definitively clarify the issue, it is conceivable that these amino acid substitutions may affect the role of sigma-1R on the establishment and maintenance of functional MAM in human neuroblastoma cells, similar to what described in motor neurons from SIGMAR1−/− mice (9). As a consequence, the global cellular Ca²⁺ signalling in cells expressing the two sigma-1R variants is significantly impaired, as revealed by the markedly decrease in the mitochondrial Ca²⁺ uptake (Fig. 5). This is expected, given the reduced tethering between the ER Ca²⁺ store and mitochondria in these conditions, and it is in line with the published data
from SIGMAR1 loss of function models (10,15). Interestingly, the regulation of ER Ca\(^{2+}\) release has a major role in the survival of neuronal cells, as its alteration has been recently seen to significantly contribute to degeneration of spinal axons (31).

To note, despite the fact that silencing of SIGMAR1 is reported to increase cytosolic Ca\(^{2+}\) level both in resting conditions (9,10) and after stimulation (14,15) in different cellular models, our findings indicated that E138Q and E150K sigma-1R mutations selectively reduced the cytosolic Ca\(^{2+}\) transients after agonist stimulation, with no alteration of the resting cytosolic Ca\(^{2+}\) level. Several explanations could account for this apparent discrepancy: i) different cell types could manifest different sensitivity of Ca\(^{2+}\) responses to stress conditions (14,32,33); ii) the effect of mutated unfunctional proteins could be different from that of the complete lack of the protein, especially in the case of molecules with multiple binding partners as the sigma-1R chaperone; iii) an increased cytosolic Ca\(^{2+}\) level has been also reported as consequence of WT sigma-1R overexpression or sigma-1R agonist stimulation (17,34), as we found in neuroblastoma cells (not shown). Thus, the reduction of cytosolic Ca\(^{2+}\) peak observed in E138Q and E150K expressing cell would result from the lack of the sigma-1R-mediated effect.

Figure 6. The expression of mutated sigma-1R proteins induces p62 and LC3-positive autophagosome formation. (A) Representative images of human SH-SY5Y cells transfected with FLAG-tagged WT, E138Q and E150K sigma-1R and immunostained with anti-FLAG and anti-p62 (A) or anti-LC3 (B) antibodies. The scale bar represents 10 \(\mu\)m. The graphs on the right represent the quantification of p62 (A) and LC3-positive (B) dots identified as autophagosomes. Data are presented as mean ± standard error from two independent experiments. At least 40 cells were measured each condition. *P < 0.05; **P < 0.01; ***P < 0.001.
tion, especially in the context of patient-derived cells, would be the sigma-1R protein in our experimental model. Further investigation of this effect may become overt due to the expression level of this protein.

The dHMN type II (37) and our data, dHMN pathology is likely due to a loss of function mechanism cannot be definitively excluded as the abnormal sigma-1R mutations identified in dHMN patients are also relevant to the more general protein folding and homeostasis. Indeed, both variants displayed an altered distribution and tended to form aggregate-like structures. A similar aberrant sigma-1R distribution was also found in cells expressing the E102Q mutation associated with jALS16 (21) and in the splice variant associated with dHMN (5). Immunofluorescence analysis of overexpressing cells revealed that the E138Q and E150K mutations induces p62 and LC3 accumulation in the cytoplasm of cells from both human and mouse origin, indicating the involvement of the autophagy pathway. In addition, sigma-1R mutations induced the up-regulation of the stress response protein HSP70. However, the correlation between the expression of sigma-1R and LC3 accumulation in the cytoplasm of cells from both human and mouse origin, indicating the involvement of the autophagy pathway or, alternatively, that this pathway is somehow impaired of the autophagy pathway. In addition, sigma-1R mutations induced the up-regulation of the stress response protein HSP70.

Notably, the E150K and to a lesser extent the E138Q mutations induced HSP70 but not p62 colocalization with sigma-1R aggregates, similarly to what was reported in motor neurons of ALS patients carrying mutations in the VAPB gene (14). This suggests the involvement of the Ubiquitin-proteasome pathway in the degradation of mutated sigma-1R and points to a similar pathogenic mechanism for SIGMAR1 and VAPB mutations. However, the correlation between the expression of sigma-1R variants and the increase of LC3 dots indicated that the autophagy pathway is also involved in our model. Indeed, the reduction of sigma-1R function has been recently associated with induction of autophagy in a breast cancer model (35) and in retinal cells (36), further supporting the hypothesis that the identified mutations lead to the loss of SIGMAR1 function. Despite the induction of autophagosome formation, the more general protein folding and homeostasis. Indeed, both variants displayed an altered distribution and tended to form aggregate-like structures. A similar aberrant sigma-1R distribution was also found in cells expressing the E102Q mutation associated with jALS16 (21) and in the splice variant associated with dHMN (5). Immunofluorescence analysis of overexpressing cells revealed that the E138Q and E150K mutations induces p62 and LC3 accumulation in the cytoplasm of cells from both human and mouse origin, indicating the involvement of the autophagy pathway. In addition, sigma-1R mutations induced the up-regulation of the stress response protein HSP70. However, the correlation between the expression of sigma-1R and LC3 accumulation in the cytoplasm of cells from both human and mouse origin, indicating the involvement of the autophagy pathway or, alternatively, that this pathway is somehow impaired.

In light of the emerging impact of protein quality control in neurodegenerative diseases, we demonstrated that the sigma-1R mutations identified in dHMN patients are also relevant to the more general protein folding and homeostasis. Indeed, both variants displayed an altered distribution and tended to form aggregate-like structures. A similar aberrant sigma-1R distribution was also found in cells expressing the E102Q mutation associated with jALS16 (21) and in the splice variant associated with dHMN (5). Immunofluorescence analysis of overexpressing cells revealed that the E138Q and E150K mutations induces p62 and LC3 accumulation in the cytoplasm of cells from both human and mouse origin, indicating the involvement of the autophagy pathway. In addition, sigma-1R mutations induced the up-regulation of the stress response protein HSP70. However, the correlation between the expression of sigma-1R and LC3 accumulation in the cytoplasm of cells from both human and mouse origin, indicating the involvement of the autophagy pathway or, alternatively, that this pathway is somehow impaired.

### Materials and Methods

#### Genome-wide SNP genotyping and analysis

The DNA of the available family members was extracted from whole blood by following the standard phenol/chloroform method. Written informed consents were obtained from all the participants in this study.

One affected and four unaffected members of family 1 were genotyped using the Mendel Nsp 250K Genechip (Affymetrix) according to the manufacturer’s protocol. Quality controls were carried out in order to filter out SNPs with Mendelian errors and high genotyping failure. HomozygosityMapper (40) was used to identify homozygous chromosomal regions in the proband of family 1 (III-1) and not present in the two healthy brothers (III-2, III-3). HomozygosityMapper calculates a homozygosity score for each marker based on the length of the homozygous blocks. Parameters for defining a homozygous stretch are optimized for the SNP array; a minimum cut-off size of 1Mb was used to reduce the influence of non-informative segments.

Identity-by-descent (IBD) chromosomal regions were searched by RELATE, a tool for detecting pairwise IBD from unphased haplotype data. (41) To prevent bias due to linkage disequilibrium (LD), the genome-wide SNP dataset was pruned to roughly 64,000 SNPs in low LD.

#### Whole-exome sequencing and Sanger sequencing

High-coverage whole-exome sequencing was performed for the proband III-1 of family 1 at BGI. Agilent SureSelect Human All Exon v4 kit for the targeted enrichment and the Illumina Hiseq2000 platform for the sequencing were used. End-paired reads were aligned against the human reference sequence (hg19) with the SOAPaligner/SOAP2 program, and all sequence variants were called by SOAppnp software. GATK and Copy Number Inference From Exome Reads (CoNIFER) software were used to detect small and large indels respectively. (42,43) Called variants were then searched in dbSNP146, 1000 Genomes, and ExAC repositories, to filter out common Single Nucleotide Polymorphisms (SNPs) with MAF > 0.5%. Variants were then prioritized according

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<th>Family 1 dHMN (this study)</th>
<th>Family 2 dHMN (this study)</th>
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<th>Arabian Family jALS16(21)</th>
<th>Chinese Family dHMN(5)</th>
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<td>+</td>
</tr>
<tr>
<td>Proximal muscle wasting and weakness</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Bulbar signs</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tr>
<tr>
<td>Respiratory signs</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>n.a</td>
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</tbody>
</table>

In conclusion, our findings represent a step forward the dissection of these mechanisms providing insights into this neurodegenerative disorder and hopefully leading to the identification of potential therapeutic strategies based on the use of sigma-1R-specific drugs (39).
to the type of mutation (deletion/insertion > nonsense > missense), amino acid conservation, prediction of pathogenicity and relevance of the candidate gene to the disease. A nucleotide position was considered highly conserved whenever the relative PhoIoP score was > 2. In silico variant effect predictions were performed by a majority vote across three independent algorithms: MutationTaster2 (44), CONDEL (45) and LRT (46).

Control DNA samples were tested for both mutations by direct sequencing on the 3100 ABI Prism Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

**Supplementary Material**

Supplementary Material is available at HMG Online.

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### References


