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Heat shock protein 27 R127W mutation: evidence of a continuum between axonal CMT and distal HMN

¹Paolo Solla, ¹Alessandro Vannelli, ²Alessandra Bolino, ¹Giovanni Marrosu, ²Silvia Coviello, ¹Maria Rita Murru, ¹Stefania Tranquilli, ¹Daniela Corongiu, ³Sara Benedetti, ¹Maria Giovanna Marrosu.

¹Centro Sclerosi Multipla, Ospedale Binaghi, Via Is Guadazzonis 2, Cagliari, Italy

²San Raffaele Scientific Institute, Milan, Italy

³Laboratory of Clinical Molecular Biology DIBIT 2, Diagnostic and Research San Raffaele, Milan, Italy

Running title: HSP27 mutation determining both CMT2 and DHMN

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Address for correspondence

Dr. Paolo Solla, MD

Centro Sclerosi Multipla, Ospedale Binaghi

Via Is Guadazzonis 2

09126 Cagliari (Italy)

Telephone number: +390706092901

Fax number: +390706092929

E-mail: paosol29@yahoo.it

Introduction. Heat shock protein 27 (HSP27), also known as HSPB1, belongs to the superfamily of small heat-shock proteins and is mainly implicated in the assembly of neurofilament network¹. HSP27 mutations have been reported to cause both Charcot-Marie-Tooth disease (CMT) type 2F and distal hereditary motor neuropathy (dHMN)¹⁻⁶, although never previously in a single family. The aim of this study was to divulge clinical and electrophysiological findings obtained in a single large Sardinian family bearing the HSP27 R127W mutation, revealing how affected subjects featured a marked interfamilial phenotypic heterogeneity varying from pure axonal CMT2F or dHMN phenotype, with one single patient clearly presenting associated features of spastic paraplegia.

Patients and methods. The index case, subject IV-2, a 44-year-old male, was investigated initially and his family tree is reported in Figure 1, clearly illustrating how other relatives were affected, leaning considerably in favor of an autosomal dominant means of inheritance. Divergent clinical features were observed in other affected family members. In spite of the index case featuring a marked association of spastic gait with peripheral neuropathy, pyramidal signs were virtually absent among the relatives, with predominant distal muscle weakness and wasting of the lower limbs, compatible with a diagnosis of peroneal muscular atrophy. Over the last three years, twenty-one members of this 5-generation Sardinian family have been studied, twenty of whom, including 13 members affected by peroneal muscular atrophy syndrome, have undergone screening for CMT2 mutations. Informed written consent was obtained from all participants.

Clinical and electrophysiological examination. Data collected included onset of symptoms, duration of disease, initial symptoms, disease course, presence of muscle cramps or pain, impairments (i.e., muscle weakness and sensory dysfunction) and other additional features. Twelve patients and 8 unaffected relatives were subjected to clinical examination and examined for: weakness and atrophy of the distal and/or proximal parts of the lower and upper limb muscles, deep tendon reflexes of the lower

and upper limb muscles, presence of extensor plantar responses and Romberg sign, presence of foot and hand deformities, nerve hypertrophy and scoliosis. Vibration, touch, joint position and pinprick sensation were examined at various sites in the lower and upper limbs. In addition, the presence of tremor, trophic alterations of the feet and knees, cranial nerve abnormalities, involvement of vocal cords, ataxia, contractures, deafness, optic nerve atrophy, pyramidal signs, dysautonomia, and dementia was assessed. To determine disease severity in terms of ability to walk and run, each patient was assessed by means of the Functional Disability Scale (FDS)⁷. A similar scale for the evaluation of a personal neurological disability affecting the upper limbs, with a six-point scale ranging from 0 to 5, was assessed and determined as follows: 0 = normal; 1 = normal but with cramps, 2 = fatigability in writing, 3 = handling difficult; 4 = need help to use hands; 5 = completely dependent. A standardized electrophysiological study was performed in eleven patients and six unaffected relatives. Motor nerve conduction velocities (MNCVs) and compound muscle action potentials (CMAPs) were investigated in the median and peroneal nerves. Antidromic sensory nerve conduction velocity (SNCV) and sensory nerve action potentials (SNAPs) were investigated in the median and in sural nerves. A concentric needle electromyography was performed using standard techniques. Data correlation tests were performed using Pearson's correlation coefficient or Spearman's rank correlations. Diagnoses of CMT and dHMN were made according to common diagnostic criteria⁸.

Molecular genetic analysis. Genomic DNA was extracted from peripheral blood by standard methods. A panel of 15 highly polymorphic STRs was used to exclude duplication or deletion of chromosome 17p11.2-p12⁹. The coding exons of mitofusin 2 (MFN2), myelin protein zero (MPZ), gap junction protein beta 1 (GJB1), neurofilament light polypeptide (NEFL), and ganglioside-induced differentiation-associated protein 1 (GDAP1) genes were analyzed by direct sequencing , ~~with no mutations being revealed~~ (Details of primer sequences used to amplify the MFN2, MPZ, GJB1, NEFL, and GDAP1 genes are available on request). In addition, only exon 3 of the Berardinelli-Seip

congenital lipodystrophy-2 (BSCL2) gene was examined for the known Silver disease mutations, ~~with the same result~~¹⁰ (conditions available on request). Finally, to analyze HSPB1 coding sequence, exon 1, 2, and 3 were amplified using primers designed on flanking intronic sequences as follows: exon 1: forward ACGGGTCATTGCCATTAATAGAGACC and reverse AGAAGCGACCCGCACTCCCAATTC; exon 2: forward TGTTAATCCCTACCAGCCTGCAG and reverse, AGGCAAGCGTTACATTACACACCG; exon 3: forward ACGCGGAAATACACGTGAGTCCTGG and reverse TGCCTGAGGCTTCCTTCCACAAACACC. PCR and sequencing reactions were performed under standard conditions. PCR purification was carried out using ExoSAP-IT (Amersham) whereas sequencing reactions using the BigDye v1.1 Terminator Cycle Sequencing Kit (Applied Biosystems). Sequences were loaded onto a 3130XL Genetic Analyzer and analyzed using the [Sequencing Analysis Software](#) version 5.2.

Results. Among the twenty subjects of the family screened for CMT2 mutations, all 13 members considered as being clinically affected by peroneal muscular atrophy syndrome, proved heterozygous for the known HSP27 p. R127W mutation in exon 2. Neither of the other genes studied in these subjects revealed the presence of pathogenic mutations or polymorphisms. Furthermore, none of the 7 remaining unaffected relatives in our series harbored a mutation in exon 2 of HSP 27 gene. Clinical features of twelve patients are listed in table 1. The age at onset was clearly available for ten of the patients examined. Mean age at onset ($\pm$ standard deviation) was 31.2 ± 7.2 years ~~(median 30, range 20 to 43). Two persons had onset at age 40 years or beyond.~~ Mean age at investigation was 45.2 ± 12.9 years ~~(median 43, range 30 to 72),~~ and mean disease duration at the time of investigation was 14 ± 12.9 years ~~(median 20, range 1 to 48).~~ Course of the disease was slowly progressive in all patients. In five patients aged from 30 to 54 years, sensory examination was entirely normal ~~revealing no deficits or abnormalities,~~ while a further two other patients featured a very mild vibration sensory loss in the feet. In the remaining 5 patients with sensory impairment, examination revealed a predominant loss of

sensation in the legs, while in the arms mild subjective [hypopallesthesia](#) ~~hypallescithesia~~ was present in one patient. One patient alone presented total loss of sensation in the lower limbs. Clinical characteristics and sensory impairments were more pronounced with age. Involvement of vocal cords, contractures, deafness, optic nerve atrophy and dementia were not observed in any patients. With the exception of the index patient, no subjects presented bladder or sexual dysfunctions. Mean FDS ($\pm$ DS) scored was 3 ± 1.8 ; ~~4 with only a single patient confined to a wheelchair, likely reflecting a more severe impairment due to the longer duration of disease (48 years)-~~, whilst [mean](#) Mean scores for neurological disability in the upper limbs was 1.8 ± 1.7 . ~~No patients achieved a score of 5 (e.g. totally disabled).~~

Electrophysiological studies (Table 2) revealed a marked predominance of motor and sensory abnormalities in the legs. Peroneal CMAP amplitudes were generally reduced and were totally absent in four patients. ~~In patients with no absence of peroneal CMAPs, MNCVs of this nerve were lower than 40 m/s but exceeded 38 m/s in three patents, and lower than 38 m/s in only one patient.~~ Evaluation disclosed normal motor conduction studies of the median nerve in almost all patients, recording MNCVs of less than 38 m/s in only one patient, [likely due \(as the reduction in peroneal conduction velocity under 38 m/s in patient III5\) to axonal loss with secondary demyelinating process.](#) Sural SNAPs were absent in only two patients, although their amplitude was markedly reduced in a further three patients. Three of the remaining affected individuals displayed sural SNAPs within normal range, although closer to lower reference limits. Median nerve SNAPs were normal except for a slightly reduced amplitude in two patients. Statistical analysis showed that duration of disease correlates both with the Sural SNAPs Amplitudes ($r = -.903$; $p < 0.001$) and Median SNAPs Amplitudes ($r = -.669$; $p < 0.05$). According to the Classification and Diagnostic Guidelines for CMT2 and dHMN⁸, of the ten patients who had undergone both clinical and neurophysiological examination, five were diagnosed as CMT2, ~~and a further~~ two as dHMN ([III:24 and IV:12](#)) and a further ~~t~~ Two patients ([IV:25 and IV:9](#) in ~~whom clinical/electrophysiological findings (very slight reduction of vibration sensory loss in the feet~~

~~but normal sensory nerve potentials at electrophysiological studies) were insufficient to warrant their inclusion in one of the above categories,~~ were labeled as an intermediate type. In fact, in the latter two patients, clinical/electrophysiological findings (mild vibration sense loss in the feet but normal sensory nerve potentials at electrophysiological studies) were insufficient to warrant their clear inclusion in one of the above categories, owing to the fact that current classification requires the absence or presence of small sensory nerve action potentials as inclusion criteria for CMT2, but at the same time formulates the detection of impaired sensation as exclusion criteria for dHMN. Finally, due to the presence of spastic paraplegia, the index patient did not meet established criteria⁸ for the diagnosis of CMT or dHMN. Indeed, these criteria⁸ exclude patients with significant involvement of CNS, including pyramidal tract. In this patient, other differential diagnoses such as spinal cord compression; HTLV-1-associated myelopathy or other disorders, were excluded.

Comment. We describe a large Sardinian family with predominant autosomal dominant axonal neuropathy in which a missense mutation (R127W) in the HSP20- α -crystallin domain of the gene encoding HSP27 was identified. The study of this large family allowed us to delineate the clinical spectrum, natural history and electrophysiological findings of this HSP27 mutation. To our knowledge, this is the first described family with HSP27 mutation where axonal CMT and dHMN were both present in different family members with a significant correlation between duration of disease and the occurrence of reduction of SNAP amplitudes, implying that sensory impairments may not be present in the initial stages of the disease, rather being manifested at a later stage. This point is of particular importance, demonstrating a difference in intensity rather than in nature between CMT2 and dHMN in the cases examined. Moreover, at least two members of the family studied here, diagnosed as affected by clear-cut CMT 2 forms, had previously been labeled as dHMN in the past, as provided for by standard criteria⁸. Furthermore, in several members of the large Sardinian family described in this paper, the clinical phenotype seemed to produce peculiar features not fully corresponding to either of

the two forms, raising important questions concerning the specificity of criteria⁸ that subdivide CMT from dHMN forms. [To this regard, the exclusively motor involvement in dHMN is still a matter of debate due to minor sensory abnormalities being described elsewhere¹¹](#). Compared with the only article² reporting the clinical features of seven Chinese patients with HSP27 R127W mutation, the present family was characterized by an earlier age at onset. In addition, while a mild to moderate sensory impairment of the hands was observed in all Chinese patients, this observation was not replicated in our study. Indeed, with one sole exception, none of our patients exhibited sensory abnormalities in the upper limbs. Bearing in mind the correlation between duration of disease and reduction of SNAP amplitudes both in the sural and median nerves, the latter findings can be easily explained by a higher age of Asian patients at examination ($63,9 \pm 10,7$) compared to that of Sardinian subjects ($45,2 \pm 12,9$). Mutations in another HSP (HSP22)^{[11](#)[12](#)[12](#)[13](#)} may present both with CMT2 and dHMN phenotypes; likewise, mutations in glycyl-tRNA synthetase gene have been reported to be associated with both ~~diseases~~^{[13](#)} [diseases^{14,1415}](#). Mechanisms whereby the same gene mutation is capable of causing different diseases remain unclear. On the grounds of the hypothesis that the primary pathologic process resides respectively only in the axons or in the neuron cell body, CMT 2 types are usually classified as axonal neuropathies (axonopathies) while dHMNs are considered as a separate entity and classified as motor neuronopathies, although considerable variations may ~~occur~~^{[11](#)} [occur¹²](#). However, as our knowledge on gene mutations increases, growing evidence is accumulating to support the notion whereby a similar distinction between axonal CMT and dHMN is somewhat dogmatic. Our findings are suggestive of the existence of a continuum between the two entities, thus advocating a reconsideration of this strict pathonological classification. The latter concept of a common spectrum is in full accordance with data reported by ~~Irobi~~^{[15](#)} [Irobi¹¹](#), underlining the arbitrary nature of the previously mentioned pathological dichotomization due to the fact that the clinical features manifested in dHMN are characterized by a well-established distal distribution indistinguishable from CMT2. Irobi

not only suggests a major involvement of axonal sites in pathologic damage, but moreover observes how, owing to lack of autopsy studies, little is known about the status of the anterior horn motor neuron ~~bodies~~¹⁵ bodies¹¹. An additional element of discussion is represented by the occurrence of spastic paraplegia in the index patient carrying the same HSP mutation. Although manifested infrequently, the presence of pyramidal features has been described in several patients with axonal form of CMT with variable severity ranging from hyperreflexia to overt spastic paraplegia. Dyck and Lambert¹⁶ classified families affected by CMT with associated spastic paraplegia as hereditary motor and sensory neuropathy (HMNS) type V, while CMT patients plus mild pyramidal signs but without spastic paraplegia were labelled as peroneal muscular atrophy with pyramidal features by Harding and Thomas¹⁷. A systematic revision of case histories¹⁸ has led to postulate that the presence of brisk reflexes in the legs and extensor plantar responses may be viewed as forming part of CMT2 phenotype. The latter opinion would seem to be in agreement with findings obtained in the present study in which a patient with brisk knee reflexes was indeed observed. Recent observations indicate that CMT with pyramidal signs is genetically heterogeneous¹⁹ and may be caused by mutations of at least two different genes: MFN2¹⁹ and N88S BSCL2 mutation²⁰. Thus, although an isolated occurrence, the certain onset of spastic paraplegia associated with CMT form in the patient examined would seem to convey an additional feature of phenotypic variability in subjects with HSP27 mutations. However, the authors of the present study were not in a conclusive position to speculate whether this occurrence was specifically linked to the single mutation rather than to other epigenetic factors. Further studies should be performed with the aim of better defining this intriguing observation. To conclude, the findings obtained in the present study, broadening the spectrum of clinical manifestations of disorders associated with HSP27 mutations, support not only the hypothesis of a continuum between CMT2 and dHMN forms, but appear moreover to suggest a possible common spectrum between these entities and several forms of CMT plus pyramidal features (HMSN V), providing important implications for

molecular genetic testing. Taken together, the data obtained suggest the need to provide for the establishing of more accurate and widely accepted working definitions to be applied in the case of CMT2/dHMN and the other CMT plus forms. These definitions should be based largely on clinical information and integrated with genetic data in order to further enhance the understanding of the pathological mechanisms involved and to provide for the development of targeted therapeutic interventions in the future.

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Pt	Sex	Age at onset	Duration of disease	FDS	Upper hands scale	Romberg sign	Weakness		Atrophy		Sensation loss				Reflexes			Plantar response	Pes cavus hammertoes	Hands Tremor	Trophic alterations	Additional symptoms
											Touch and pinprick	Vibration and joint position		Upper limb	Ankle jerks	Knee jerks						
		years	years				UL	LL	UL	LL	UL	LL	UL	LL								
IV:2	M	27	17	4	2	+	+	+	+	+	−	+	−	+	N	↑↑	↑↑	E	−	+	+	spastic paraplegia, bladder and sexual dysfunctions, cramps
IV:22	F	34	4	1	1	+	+	+	+	+	−	+	−	+	−	−	−	NE	+	+	−	
III:15	F	43	14	4	4	+	+	+	+	+	−	+	+	+	↓	−	−	NE	−	+	−	hands and feet paraesthesias cramps
III:24	F	42	12	4	3	+	+	+	−	+	−	−	−	−	↓	−	−	NE	−	+	−	
IV:19	M	30	15	3	3	+	+	+	−	+	−	+	−	+	N	−	−	P	+	+	−	cramps
IV:25	F	20	10	3	3	−	+	+	+	+	−	−	−	±	N	−	↑	NE	+	+	+	
IV:6	M	30	12	3	0	+	−	+	−	+	−	−	−	−	−	−	−	NE	−	+	−	
IV:9	F	33	7	1	0	−	−	+	−	−	−	−	−	±	N	−	↓	NE	−	−	−	
IV:12	F	29	1	0	0	−	−	−	−	−	−	−	−	−	N	−	−	NE	−	−	−	cramps
III:2	F	24	48	7	4	NS	+	+	+	+	−	+	−	+	−	−	−	NS	+	+	−	
III:9	F	NA	NA	3	NS	-	+	+	+	+	-	-	-	-	-	-	-	NS	NS	−	-	
III:5	F	NA	NA	3	0	-	-	+	-	+	-	-	-	-	+	-	↓	NE	+	+	-	

Table 1. Clinical features of patients with HSP27 R127W mutation.
Legend. Pt, patient; FDS, Functional Disability Scale; UL, Upper limbs, LL, Lower limbs; m, male; f, female; +, present; -, absent; ±, mild feet hypopallaesthesia; N, Normal; ↑↑, clonus; ↑, brisks; ↓, reduced; E, Extension, F, Flexion; NE, Neutral; NS, Not studied; NA, not available.

Table 2: Electrophysiological findings of Sardinian patients with the HSP27 mutation.

Patient	Median nerve		Median nerve		Peroneal nerve		Sural nerve		EMG
	MNCV	CMAP	SNCV	SNAP	MNCV	CMAP	SNCV	SNAP	
	(m/s)	(mV)	(m/s)	(µV)	(m/s)	(mV)	(m/s)	(µV)	
	NV (SD)	48.8 (38)	6.9 (3.9)	56.2 (44)	39.0 (20)	48.3 (40)	5.1 (2.5)	52.5 (5.6)	18.9 (9.5)
IV:2	58,3	8,9	63,4	22	39	2,9	50	2,8	LL chronic denervation
IV:22	56,4	19,2	62,5	49,5	40,5	0,3	53,8	12,3	LL severe chronic denervation
III:15	30,7	0,4	ND	5,2	39,3	0,7	ND	-	LL and UL severe chronic denervation
IV:19	53,8	12,5	60,2	33,2	ND	-	29,8	0,61	LL severe chronic denervation
IV:6	47,5	5,4	55,8	27,2	ND	-	32,9	3,8	LL severe chronic denervation
III:24	51,1	6	59,7	40,7	39,5	0,3	41,2	9,6	LL chronic denervation
IV:25	50,6	4,7	61,3	68,6	ND	-	51,9	9,7	LL severe chronic denervation
IV:9	53,9	11,9	60,8	57,3	44,9	2,5	48,1	12,4	LL chronic denervation
IV:12	58,8	13,1	71,2	54,5	43,1	3,7	45,3	16,3	LL chronic denervation
III:9	46	↓	NS	NS	ND	-	NS	NS	LL severe chronic denervation
III:5	NS	NS	ND	5	31	↓	ND	-	LL > UL severe chronic denervation

Abbreviations: NV, normal value; SD, standard deviation, MNCV, Motor nerve conduction velocity; SNCV, Sensor nerve conduction velocity; ND, not determined; NS, Not studied; CMAP, compound muscle action potential; SNAP, sensory nerve action potentials; -, absent; ↓, reduced; LL, lower limb; UL, upper limb.

FIGURE 1: Pedigree of the family with HSP27 p. R127W mutation.

Legend. Black symbols indicate affected family members; slash, deceased; circles, females; squares, males; *, genotyped individual; black arrows, subjects undergoing clinical and electrophysiological studies; white arrows, subjects studied clinically. Index patient - subject IV:2.