ORIGINAL CONTRIBUTION

Mutation Analysis of the Small Heat Shock Protein 27 Gene in Chinese Patients With Charcot-Marie-Tooth Disease

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Background: Charcot-Marie-Tooth (CMT) disease, the most common hereditary peripheral neuropathy, is highly clinically and genetically heterogeneous, and mutations in at least 18 genes have been identified. Recently, mutations in small heat shock protein 27 (*Hsp*27) were reported to cause CMT disease type 2F and distal hereditary motor neuropathy.

Objective: To investigate the frequency and phenotypic features of an Hsp27 mutation in Chinese patients with CMT disease.

Design: DNA samples from 114 unrelated patients with CMT disease were screened for mutations in Hsp27 by polymerase chain reaction and direct sequencing. A cosegregated study was performed using the *Mbi*I restriction endonuclease, and 50 healthy control subjects were analyzed. Haplotype analysis was performed using 5 short tandem repeat markers to analyze whether the families with the same mutation probably had a common ancestor.

Results: One missense mutation, C379T, was detected in 4 autosomal dominant families with CMT disease type 2, and haplotype analysis indicated that the 4 families probably had a common founder. The frequency of the Hsp27 mutation is 0.9% (1/111) in Chinese patients with CMT disease in our study, and the phenotypes were characterized by later onset (age, 35-60 years) and mild sensory impairments. Electrophysiological findings showed moderately to severely slowed nerve conduction velocities in lower limb nerves but normal or mildly reduced velocities in upper limb nerves.

Conclusions: To our knowledge, this is the first report of an Hsp27 mutation in the People's Republic of China. The C379T mutation in Hsp27 also causes CMT disease type 2, except for distal hereditary motor neuropathy, and the phenotypes are distinct from the family with CMT disease type 2F described previously. A mutation of Hsp27 may be uncommon in Chinese patients with CMT disease.

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CHARCOT-MARIE-TOOTH (CMT) disease is the most common hereditary peripheral neuropathy, with a prevalence of approximately 1 in 2500,[1] and causes progressive weakness and atrophy of the distal legs and arms, with decreased or absent tendon reflexes. According to the electrophysiological and pathological investigations, it can be further divided into 2 types: CMT disease type 1, the demyelinating form, characterized by a slow motor median nerve conduction velocity (NCV) (<38 m/s); and CMT disease type 2, the axonal form, with a normal or slightly reduced NCV (≥38 m/s).[2,3] Charcot-Marie-Tooth disease is highly clinically and genetically heterogeneous, and mutations have been reported in at least 18 genes (information available at: Inherited Peripheral Neuropathies Mutation Database [http://molgen-www.uia.ac.be/CMTMutations/]).

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Recently, 5 missense mutations in small heat shock protein 27 (Hsp27) have been identified to cause CMT disease type 2F and distal hereditary motor neuropathy (dHMN), most of which occur in the highly conserved Hsp20 α-crystallin domain of the protein.[4] Small heat shock protein 27 is a member of the Hsp superfamily and is abundant in different nerve cells.[5] Several studies showed that wild-type Hsp27 has neuroprotective action. It can protect neurons against cell apoptosis induced by kainic acid, retinoic acid, or nerve growth factor withdrawal,[6-9] and its up-regulation and phosphorylation is

Table 1. Sequence of the Primers*

Exon	Segment	Primer Sequence (5′-3′) Forward	Primer Sequence (5′-3′) Reverse	Fragment Size, Base Pairs
1	1	CCGACTGGAGGAGCATAAAA	GGGCGAAGTGGTTGACAT	398
	2	GAGTGGTCGCAGTGGTTAGG	CTGAGCAAGGGAATCAGGAG	395
2	1	CCAACCCCCTCTGTTAATCC	GAGGAAAGGCAAGCGTTACA	300
3	1	ACGAGCATGGCTACATCTCC	GTGACAGGTGGTTGCTTTGA	476

*The annealing temperature was 60°C for all primers.

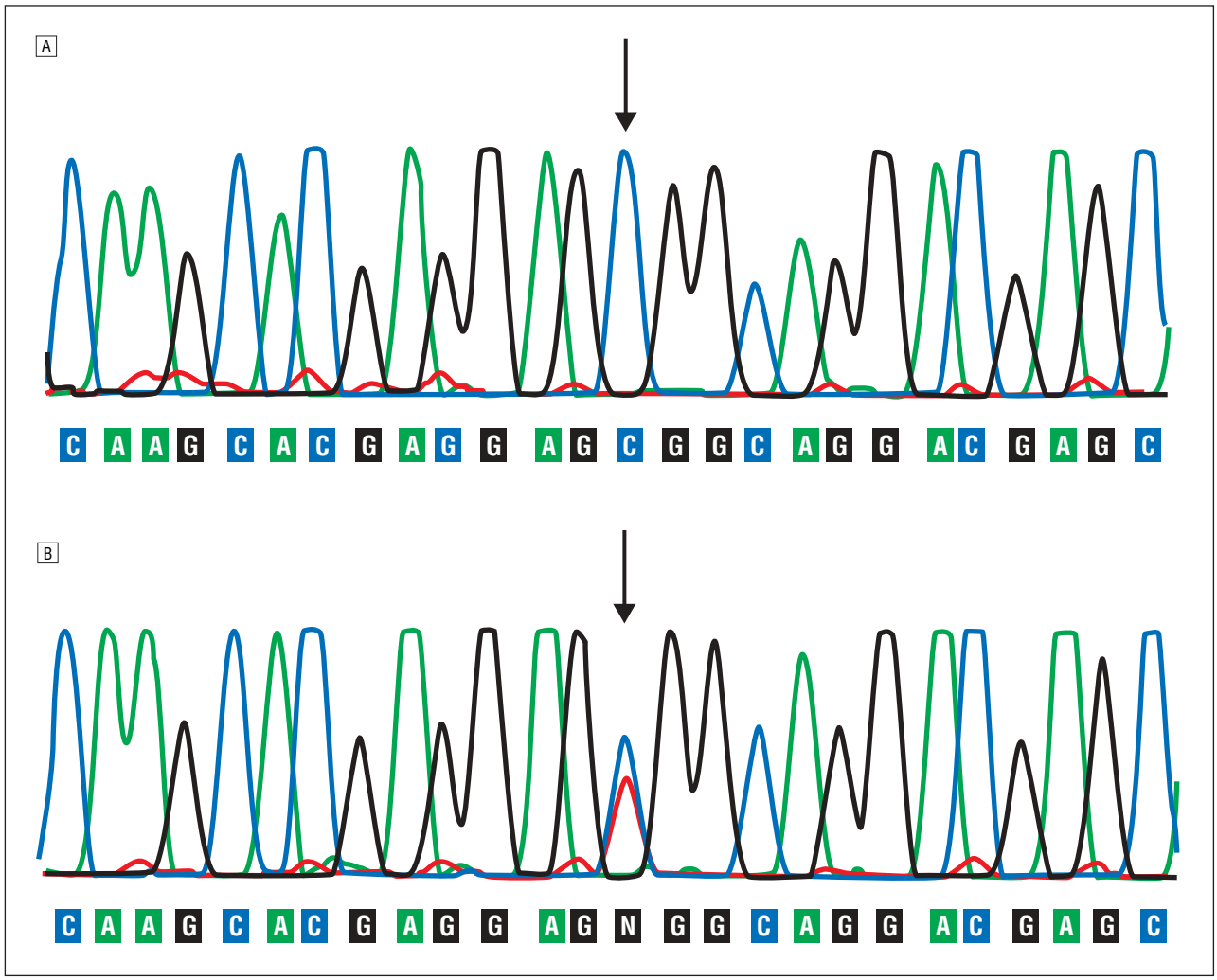

Figure 1. Wild-type sequence (with the corresponding base of the mutation indicated [arrow]) (A) and sequence that identified a C379T mutation (arrow) in small heat shock protein 27 (B).

necessary for sensory and motor neuron survival following peripheral nerve injury.[10] Reversely, mutant Hsp27 can reduce the viability of neuronal cells and impaired neurofilament assembly.[4]

To study the Chinese patients with CMT disease for mutations in Hsp27 and to analyze its phenotypic features, we screened 114 individuals and detected a missense mutation (C379T) in 4 autosomal dominant families with CMT disease type 2. We also reported the clinical electrophysiological features of the patients with the detected mutation.

METHODS

PATIENTS

This study included 114 unrelated individuals with CMT disease. All patients were diagnosed as having the disease by 2 neurologists (B.T., X.L., G.Z., W.L., C. Z., B.C., F.Z., L.S., R.Z., or H.J.) according to the diagnosis criteria of CMT disease type 2, and they were all of Han nationality. Informed consent was obtained, and 50 healthy control subjects were screened to determine whether the detected sequence variation was a polymorphism or a mutation. Following detection of the mutation in an

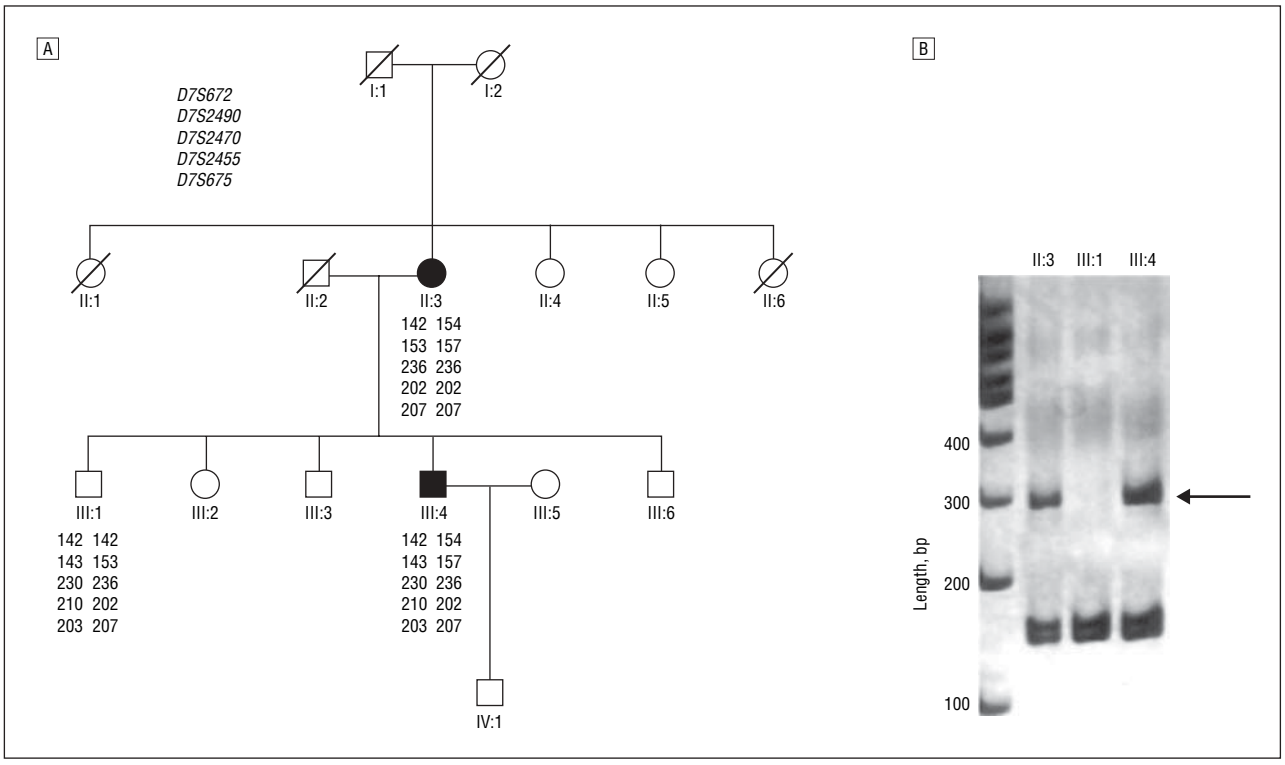

Figure 2. Family 1 and haplotype analysis (A) and the restricted digest of the family members (B). In A, circles indicate females; squares, males; shaded symbols, subjects with the C379T mutation in small heat shock protein 27; unshaded symbols, subjects without the mutation; and symbols with diagonal lines, deceased subjects. In B, mutated fragments (arrow) are not digested and are 300 base pairs (bp) long, whereas digested healthy fragments are 155 and 145 bp long.

index patient, additional family members (if available) were analyzed to determine whether the observed sequence variation cosegregated with the disease.

PROCEDURES

Standard procedures were used to extract DNA from peripheral blood samples. Three exons of the *Hsp*27 gene were amplified as 4 polymerase chain reaction (PCR) fragments. Primers were designed to amplify each entire exon with its flanking sequence; the sequences are listed in **Table 1**. All fragments were amplified using polymerase (Hotstar Taq DNA polymerase; Qiagen, Studio City, Calif) according to the manufacturer's protocol. The PCR products were performed in thermocyclers (Perkin-Elmer, Foster City, Calif), starting with an initial denaturation of 15 minutes at 95°C; followed by 32 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute; followed by one last step of extension at 72°C for 5 minutes. Electrophoresis of the PCR products was performed on 6% polyacrylamide gel in 0.5% Tris borate EDTA, followed by silver staining. For the 114 index patients, PCR products were gel purified and sequenced directly on a sequencer (ABI3100; Applied Biosystems, Changsha). The data were collected and analyzed using sequencing analysis software (DNASTAR, Inc, Madison, Wis). The detected sequence variation abolishes an *Mbi*I restriction endonuclease site in the DNA of the affected patients. Therefore, DNA obtained by PCR amplification of Hsp27 exon 2 was digested with *Mbi*I and separated by electrophoresis on polyacrylamide gel for the cosegregation study in family members and for excluding the polymorphism study in 50 controls.

Haplotype analysis was performed in the 4 mutation-detected families using 5 short tandem repeat markers (*D7S672*, *D7S2490*, *D7S2470*, *D7S2455*, and *D7S675*).[4] Markers were amplified by multiplexed PCR from genomic DNA using DNA polymerase (HotGoldstar; Eurogentec, Seraing, Belgium) according to the manufacturer's protocol. The reaction started with an initial denaturation of 12 minutes at 94°C; followed by 15 cycles of denaturation (94°C for 30 seconds), annealing (starting at 63°C and reducing by 0.5°C after every cycle, for 1 minute), and extension (72°C for 1 minute 50 seconds); then processed through 24 cycles of denaturation (94°C for 30 seconds), annealing (56°C for 1 minute), and extension (72°C for 1 minute 50 seconds); followed by one last step of extension (72°C for 15 minutes). Amplifications were performed in a 96-well reaction plate using thermocyclers (Perkin-Elmer), and the products were separated by capillary electrophoresis on an automated DNA sequencer (ABI3100; Applied Biosystems). The data were collected by computer software (ABI3100 Data Collection Software, version 1.01) and analyzed by different software (GeneScan and Genotyper analysis software; Applied Biosystems).

RESULTS

MOLECULAR AND HAPLOTYPE ANALYSES

We performed mutation screening of the entire coding region, exon/intron boundaries of Hsp27, in a group of 114 unrelated patients with CMT disease, and discovered a C379T mutation that generated the amino acid alterations of Arg127Trp in 4 families with autosomal dominant CMT disease type 2 (**Figure 1**). These 4 families all came from the Hunan province. The mutation was heterozygous, segregated perfectly with the CMT disease phenotype, and was absent in 50 healthy controls (**Figures 2**, **3**, **4**, and **5**). We also compared the disease-segregating haplotypes in relatives of the 4 families (if available). Haplotype analysis indicated that 3 families (1, 3, and 4) had a common ances-

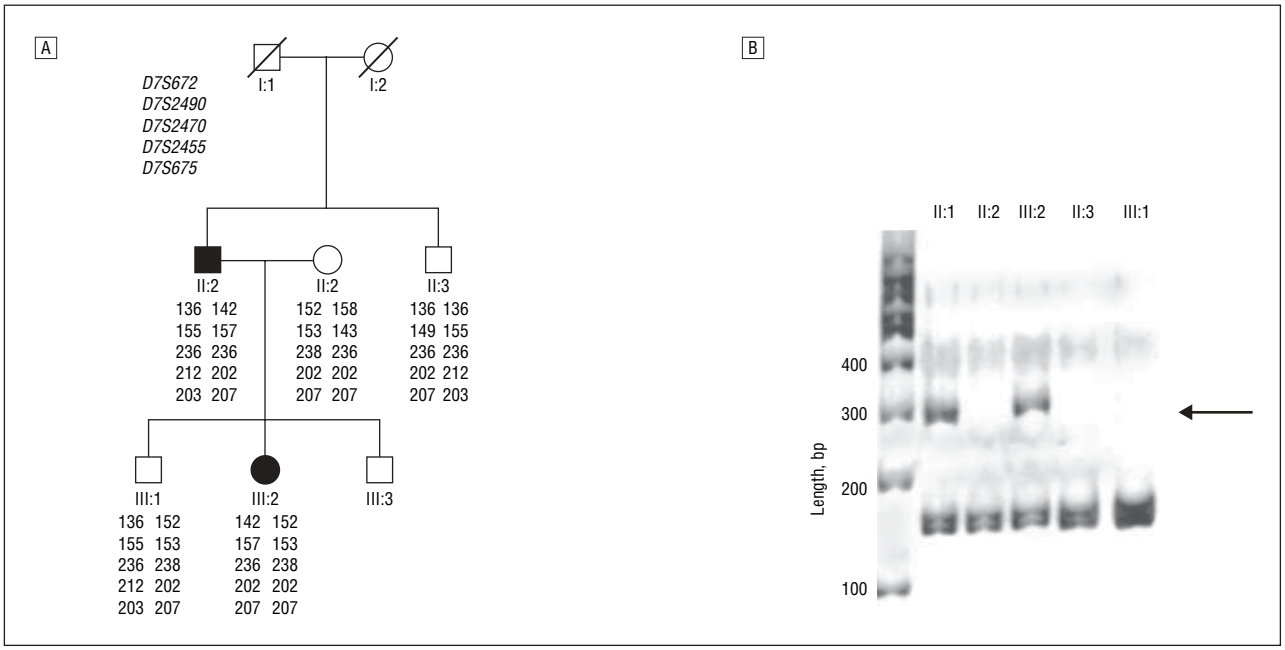

Figure 3. Family 2 and haplotype analysis (A) and the restricted digest of the family members (B). In B, the abnormal migration alteration is indicated (arrow). An explanation of all symbols/abbreviations is given in the legend to Figure 2.

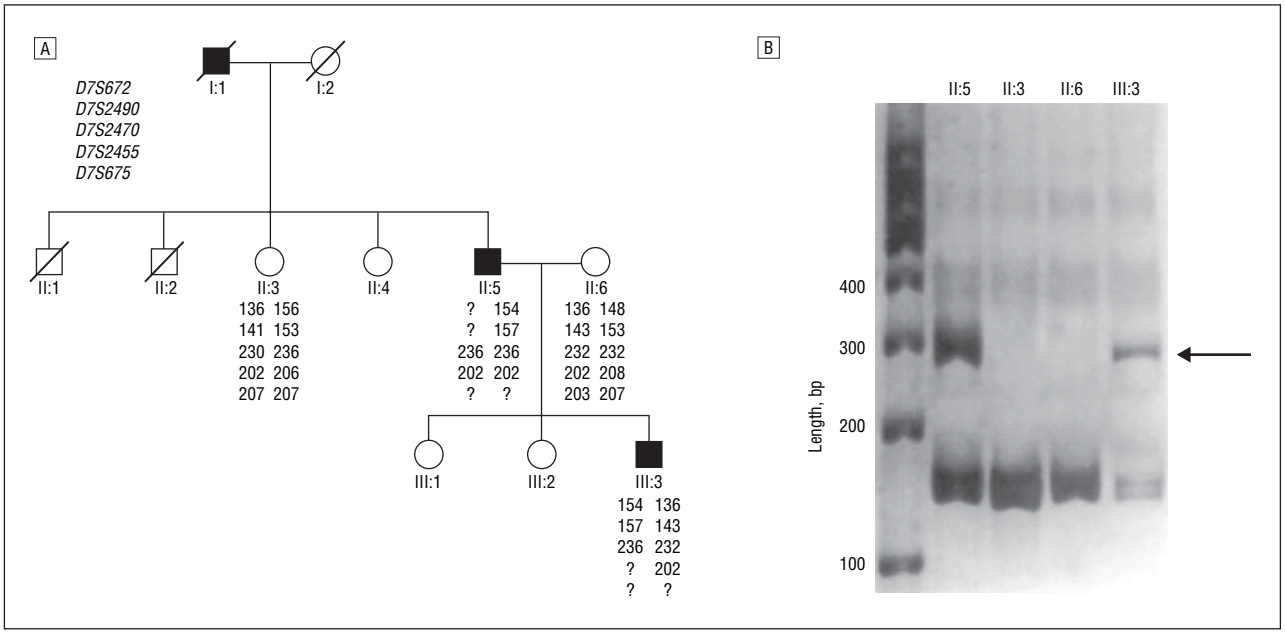

Figure 4. Family 3 and haplotype analysis (A) and the restricted digest of the family members (B). In B, the abnormal migration alteration is indicated (arrow). An explanation of all symbols/abbreviations is given in the legend to Figure 2.

tor and the other family (2) was also closely related to them, suggesting that the 4 families probably had a common founder.

CLINICAL INFORMATION OF PATIENTS WITH THE HSP27 MUTATION

At least 1 affected member of each family was examined. According to the diagnosis criteria,[11] families 1, 2, and 4 were diagnosed as having CMT disease type 2. Family 3 cannot be divided according to present records. The clinical and electrophysiological features of the 4 families are summarized in **Table 2** and **Table 3** in detail. The initial symptoms in all patients were difficulty in walking, followed by weakness and atrophy of the distal parts of the limbs. Tendon reflexes were depressed or absent, with mild stocking sensory loss to pricking pain or vibration in the feet. Talipes cavus or clawhand deformity was observed in most of the patients. Other symptoms, including cramps and fasciculations in the lower limbs and numbness and tingling in the feet, were seen in 1 patient each. There was no cranial nerve involvement and no cerebellar or pyramidal signs in all patients. Patient III:2 of family 2, aged

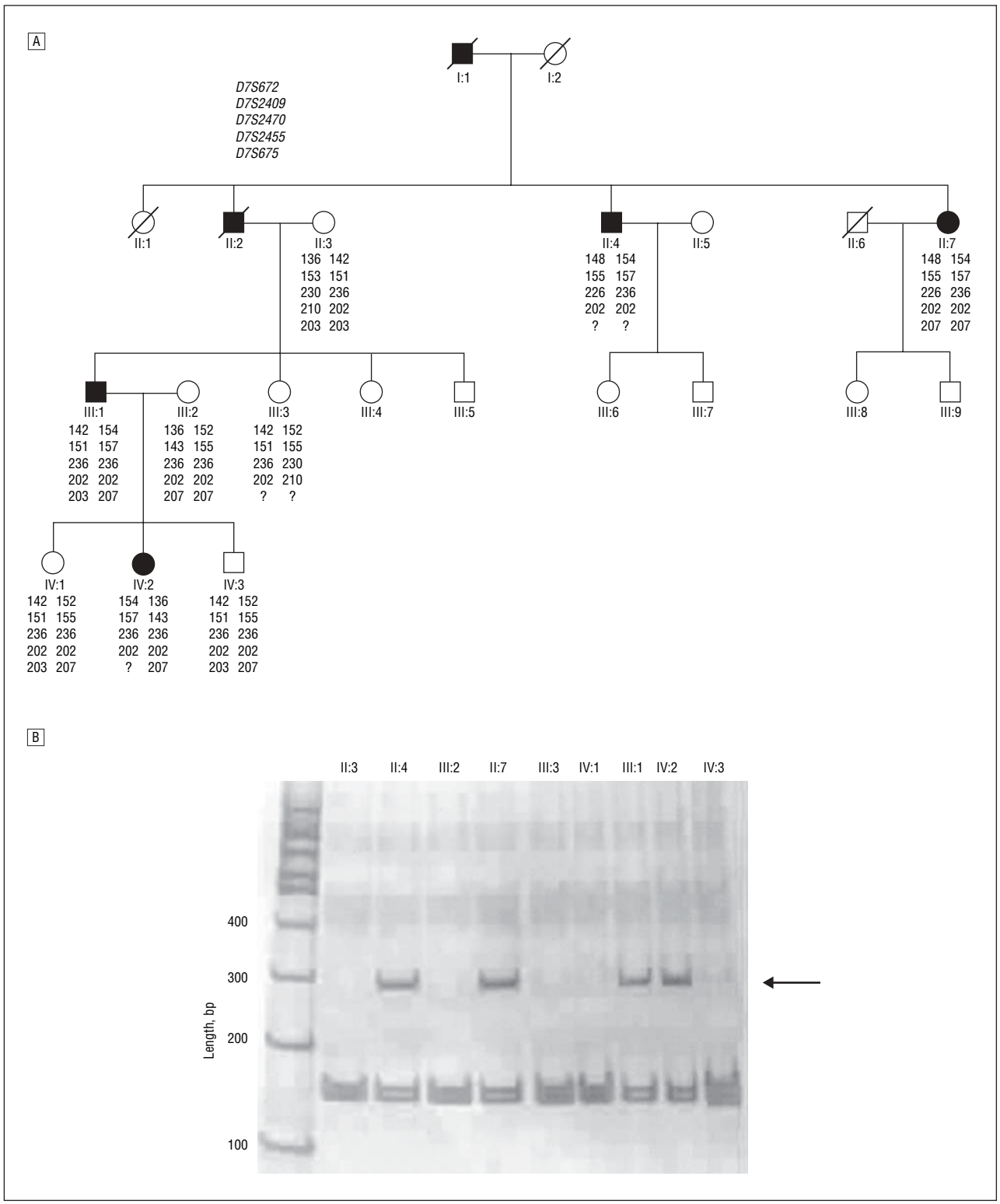

Figure 5. Family 4 and haplotype analysis (A) and the restricted digest of the family members (B). In B, the abnormal migration alteration is indicated (arrow). An explanation of all symbols/abbreviations is given in the legend to Figure 2.

37 years, patient III:3 of family 3, aged 23 years, and patient IV:2 of family 4, aged 29 years, who were mutation carriers, were asymptomatic and might be presymptomatic patients, which could be due to age-dependent penetrance. The mean±SD age of onset was 46.29±9.01 years, and the distal arms were involved a mean±SD of 13.17±6.68 years later. Patients required a walking stick after a mean±SD of 12.60±4.93 years and a wheelchair after a mean±SD of 20.67±5.69 years after the onset. The duration of the disease was a mean±SD of 17.57±6.63 years.

Table 2. Clinical Features of the Patients With the Hsp27 Mutation

Family	Patient	Age at Onset, y	Duration of the Disease, y	Hands Involved, y	Requiring Walking Stick, y	Requiring Wheelchair, y	Upper Limbs		Lower Limbs		Sensory Disorders	Talipes Cavus	Clawhands	Additional Symptoms
							Tendon Reflexes	Atrophy	Tendon Reflexes	Atrophy				
1	II:3	54	29	24	20	27	↓	+	–	+	+	+	+	None
	III:4	35	14	13	0	0	N	+	–	+	+	+	–	Cramps and fasciculations in the lower limbs
2	II:1	49	19	8	11	16	↓	+	–	+	+	+	+	None
3	II:5	42	17	9	15	0	↓	+	–	+	+	+	+	Numbness and tingling in the feet
4	II:4	60	7	7	0	0	N	+	↓	+	–	–	–	None
	II:7	47	17	0	9	0	N	–	–	+	+	+	–	None
	III:1	37	20	18	8	19	↓	+	–	+	+	+	–	None

Abbreviations: Hsp, small heat shock protein; N, normal; +, present; –, absent.

Table 3. Electrophysiological Data of Patients With the Hsp27 Mutation

Family	Patient	MCV, m/s								SCV, m/s					
		Tibial		Peroneal		Median		Ulnar		Median		Ulnar		Sural	
		Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
1	III:4	–	31.5	–	26.3	51.2	ND	50.0	ND	ND	60.0	ND	59.1	ND	33.3
2	II:1	ND	26.7	ND	ND	ND	54.8	ND	47.8	53.1	ND	46.1	ND	29.1	ND
4	II:4	41.6	44.7	34.4	44.0	ND	48.7	51.0	ND	ND	ND	ND	ND	28.4	30.2
	III:1	–	–	–	–	52.6	ND	49.8	ND	ND	61.0	ND	59.5	–	–

Abbreviations: Hsp, small heat shock protein; MCV, motor nerve conduction velocity; ND, not determined; SCV, sensory nerve conduction velocity; –, absent.

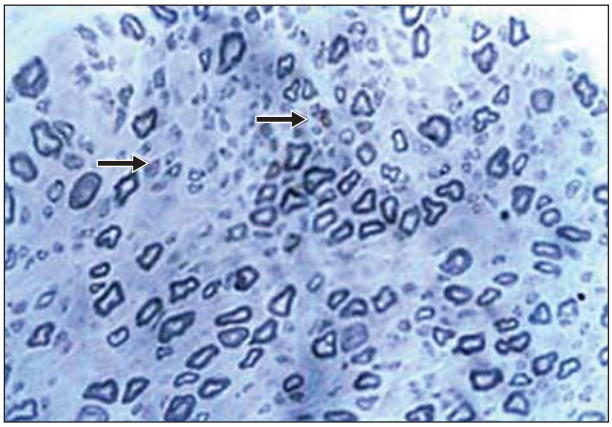

Figure 6. The results of a sural nerve biopsy performed on patient II:1 of family 2. The transverse semithin section shows chronic atrophy, loss, and regeneration of myelinated axons (arrows) without signs of obvious demyelination and a reduction in the density of myelinated fibers (toluidine blue, original magnification ×400).

The electrophysiological investigations showed moderately to severely diminished motor and sensory NCVs or no nerve action potentials in the lower limb nerves but normal or mildly reduced velocities and potentials in the upper limb nerves. The result of a sural nerve biopsy performed on patient II:1 of family 2 (**Figure 6**) was consistent with chronic axonal neuropathy.

COMMENT

Small heat shock proteins are a superfamily of proteins that vary from 15 to 30 kDa and share the α-crystallin domain, a conserved sequence of 85 to 100 amino acid residues in the C-terminus.[12] Ten Hsps are identified: HspB1 through HspB10.[13] Mutations in 4 Hsps (Hsp27, Hsp22, αA-crystallin, and αB-crystallin) are associated with diseases, including CMT disease type 2, dHMN, congenital cataract, myofibrillar myopathy, and desmin-related myopathy,[4,14-17] suggesting that the Hsp family may play an important role in the pathogenesis of neurological and muscular disorders. Small heat shock proteins are part of signal transduction cascades and have molecular chaperone-like properties, and they can increase cell survival under stress conditions by inhibiting apoptosis.[5]

In this study, we screened 114 Chinese patients with CMT disease phenotypes and identified a missense mutation (C379T) in Hsp27 that has been reported recently.[4] This mutation was found in 4 autosomal dominant families with CMT disease type 2, and haplotype analysis indicated that the 4 families probably had a common founder. To our knowledge, this is the first report of the Hsp27 mutation in the People's Republic of China. The frequency of the Hsp27 mutation is 0.9% (1/111) in Chinese patients with CMT disease in our study. Evgrafov et al[4] also screened 301 unrelated individuals with CMT

disease and confirmed one family with the Hsp27 mutation, suggesting that a mutation of the *HSP27* gene in patients with CMT disease may not be common.

In our study, the Hsp27 C379T mutation causes CMT disease type 2 phenotypes. However, this mutation in Hsp27 also causes dHMN, a disease of pure motor neuropathy, also known as spinal CMT disease, characterized by normal motor and sensory NCVs and degeneration of spinal cord anterior horn cells, which is similar to CMT disease clinically but without sensory abnormalities.[18] A similar phenomenon is observed for the C404T mutation in Hsp27, which is also associated with CMT disease type 2 and dHMN,[4] suggesting that even the same mutation in the same gene may lead to a variation in clinical phenotypes, but the mechanisms remain unclear.

Because CMT disease type 2 is clinically and genetically heterogeneous, it is important to describe the clinical features of the 4 mutation-detected families studied herein and to compare them with those of families with CMT disease type 2 described in the literature. Evgrafov et al[4] found a C404T missense mutation in Hsp27 in a Russian family with CMT disease type 2F, which was reported previously.[19] In this family, disease onset occurred between the ages of 15 and 25 years. Mild to moderate sensory impairments were observed in the feet and hands in all the patients. However, in our study, the 4 mutation-detected families were clinically characterized by late onset (range, 35-60 years) and mild sensory impairments in the feet. In addition, cramps and fasciculations in the lower limbs that were absent in the Russian family with CMT disease type 2F were seen in our study, suggesting clinical variation caused by different mutations in the same gene. The clinical manifestations, including variable ages at onset, weakness and/or atrophy in the distal muscles, reduced or absent tendon reflexes, no nerve enlargement, normal or slightly decreased motor NCVs in the median nerve, and chronic myelinated axonal atrophy, loss, and regeneration, of the 4 mutation-detected families were similar to those of patients clinically diagnosed as having CMT disease type 2.[20] Cramps or fasciculations in the limbs in patients with CMT disease type 2 were also reported by other researchers.[21,22]

In conclusion, our study confirmed that the Hsp27 mutation can cause a late-onset CMT disease type 2 phenotype with mild sensory disorder. And, we concluded that the frequency of the Hsp27 mutation may not be common in Chinese patients with CMT disease. Further efforts should include determining the molecular mechanism of mutant Hsp27 causing CMT disease and dHMN, which would be helpful for future therapeutic strategies in patients with hereditary peripheral neuropathy.

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