

Emmanuelle Plassart^a
Jocelyne Reboul^a
Claire-Sophie Rime^a
Dominique Recan^b
Philippe Millasseau^c
Bruno Eymard^d
Jean Pelletier^e
Charles Thomas^f
Françoise Chapon^g
Claude Desnuelle^h
*Christian Confavreux*ⁱ
*Bernadette Bady*ⁱ
Jean-Jacques Martin^k
*Gilbert Lenoir*ⁱ
Georges Serratrice^e
Michel Fardeau^{d,j}
Bertrand Fontaine^{a, d}

- ^a INSERM U-134, Hôpital de la Salpêtrière, Paris;
^b Service de Biochimie Génétique, Hôpital Cochin, Paris;
^c Généthon, Evry;
^d Services de Neurologie, Hôpital de la Salpêtrière, Paris;
^e CHU La Timone, Marseille;
^f Service de Neurologie, Thionville;
^g CHU de Caen;
^h CHU de Nice;
ⁱ Hôpitaux de Lyon;
^j INSERM U-153, Paris, France;
^k Universiteit Antwerpen, Belgium

Mutations in the Muscle Sodium Channel Gene (SCN4A) in 13 French Families with Hyperkalemic Periodic Paralysis and Paramyotonia Congenita: Phenotype to Genotype Correlations and Demonstration of the Predominance of Two Mutations

Key Words

Periodic paralysis
Hyperkalemia
Myotonia
Sodium channel

Abstract

Hyperkalemic periodic paralysis (hyperPP), paramyotonia congenita (PC) and PC with myotonia permanens are closely related muscle disorders of genetic origin due to allelic mutations in the muscle sodium channel gene, SCN4A. Seven families of French origin with hyperPP were studied. Five of these had the Thr704Met mutation, but 2 families, genetically linked to SCN4A, failed to show any of the known mutations of SCN4A. Correlations between the phenotype and the genotype were made for patients with the Thr704Met mutation. All 12 patients over 30 years old with the Thr704Met mutation presented muscle weakness due to degeneration of muscle fibers in addition to periodic paralysis. Only approximately 12.5% of patients with the Thr704Met mutation presented with clinical myotonia and about 50% with hyperkalemia. One family with PC displayed the Gly1306Val mutation with a phenotype similar to the one already reported for this mutation. Five families with either PC or PC with myotonia permanens had the Thr1313Met mutation indicating that the severity of myotonia and its permanence were variable. Two mutations of SCN4A were found to be predominant in these 13 families: the Thr704Met and the Thr1313Met mutations. Only 2 families with the Thr704Met mutation and 3 families with the Thr1313Met shared the same SCN4A haplotype determined with intragenic dinucleotide repeats. Recurrent mutations of SCN4A may contribute to the predominance of these two mutations in the French population.

Introduction

Periodic paralysis and nondystrophic myotonias are a group of hereditary diseases which implicate abnormal function of muscle membrane ion channels. Myotonia congenita, a muscle disorder with both autosomal dominant and recessive modes of transmission, is a chloride channelopathy [1, 2]. Hyperkalemic periodic paralysis (hyperPP), paramyotonia congenita (PC) and PC with myotonia permanens are sodium channelopathies [3–5]. The gene defect in hypokalemic periodic paralysis is still unknown but does not affect the chloride or the sodium channels [6, 7]. The mode of inheritance of hyperPP, PC and PC with myotonia permanens is autosomal dominant with almost complete penetrance. The onset of the disease is usually in early childhood. HyperPP is characterized by transient episodes of muscle weakness lasting from minutes to hours. During crises, blood potassium levels rise. Patients with hyperPP may also develop permanent muscle weakness in the fourth decade of life due to myopathic degeneration of muscle fibers [8–12]. In PC, patients present with ‘paradoxical’ myotonic symptoms, i.e. myotonic symptoms aggravated by muscle exercise. Sensitivity to exposure to the cold, ‘cold-induced stiffness’, is another hallmark of PC.

HyperPP and PC are genetically homogeneous disorders due to allelic mutations of the muscle sodium channel gene, SCN4A. As in other excitable cells, muscle sodium channels play a key role in the generation of action potentials in myofibers. The muscle sodium channel is composed of two subunits: a large α subunit (approximately 260 kD) and a small β subunit (approximately 30 kD). The α subunit (SCN4A) is a voltage-dependent regulator of sodium ion exchange through the sarcolemmal membrane [13–16]. SCN4A encodes an approximately 1,800-amino-acid protein

composed of four homologous domains (DI–DIV), with six putative transmembrane segments each (S1–S6) [13–19]. Evidence that SCN4A was the primary defect in hyperPP and PC [reviews in ref. 10, 16, 19–21] included data from electrophysiological recordings of sodium currents in patients’ muscle fibers [22–26], linkage studies with SCN4A [3–5, 27], mutation analysis of SCN4A [28–31], and *in vitro* expression of mutated SCN4A [32, 33].

Four different mutations of SCN4A have been reported to be associated with hyperPP in humans [10, 28, 29]. One mutation in the gene homolog to SCN4A in horses was reported to be associated with hyperPP [34]. Nine different mutations of SCN4A have been implicated in PC or related disorders [10, 30, 31, 35].

Several variants of hyperPP (hyperPP without myotonia, with myotonia and with paramyotonia) and of PC (pure paramyotonia congenita, myotonia fluctuans, myotonia permanens) have been described and considered to breed true within families [8–11]. Some of these variants have already been associated with specific mutations of SCN4A [10, 26, 28–31, 35–37]. Clinical variability is however present within families (for example the presence or absence of hyperkalemia, the presence or absence of a permanent muscle weakness, the degree of myotonia and cold sensitivity). A strict correlation between the SCN4A mutation and the clinical phenotype may, therefore, be difficult to establish when considering small families. One way to circumvent this obstacle is to compare different families with the same SCN4A mutation. This was the first goal of the present study. In addition, the issue of a founder effect in hyperPP and in PC was addressed.

Table 1. Clinical phenotypes displayed in seven families with dyskalemic periodic paralysis

Individual	Sex	Age of onset years	Duration years	Clinical myotonia	Para-myotonia ¹	Permanent muscle weakness	Serum potassium	EMG	Muscle biopsy
A1	M	1	40	no	no	yes	NL	NL	TA, VAC, MP
A2	F	1	36	no	no	yes	NL	NL	MP
A3	M	1	36	no	no	yes	NL	ND	ND
A4	F	1	51	no	no	yes	ND	ND	ND
A5	M	1	3	no	no	no	ND	ND	ND
A6	F	1-2	18	no	no	no	NL	NL	ND
A7	F	1-2	10	no	no	no	ND	ND	ND
B1	M	1	30	no	no	yes	NL	NL	VAC, TA
C1	M	1-2	36	no	no	yes	I	NL	VAC, MP
D1	F	3-4	56	no	no	yes	I	MT	ND
D2	M	2-3	43	no	no	yes	ND	ND	ND
D3	F	<1	10	no	no	no	ND	ND	ND
E1	F	<1	67	yes	no	yes	I	MT	VAC, MP
E2	M	2	40	yes	no	yes	I	MT	VAC, MP
E3	M	<1	60	no	no	yes	I	ND	VAC, TA, MP
E4	F	1	60	no	±	yes	I	MT	MP
F1	F	1	67	yes	yes	no	ND	ND	ND
F2	M	1	45	yes	yes	no	ND	ND	ND
F3	F	1	44	yes	yes	no	ND	ND	ND
F4	M	1	41	yes	±	no	NL	MT	VAC, MP
F5	F	2	6	yes	yes	no	ND	ND	ND
F6	M	1	40	yes	yes	no	ND	ND	ND
F7	M	1	38	yes	yes	no	ND	ND	ND
G1	F	3	61	yes	yes	no	I	MT	NL
G2	M	2	35	yes	yes	no	ND	ND	ND
G3	F	3	34	yes	yes	no	ND	ND	ND

Individuals who could be examined are listed in the first column. All patients presented episodes of transient muscle weakness. The letter indicates the family, and the number, the studied individual. Clinical myotonia indicates that myotonia was present either on clinical examination or when the muscle was struck. The levels of blood potassium were measured during acute episodes of muscle weakness. NL = Normal; ND = not determined; I = increased over the normal range of the laboratory where the determination was performed; MT = myotonia revealed by electromyographic (EMG) examination; TA = tubular aggregates; VAC = vacuoles; MP = myopathic changes.

¹ Paramyotonia cold-induced weakness.

Table 2. Clinical phenotypes displayed in 6 families with PC or PC with myotonia permanens

Individual	Sex	Age of onset years	Duration years	Cold-induced weakness	Myotonia permanens	Muscle pain and cramps	EMG	Muscle biopsy
H1	F	18	54	no	no	yes	MT	TA
H2	F	<1	62	no	no	yes	MT	ND
I1	M	1–2	26	yes	yes	yes	MT	VAC
I2	F	1–2	58	no	yes	yes	ND	ND
J1	F	7–8	38	yes	yes (diaphragmatic myotonia)	no	MT	NL
K1	F	1	40	no	no	yes	MT	NL
K2	F	2–3	10	no	no	yes	MT	ND
L1	M	3–4	38	yes	yes (calf hypertrophy)	yes	MT	NL
M1	M	2–3	63	yes	yes (global muscle hypertrophy)	no	MT	NL
M2	M	2–3	27	no	yes (global muscle hypertrophy)	no	MT	NL

Individuals who could be examined are listed in the first column. All patients presented paradoxical myotonia and cold-induced stiffness. The letter indicates the family, the number the studied individual. EMG = Electromyograph; MT = myotonia; VAC = vacuoles; TA = tubular aggregates; NL = normal; ND = not determined.

Subjects, Materials and Methods

Patients

Thirty-seven patients with either hyperPP or PC (tables 1, 2) were diagnosed according to classical criteria [8–11]. Families A, B, C, F, G, and J originated in the Paris area, family D in northern France (Lille), family E in eastern France (Thionville), families H and I in western France (Caen, Brest), family K in southern France (Marseille), family L in southeastern France (Grenoble) and family M in central France (Limoges). According to the families, there had been no recent migrations for at least the last three generations, except for an unaffected grandparent of family H who came from Spain and an unaffected grandparent of family K who came from Italy. Informed consent, according to French law, was obtained from family members who agreed to collaborate in our study (130 individuals).

Blood samples were collected and lymphoblastoid cell lines and DNA were prepared using standard procedures [38].

Typing of the Dinucleotide Repeat Polymorphisms at the *SCN4A* Locus

We used the recently reported *SCN4A*(GA)_n and *SCN4A*(GT)_n polymorphisms [39]. Polymerase chain reaction (PCR) amplifications were performed with primers as described [39], with the following modifications. Polyacrylamide gels were transferred onto a nylon membrane (Hybond N+, Amersham) and hybridized with one of the radioactive primers according to the recommendations of the membrane manufacturer and standard procedures [40]. To better distinguish *SCN4A* alleles, PCR products were resolved on 8 and 4% denaturing polyacrylamide gels, for *SCN4A*(GA)_n and *SCN4A*(GT)_n, respectively. Alleles

Table 3. Frequency of SCN4A alleles and haplotypes in the population studied

	SCN4A alleles	Number of chromosomes	Frequency
SCN4A(GA) _n	1	31	0.38
	2	7	0.09
	3	15	0.19
	4	28	0.34
SCN4A(GT) _n	1	3	0.04
	2	5	0.06
	3	8	0.09
	4	2	0.02
	5	4	0.05
	6	32	0.37
	7	0	0
	8	0	0
	9	31	0.36
	10	1	0.01
SCN4A haplotype	GA1/GT6	16	0.28
	GA4/GT9	16	0.28
	GA3/GT3	6	0.1
	GA3/GT6	5	0.08
	GA1/GT5	4	0.07
	GA1/GT4	2	0.03
	GA2/GT6	5	0.08
	GA3/GT1	1	0.02
	GA2/GT9	1	0.02
	GA1/GT0	1	0.02
GA1/GT1	1	0.02	

The frequency of SCN4A alleles and haplotypes was estimated by characterizing and counting the alleles and haplotypes for the spouses in the families and in unrelated individuals (total number of chromosomes studied in the table).

were visualized by autoradiography using MP films (Amersham).

Genetic Analysis

As each of our families was of French origin, the frequencies of SCN4A alleles were estimated by typing the unaffected spouses in our pedigrees and a number of unrelated controls (table 3). The frequencies of the sodium channel haplotypes were also determined (table 3). To calculate lod scores, we used the frequencies

of SCN4A estimated in our study, and those already reported [39]. The results were similar. Lod scores were calculated using the MLINK program of the LINKAGE package [41], assuming a penetrance of 100%. Genetic homogeneity in our families was tested with the program HOMOG [41], taking into account lod scores for families already reported [39]. Linkage disequilibrium between the two SCN4A dinucleotide polymorphisms was demonstrated using a χ^2 test [42] between the expected and the observed population.

Single Strand Conformational Polymorphism (SSCP) analysis

According to the genomic structure of SCN4A [43, 44], the coding sequence of SCN4A was divided into segments of approximately 300 bp. Primers were designed to amplify these segments from genomic DNA (sequence available on request). SSCP analysis of the exons covering the four domains, and interdomains II-III and III-IV of SCN4A were performed for at least one affected individual per family. The chosen domains and interdomains were those in which mutations have already been described and those suggested by functional studies of the sodium channel [14-17]. PCR reactions were performed using standard procedures in a final volume of 50 μ l [40]. The Taq polymerase was from ATGC. The following conditions were used: 200 ng of genomic DNA, 100 ng of each primer, 40 cycles with a denaturation step (94°C, 45 s), an annealing step (61°C, 45 s) and an extension step (72°C, 45 s) preceded by a hot start (96°C, 5 min) (thermal cycler PHC-3 from Techne). SSCP analysis was done as previously described [45] with the following modifications. After heat denaturation in 10 mM EDTA and 0.1% SDS, PCR products were separated by electrophoresis (14 h at 8 W) on a Hydrolink MDE gel (Bioprobe systems) and transferred onto a nylon membrane (Hybond N+, Amersham). Membranes were hybridized with both radioactive primers which were used to perform the PCR. SSCP conformers were revealed by autoradiography using MP films (Amersham). When an aberrant conformer was revealed, segregation of the abnormal conformer with the disease was verified in the family.

Sequencing SCN4A Mutations

Sequences were determined by direct sequencing of PCR-amplified genomic DNA products. PCR products were purified on Centricon 100 columns (Amicon). Sequencing was performed in the sense and the antisense DNA strands with the primers used for the PCR and fluorescent base analogs as recommended by the supplier (Taq DyeDeoxy terminator Cycle Se-

Table 4. SCN4A haplotypes and mutations in 11 families

Family	Diagnosis	SCN4A haplotype	Exon	Nucleotide	Mutation
A	NormoPP	GA4/GT10	13	C2188T	Thr704Met
B	HyperPP	GA2/GT6	13	C2188T	Thr704Met
C	HyperPP	GA1/GT5	13	C2188T	Thr704Met
D	HyperPP	GA3/GT3	13	C2188T	Thr704Met
E	HyperPP	GA3/GT3	13	C2188T	Thr704Met
H	PC	GA1/GT5	22	C3917T	Gly1306Val
I	PC	GA4/GT9	22	C3938T	Thr1313Met
J	PC with MP	GA1/GT5	22	C3938T	Thr1313Met
K	PC	GA4/GT9	22	C3938T	Thr1313Met
L	PC with MP	GA1/GT2 or GA2/GT2	22	C3938T	Thr1313Met
M	PC with MP	GA4/GT9	22	C3938T	Thr1313Met

The letter in the first column refers to the family studied. The clinical diagnosis is indicated in the second column. The SCN4A haplotype segregating with the disease is indicated in the third column. Mutations in the coding sequence of SCN4A were screened as indicated in Subjects, Materials and Methods. The exons where mutations were found, the nucleotide and the resulting amino acid changes are given in the fourth, fifth, and sixth columns, respectively. NormoPP = Normokalemic periodic paralysis; MP = myotonia permanens.

quencing Kit, Applied Biosystems). Products of the sequencing reaction were purified on Bio Spin Chromatography Columns (Biorad). DNA sequences were read on an automat sequencer (Applied Biosystems). Sequencing was also performed with the Sequenase kit, version 2.0 (United States Biochemicals) with [α - 35 S]-dATP (1,000 Ci/mmol, Amersham). Segregation of the mutation with the disease was verified in the family. Primers used were for the Thr704Met mutation: 13A1 (5'-TGGGTGGTGGTCCCTGGGCC-3') and 13A2 (5'-GCCACCCAGCCAGCCTCACT-3') and for the Gly1306Val and the Thr1313Met mutations: 22A1 (5'-TGGAGGCAGGAAGGGGAAGT-3') and 22A2 (5'-GGCAGCACACAGGACAGG-3') [30]. Each of 14 known SCN4A mutations was screened for in at least one individual per family [10, 28–31, 34–36] (primer sequences available on request).

Results

Families

Seven families with hyperPP were analyzed and the clinical characteristics are listed in table 1. All patients presented episodes of

transient muscle weakness. The mean age of onset was 1.5 ± 0.8 (SD) years ($n = 26$). There were 13 females and 13 males. Loading with potassium salts in family A had no effect (individuals A1–A3). Clinical and electromyographic examination of the asymptomatic mother, brother and sister of patient B1 lead to the unexpected finding of rare myotonic discharges on the EMG of his 60-year-old mother. Although asymptomatic, she was found to have a deleterious mutation in SCN4A. Complete clinical description of family E (table 1) has already been published [46, 47]. Final diagnoses are given in table 4.

None of the index cases of the PC families showed permanent muscle weakness. The mean age of onset was 4.1 ± 4 years ($n = 10$). There were 6 females and 4 males. According to classical criteria, they all presented with paradoxical myotonia and cold-induced stiffness (table 2) [8–11]. Clinical details are listed in table 2 and will be discussed in relation to

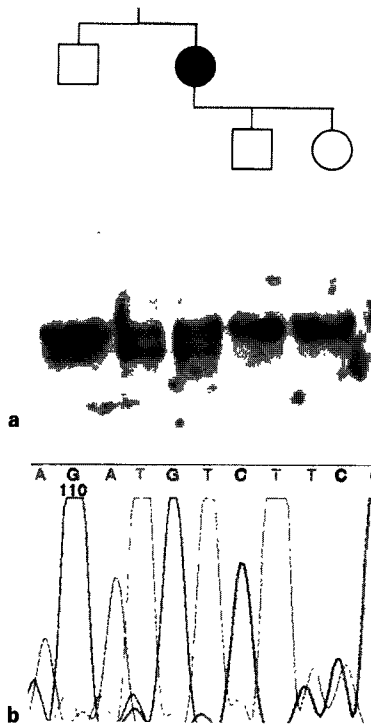


Fig. 1. Analysis of the Gly1306Val mutation in a family with PC. **a** Segregation of an abnormal conformer of SCN4A exon 22 with the disease. SSCP analysis was performed with the primers 22A1 and 22A2 as described in the text. **b** A G→T transversion at nucleotide 3917 (exon 22) resulting in a Gly1306Val mutation. Sequencing of both sense and antisense strands of amplified genomic DNA was performed as indicated in the text with the primers 22A1 and 22A2. Both alleles were sequenced: at position 3917, both a normal G and an abnormal T are present (arrow). The figure shows the sequence of the antisense DNA strand. Segregation of the mutation with the disease was verified.

SCN4A mutations. Patient J1 had very mild symptoms of myotonia with features of both PC and PC with myotonia permanens. She presented with severe episodes of diaphragmatic myotonia provoked by exposure to the cold or forced respiration. Diaphragmatic myotonic discharges were documented by electromyography (EMG). One of these diaphragmatic episodes was so severe that she was hospitalized in an intensive-care unit. Myotonia permanens was also found in families L and M (table 4).

Genetic and Molecular Analysis of SCN4A

Three mutations of SCN4A were found: Thr704Met, Gly1306Val, and Thr1313Met (fig. 1, 2) [28, 31]. Aberrant conformers and mutations segregated with the disease. An ex-

ample from family H is shown in figure 1. None of the mutations were found in normal controls, as already reported [28, 31]. All the mutations were traced to previous generations and there was no evidence of a neomutation. One asymptomatic case was documented: the mother of patient B1 carried the same Thr704Met mutation as her son (table 4). In the course of this study, polymorphic conformers were also demonstrated. An example, shown in figure 3a, is the A→G transversion at nucleotide 4817 resulting in a conservative change at the amino acid level, Thr1623Thr (fig. 3b) [10]. Another polymorphism, a G→A transversion at nucleotide 4126, resulting in a conservative change at the amino acid level, Asn1376Asp [10], was also found.

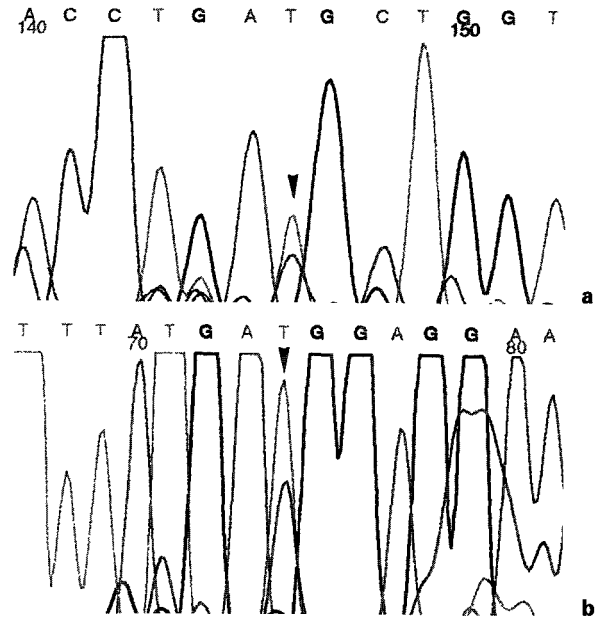


Fig. 2. The Thr704Met and Thr1313Met mutations in SCN4A. Sequencing of both sense and antisense amplified genomic DNA was performed as indicated in the text. The figures show the sequence of the sense DNA strand. Segregation of the mutation with the disease was verified. **a** With primers 13A1 and 13A2, C→T transversion at nucleotide 2188 (exon 13; normal C and abnormal T arrowed) results in a Thr704Met mutation. **b** With primers 22A1 and 22A2, a C→T transversion at nucleotide 3938 (exon 22; normal C and abnormal T arrowed) results in a Thr1313Met mutation.

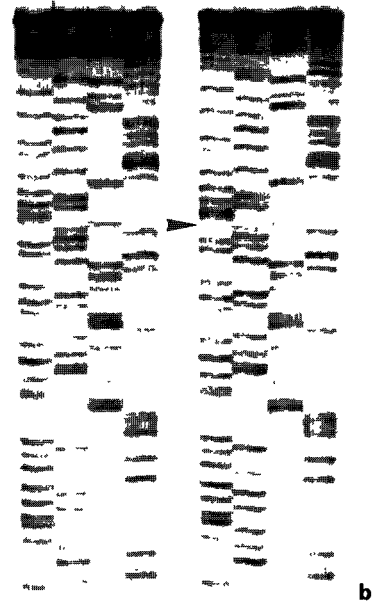
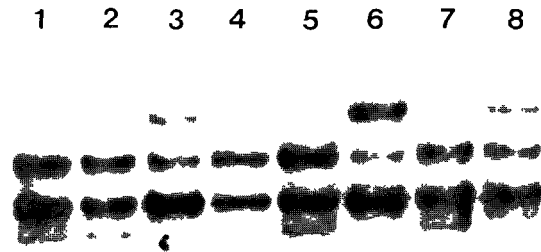


Fig. 3. A frequent polymorphic A→G transversion at nucleotide 4869. **a** Conformers revealed by SSCP corresponding to an A→G transversion at nucleotide 4869 in 8 unrelated individuals. SSCP analysis was performed as described in the text (primer sequences available on request). **b** Sequence of an A→G transversion at nucleotide 4869. Sequencing of both sense and antisense amplified genomic DNA was performed as described in the text. The arrowhead indicates an individual heterozygous for an A→G transversion at nucleotide 4869. The figure shows the sequence of the sense DNA strand. The A→G transversion at position 4869 was present in affected and unaffected individuals and did not segregate with the disease. The A→G transversion at nucleotide 4869 results in a conservative Thr1313Met amino acid change in SCN4A.

Table 5. Linkage studies with the SCN4A microsatellite polymorphisms in 2 families with hyperPP with PC

Family	Diagnosis	Lod scores, θ					SCN4A haplotype
		0.00	0.10	0.20	0.30	0.40	
F	hyperPP with PC	2.10	1.73	1.32	0.87	0.38	GA1/GT4
G	hyperPP with PC	4.04	3.22	2.55	1.71	0.84	GA4/GT9

The letter in the first column refers to the family studied. The clinical diagnosis is indicated in the second column. Lod scores are given in the third column. The SCN4A haplotype segregating with the disease is indicated in the fourth column.

Table 6. Linkage disequilibrium between the two SCN4A microsatellite polymorphisms in the control population and studied families

		GA1/GT6	GA4/GT9	GA3/GT3	GA1/GT9
Haplotypes in the control population	expected	6.20	6.37	0.64	7.06
	observed	16	16	6	1
Haplotypes in the studied families	expected	3.64	3.64	1.30	0.26
	observed	0	4	2	0

The difference between the expected frequencies and the observed frequencies of the 4 indicated haplotypes was tested with the χ^2 test of independence. For the population, in all 4 cases $p < 0.001$. The expected haplotypes in the studied families were calculated from the frequencies of normal haplotypes. The distribution of the haplotype GA1/GT6 on mutated chromosomes was not that expected from normal haplotype frequencies ($p < 0.02$).

Lod scores for SCN4A versus the disease were calculated for the two families (F and G) showing none of the already reported SCN4A mutations. Peak lod scores were for a recombination fraction of $\theta = 0.00$ (table 5). For family G, the lod score at $\theta = 0.00$ is 4.04. The lod score for family F is 2.10 at $\theta = 0.00$, indicating the absence of recombinants and the existence of linkage between the disease and the markers. The disease segregated with two different SCN4A haplotypes: GA1/GT4 and GA4/GT9 (table 5) suggesting that these two families were probably unrelated. Altogether,

these results indicate that undescribed SCN4A mutations are likely to be discovered in these two families.

SCN4A haplotypes were determined in the French population and in the studied families (tables 3, 4, 6). The frequencies of four haplotypes are clearly in disequilibrium ($p < 0.001$) in the population: GA1/GT6, GA4/GT9 and GA3/GT3, which are frequent haplotypes in the control population, and GA1/GT9, which is rare (table 6). Two frequent haplotypes, GA3/GT3 and GA4/GT9 (tables 3, 6) were found in linkage disequilibrium in 6 families

(tables 4, 5). GA3/GT3 was present in two apparently unrelated families with the Thr704Met mutation (tables 3, 4). GA4/GT9 was demonstrated in 3 apparently unrelated families with the Thr1313Met mutation (tables 3, 4). The other 7 families displayed rare haplotypes (tables 3, 4). The haplotype GA1/GT6, in linkage disequilibrium, was not found in the families. The distribution of this haplotype in the control population and in the studied families differed significantly ($p < 0.02$; table 6). This difference should be interpreted with caution however, given the small number of families with SCN4A mutations. No significant difference between the normal and the studied families was observed for the other haplotypes.

Genotype to Phenotype Correlations in HyperPP

Six males and seven females displayed permanent muscle weakness. The mean duration of the disease for this group of patients was 46 ± 11 years. Permanent muscle weakness was due to a myopathic process as shown by muscle biopsies (table 1). Therefore, we classified our patients presenting the Thr704Met mutation into two groups: those under the age of 30 and those over the age of 30. In the group of patients under 30 years old, 0/4 had permanent muscle weakness. In the group of patients over the age of 30, every patient had permanent muscle weakness (12/13, tables 1, 4), except for the 60-year-old mother of patient B1, who was totally asymptomatic. In patients over the age of 30 presenting with hyperPP with paramyotonia, only 1 out of 9 had permanent muscle weakness. Thus, although both groups of patients presented with periodic paralysis. Thr704Met was the only mutation associated with permanent muscle weakness in a majority of patients over the age of 30.

In patients with the Thr704Met mutation, only 2/16 had clinical myotonia and 5/9 myotonic discharges on EMG examination (tables 1, 4). Visible modifications of blood potassium levels during acute episodes of periodic paralysis were found in 5/11 patients with this mutation (tables 1, 4).

Genotype to Phenotype Correlations in PC

Cold-induced weakness was present in 0/2 patients with the Gly1306Val mutation and in 4/8 patients with the Thr1313Met mutation (families I, K, L, M). Permanent myotonia was present in 0/2 patients with the Gly1306Val mutation and in 6/8 patients with the Thr1313Met mutation (tables 2, 4). Muscle pain and cramps were present in 2/2 patients with the Gly1306Val mutation and in 5/8 patients with the Thr1313Met mutation (tables 2, 4). Muscle hypertrophy was present in 0/2 patients with the Gly1306Val mutation and 3/8 in patients with the Thr1313Met mutation (tables 2, 4). Clinical manifestations of the Thr1313Met mutation are therefore variable, particularly regarding the severity of myotonia and the permanence of myotonic manifestations.

Discussion

Five of 7 families of French origin with hyperPP had the Thr704Met mutation [28]. In the other 2 families, a variant of hyperPP with PC was genetically linked to SCN4A but showed none of the known SCN4A mutations. Although the lod score in family F was below the threshold of 3.00, HOMOG analysis provided evidence of true linkage to SCN4A with only a 1% chance of a false-positive lod score. Before the identification of the SCN4A mutations, we cannot, however, totally exclude the possibility of a gene different from SCN4A but located in the same chromo-

somal region in families F and G. We found no family with the Met1592Val mutation [29], although it has been demonstrated in about 25% of hyperPP families analyzed by two different laboratories [36, 37]. All the families displaying the Met1592Val mutation were of North American origin of unspecified ancestry [36, 37]. The Thr704Met mutation has now been detected in families of French, Japanese, Polish, German, and Swedish origin as well as in North Americans [10, 36, 37, and the present study]. Altogether, the Thr704Met mutation has been found in 14 hyperPP families and the Met1592Val mutation in 5 [36, 37, and the present study]. The Thr704Met mutation may therefore be the most frequent SCN4A mutation in hyperPP, and is the only mutation found as yet in the European population.

What clinical characteristics are common to patients with the Thr704Met mutation? In our series, episodes of transient muscle weakness were always associated with permanent muscle weakness in patients over 30 years old. Permanent muscle weakness is due to myopathic degeneration of muscle fibers and may therefore be considered as a symptom of late onset associated with the Thr704Met mutation. Expression studies of SCN4A with the Thr704Met mutation showed abnormal inactivation and a shift in the activation curve of the mutated channel [32, 33]. How these properties can induce degeneration of myofibers is unknown. A long-term increase in sodium flux or in calcium permeability resulting in abnormal muscle metabolism or an activation of proteases have been proposed as possible mechanisms [12]. Clinical myotonia, although always detected in patients suffering from hyperPP with paramyotonia but without the Thr704Met mutation, was found in only 12.5% of patients with the Thr704Met mutation. Using myotonia as the only diagnostic criterion may therefore lead to an observation

bias. For example, myotonia was noted in family F and was absent in family E, although both families shared the same haplotype of SCN4A, GA3/GT3, associated with the Thr704Met mutation. Increased blood potassium levels were demonstrated in only 50% of patients with the Thr704Met mutation. In family A, all patients presented the Thr704Met mutation but it was not possible to document any increase in blood potassium levels during the episodes of transient muscle weakness. Moreover, potassium loading did not induce attacks. As already suggested by clinical observations [8–11] and now confirmed by a molecular analysis [48, and the present study], hyperkalemia is not a constant feature in hyperPP. Therefore, hyperPP and normokalemic periodic paralysis should not be considered as distinct entities. In conclusion, our results suggest that myotonia and hyperkalemia are not always present in patients with the Thr704Met mutation, and should be used with caution to classify patients with dyskalemic periodic paralysis.

In PC, the question of clinical variability could not be addressed for the Gly1306Val mutation, since only 1 family had this mutation. Specific clinical phenotypes have been associated with mutations at amino acid 1306: PC, myotonia permanens and myotonia fluctuans with the Gly1306Val, Gly1306Glu, and Gly1306Ala mutations, respectively [10, 26, 31]. The phenotypes displayed by our family and the three previously reported families were similar [10, 26, 31]. Myotonia permanens was the feature most constantly associated with the Thr1313Met mutation. Altogether, 9 families with the Thr1313Met mutation have been reported [31, 36, and the present study]. Clinical variability might be a distinctive feature of the amino acid 1313 mutation, which was observed both within families and among

groups of muscles in the same patient, as illustrated by patient J1 who presented severe diaphragmatic myotonia and mild myotonia of the other muscle groups [10, 26, 31]. How might such variability in patients with the same mutation be explained? Recently Lerche et al. [26] suggested that the degree of myotonia is related to parameters of sodium channel inactivation. However, the interaction of the mutated channel with other muscle ion channels might also be important. Only a small fraction of sodium channels in muscle fibers of hyperPP patients are abnormally inactivated [24, 25]. Further electrophysiological and expression studies will be needed to resolve the problem of clinical variability.

Complete penetrance was found for the Gly1306Val and the Thr1313Met mutations. One case of incomplete penetrance for the Thr704Met mutation (mother of patient B1) was observed, however, and incomplete penetrance has already been reported for the Ala1156Thr mutation [35]. The asymptomatic case reported here is the first for the Thr704Met mutation, the most frequent SCN4A mutation causing hyperPP. Factors which might explain incomplete penetrance have been extensively discussed by McClatchey et al. [35].

The Thr704Met and the Thr1313Met mutations were both found in 5 unrelated families. Pathogenic mutations affecting the same amino acids suggest that these particular amino acids are important for the normal function of the muscle sodium channel *in vivo*. These two mutations predominate among SCN4A mutations in families of French origin. Both a founder effect or recurrent mutations in SCN4A-associated muscle disorders are possible. We found no evidence, however, of a new mutation which would corroborate the recurrent-mutation hypothesis, and genotyping with the SCN4A dinucleotide repeat polymorphisms in different families with the

same SCN4A mutation did not support a founder effect [39, 49]. Wang et al. [49] suggested, however, that these results should be interpreted with caution, since the mutation rate of dinucleotide repeats is presumed to be high [50–52]. On the other hand, dinucleotide repeats have been successfully utilized to demonstrate linkage disequilibrium in fragile X syndrome [53] and torsion dystonia [54], and a founder effect of Friedreich's ataxia in Louisiana Acadians [55]. SCN4A dinucleotide repeat polymorphisms are contained within introns of the SCN4A gene [24, 39], a small gene spanning about 35 kb of genomic DNA on chromosome 17q [43, 44]. In our study, two families with the GA3/GT3 SCN4A haplotype and three families with GA4/GT9 shared the Thr704Met and the Thr1313Met mutation, respectively. GA3/GT3 and GA4/GT9 are frequent haplotypes which are in linkage disequilibrium, suggesting that these two microsatellites are stable in the French population. The other SCN4A haplotypes associated with the two predominant mutations were rare haplotypes (6 families). Given the presumptive high rate of dinucleotide mutations, it is possible that the two mutations arose before haplotype divergence. However, most haplotypes differ in both the (GA)_n and the (GT)_n repeats, whereas neomutations in dinucleotide repeats are usually simple events consisting of an addition or deletion of one repeat unit [51, 52]. The mutation rate is about 10⁻³ for each dinucleotide repeat [51, 52]. If all SCN4A haplotypes associated with the two predominant SCN4A mutations had a common origin, a very high number of generations would be required to generate such diversity, rendering it unlikely. Taken together, our results may indicate that recurrent mutations contribute to the predominance of two SCN4A mutations in the French population. However, we cannot exclude the possibility of founding chromo-

somes for the families sharing the same SCN4A haplotypes. Can the recurrence of mutations at the same sites in SCN4A be explained? Benign polymorphisms have been reported [10, 18, 19, 43, 44], and two were found in the families studied here, indicating that mutations do occur in the SCN4A gene. The selection of patients for either periodic paralysis or myotonic symptoms may introduce a bias towards a particular mutation, since other clinical phenotypes such as late-onset myopathy might be linked to other mutations. Moreover, the muscle sodium channel gene is so crucial in generating action potentials that the existence of lethal mutations of SCN4A is possible. If demonstrated, they might help us to evaluate the mutation rate of SCN4A.

In conclusion, the present study has helped to clarify the clinical phenotypes associated with two predominant mutations of SCN4A in families of French descent: the Thr704Met and the Thr1313Met mutations. Recurrent

mutations may contribute to the predominance of two SCN4A mutations in the French population.

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References

- 1 Koch MC, et al: The skeletal muscle chloride channel in dominant and recessive human myotonia. *Science* 1992;257:797-800.
- 2 George AL Jr, Crackower MA, Abdalla JA, Hudson AJ, Ebers GC: Molecular basis of Thomsen's disease (autosomal dominant myotonia congenita). *Nature Genet* 1993;3:305-309.
- 3 Pt cek LJ, Trimmer JS, Agnew WS, Roberts JW, Petajan JH, Leppert M: Paramyotonia congenita and hyperkalemic periodic paralysis map to the same sodium channel gene locus. *Am J Hum Genet* 1991;49:851-854.
- 4 Koch MC, Ricker K, Otto M, Grimm T, Bender K, Zoll B, Harper PS, Lehmann-Horn F, R udel R, Hoffman EP: Linkage data suggesting allelic heterogeneity for paramyotonia congenita and hyperkalemic periodic paralysis on chromosome 17. *Hum Genet* 1991;88:71-74.
- 5 Ebers GC, George AL, Barchi RL, Ting-Pasador SS, Kallen RG, Lathrop GM, Beckman JS, Hahn AF, Brown WF, Campbell RD, Hudson AJ: Paramyotonia congenita and hyperkalemic periodic paralysis are linked to the adult muscle sodium channel gene. *Ann Neurol* 1991;30:810-816.
- 6 Fontaine B, Trofatter J, Rouleau GA, Khurana TS, Haines J, Brown R, Gusella JF: Different gene loci for hyperkalemic and hypokalemic periodic paralysis. *Neuromusc Disord* 1991;1:235-238.
- 7 Casley WL, Allon M, Cousin HK, Ting SS, Crackower MA, Hashimoto L, Cornelis F, Beckman JS, Hudson AJ, Ebers GC: Exclusion of linkage between hypokalemic periodic paralysis (Hokpp) and three candidate loci. *Genomics* 1992;14:493-494.
- 8 Buruma OJS, Schipperheyn JJ: Periodic paralysis; in Vinken PJ, Bruyn GW (eds): *Handbook of Clinical Neurology*. Amsterdam, North Holland Publishing, 1979, vol 41, pp 147-174.

- 9 Rüdell R, Ricker K: The primary periodic paralyses. *Trends Neurosci* 1985;8:467-470.
- 10 Lehmann-Horn F, Rüdell R, Ricker K: Workshop report: Non-dystrophic myotonias and periodic paralyses. *Neuromusc Disord* 1993;3:161-168.
- 11 McKusick VA: *Mendelian Inheritance in Man*. Baltimore, Johns Hopkins University Press, 1990.
- 12 Bradley WG, Taylor R, Rice DR, Hausmanowa-Petrusewicz I, Adelman LS, Jenkison M, Jedrzejowska H, Drac H, Pendlebury WN: Progressive myopathy in hyperkalemic periodic paralysis. *Arch Neurol* 1990;47:1013-1017.
- 13 Noda M, Ikeda T, Kayano T, Suzuki H, Takeshima H, Kurasaki M, Takahashi H, Numa S: Existence of distinct sodium channel messenger RNAs in rat brain. *Nature* 1986;320:188-192.
- 14 Catterall WA: Structure and function of voltage-sensitive ion channels. *Science* 1988;242:50-61.
- 15 Strühhmer W: Structure-function studies of voltage-gated ion channels. *Annu Rev Biophys Chem* 1991;20:65-78.
- 16 Barchi RL: Sodium channel gene defects in the periodic paralyses. *Curr Opin Neurobiol* 1993;6:40-47.
- 17 Trimmer JS, Cooperman SS, Tomiko SA, Zhou J, Crean SM, Boyle MB, Kallen RG, Sheng Z, Barchi RL, Sigworth FJ, Goodman RH, Agnew WS, Mandel G: Primary structure and expression of a mammalian skeletal muscle sodium channel. *Neuron* 1989;3:33-49.
- 18 George AL, Komisarof J, Kallen RG, Barchi RL: Primary structure of the adult human skeletal muscle voltage-dependent sodium channel. *Ann Neurol* 1992;31:131-137.
- 19 Wang J, Rojas CV, Zhou J, Schwartz LS, Nicholas H, Hoffman EP: Sequence and genomic structure of the adult skeletal muscle sodium channel α subunit gene on 17q. *Biochem Biophys Res Commun* 1992;182:794-801.
- 20 Ptáček LJ, Johnson KJ, Griggs RC: Genetics and physiology of the myotonic muscle disorders. *N Engl J Med* 1993;328:482-489.
- 21 Fontaine B: Periodic paralysis, myotonia congenita and sarcolemmal ion channels: A success of the candidate gene approach. *Neuromusc Disord* 1993;3:101-107.
- 22 Lehmann-Horn F, Kütther G, Ricker K, Grafe P, Ballanyi K, Rüdell R: Adynamia episodica hereditaria with myotonia: A non-inactivating sodium current and the effect of extracellular pH. *Muscle Nerve* 1987;10:363-374.
- 23 Lehmann-Horn F, Rüdell R, Ricker K: Membrane defects in paramyotonia congenita (Eulenburg). *Muscle Nerve* 1987;10:363-374.
- 24 Lehmann-Horn F, Iazzo PA, Hatt H, Franke C: Altered gating and conductance of Na⁺ channels in hyperkalemic periodic paralysis. *Pflügers Arch* 1991;418:297-299.
- 25 Cannon SC, Brown RH, Corey DP: A sodium channel defect in hyperkalemic periodic paralysis: Potassium-induced failure of inactivation. *Neuron* 1991;6:619-626.
- 26 Lerche H, Heine R, Pika U, George AL Jr, Mitrovic N, Browatzki M, Weiss T, Rivet-Bastide M, Franke C, Lomonaco M, Ricker K, Lehmann-Horn F: Human sodium channel myotonia: Slowed channel inactivation due to substitutions for a glycine within the III/IV linker. *J Physiol* 1993;470:13-22.
- 27 Fontaine B, Khurana TS, Hoffman EP, Bruns GAP, Haines JL, Trofatter JA, Hanson MP, Rich J, McFarlane H, Yasek DM, Romano D, Gusella JF, Brown RH Jr: Hyperkalemic periodic paralysis and the adult muscle sodium channel α -subunit gene. *Science* 1990;250:1000-1002.
- 28 Ptáček LJ, George AL, Griggs RC, Tawil R, Kallen RG, Barchi RL, Robertson M, Leppert MF: Identification of a mutation in the gene causing hyperkalemic periodic paralysis. *Cell* 1991;67:1021-1027.
- 29 Rojas CV, Wang J, Schwartz LS, Hoffman EP, Powell BR, Brown RH Jr: A Met to Val mutation in the skeletal muscle Na⁺ channel α -subunit in hyperkalemic periodic paralysis. *Nature* 1991;354:387-389.
- 30 Ptáček LJ, George AL Jr, Barchi RL, Griggs RC, Riggs JA, Robertson M, Leppert MF: Mutations in an S4 segment of the adult skeletal muscle sodium channel causes paramyotonia congenita. *Neuron* 1992;8:891-897.
- 31 McClatchey AI, Van den Berg P, Pericak-Vance MA, Raskind W, Verellen C, McKenna-Yasek D, Rao K, Haines JL, Bird T, Brown RH Jr, Gusella JF: Temperature-sensitive mutations in the III-IV cytoplasmic loop region of the skeletal muscle sodium channel gene in paramyotonia congenita. *Cell* 1991;68:769-774.
- 32 Cummins TR, Zhou J, Sigworth FJ, Ukomadu C, Stephan M, Ptáček LJ, Agnew WS: Functional consequences of a Na⁺ channel mutation causing hyperkalemic periodic paralysis. *Neuron* 1993;10:667-678.
- 33 Cannon SC, Strittmatter SM: Functional expression of sodium channel mutations identified in families with periodic paralysis. *Neuron* 1993;10:317-326.
- 34 Rudolph JA, Spier SJ, Byrns G, Rojas CV, Bernoco D, Hoffman EP: Periodic paralysis in quarter horses: A sodium channel mutation disseminated by selective breeding. *Nature Genet* 1992;2:144-147.
- 35 McClatchey AI, McKenna-Yasek D, Cros D, Worthen HG, Kuncel RW, De Silva SM, Cornblath DR, Gusella JF, Brown RH Jr: Novel mutations in families with unusual and variable disorders of the skeletal muscle sodium channel. *Nature Genet* 1992;2:148-152.
- 36 Ptáček LJ, Gouw L, Kwiecinski H, McManis P, Mendell JR, Barohn RJ, George AL, Barchi RL, Robertson M, Leppert MF: Sodium channel mutations in paramyotonia congenita and hyperkalemic periodic paralysis. *Ann Neurol* 1993;33:300-307.
- 37 Feero WG, Wang J, Barany F, Zhou J, Todorovic SM, Convit R, Gallo-way G, Hartlage P, Hayakawa H, Hoffman EP: Hyperkalemic periodic paralysis: Rapid molecular diagnosis and relationship of genotype to phenotype in 12 families. *Neurology* 1993;43:668-673.
- 38 Gusella JF: DNA polymorphism and human diseases. *Ann Rev Biochem* 1986;55:831-854.

- 39 McClatchey AI, Trofatter J, McKenna-Yasek D, Raskind W, Bird T, Pericak-Vance M, Gilchrist J, Arahata K, Radosavljevic D, Whorthen HG, Van den Bergh P, Haines JL, Gusella JF, Brown RH Jr: Dinucleotide repeat polymorphisms at the SCN4A locus suggest allelic heterogeneity of hyperkalemic periodic paralysis and paramyotonia congenita. *Am J Hum Genet* 1992;50:896-901.
- 40 Maniatis T, Fritsch EF, Sambrook J: *Molecular Cloning: A Laboratory Manual*, ed 2. Cold Spring Harbor, Cold Spring Harbor Laboratory, 1989.
- 41 Ott J: *Analysis of Human Genetic Linkage*. Baltimore, Johns Hopkins University Press, 1991.
- 42 Jennings HS: The numerical result of diverse systems of breedings with respect to two pairs of characters. *Genetics* 1917;2:97-106.
- 43 McClatchey AI, Lin CS, Wang J, Hoffman EP, Rojas C, Gusella JF: The genomic structure of the human skeletal muscle sodium channel gene. *Hum Mol Genet* 1992;1:521-527.
- 44 George AL Jr, Iyer GS, Kleinfeld R, Kallen RG, Barchi RL: Genomic organization of the human skeletal muscle sodium channel gene. *Genomics* 1993;15:598-606.
- 45 Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T: Detection of polymorphisms of human DNA by gel electrophoresis as single strand conformation polymorphisms. *Proc Natl Acad Sci USA* 1989;86:2766-2770.
- 46 Thomas C, Schweitzer R, Isch F, Collin H, Fardeau M: Données nouvelles sur la généalogie, la clinique et l'histologie d'une famille atteinte de paralysie périodique hyperkaliémique. *Rev Neurol (Paris)* 1978;134:45-58.
- 47 Serratrice G, Desnuelle C: Paramyotonie congénitale, adynamie épisodique héréditaire ou paralysie périodique familiale paramyotonique et hyperkaliémique. *Semaine Hôp Paris* 1982;58:841-848.
- 48 Lehmann-Horn F, Heine R, Pika U, Steinbach P, Golla A, Dobler M, Ricker K: The same SCN4A mutation is present in non-myotonic and in myotonic hyperkalemic and normokalemic periodic paralysis patients. *Neuromusc Disord* 1993, in press.
- 49 Wang J, Zhou J, Todorovic SM, Feero WG, Barany F, Conwit R, Hausmanowa-Petrusewicz I, Fidzianska A, Arahata K, Wessel HB, Sillen A, Marks HG, Hartlage P, Galloway G, Ricker K, Lehmann-Horn F, Hayakawa H; Hoffman EP: Molecular genetic and genetic correlations in sodium channelopathies: Lack of founder effect and evidence for a second gene. *Am J Hum Genet* 1993;52:1074-1084.
- 50 Weber JL: Informativeness of human (dC-dA)_n polymorphism. *Genomics* 1990;7:524-530.
- 51 Weissenbach J, Gyapay G, Dib C, Vignal A, Moricette J, Millasseau P, Vaysseix G, Lathrop M: Second generation linkage map of the human genome. *Nature* 1992;359:794-801.
- 52 Weber JL, Wong C: Mutation of human short tandem repeats. *Hum Mol Genet* 1993;2:1123-1128.
- 53 Oudet C, Mornet E, Serre JL, Thomas F, Lentès-Zengerling S, Kretz C, Deluchat C, Tejada I, Boué J, Boué A, Mandel JL: Linkage disequilibrium between the fragile X mutation and two closely linked CA repeats suggest that fragile X chromosomes are derived from a small number of founder chromosomes. *Am J Hum Genet* 1993;52:297-304.
- 54 Ozelius LJ, Kramer PL, de Leon D, Risch N, Bressman SB, Schubach DE, Brin MF, Kwiatkowski DJ, Burke RE, Gusella JF, Fahn S, Breakefield XO: Strong allelic association between the torsion dystonia gene (DYT1) and loci on chromosome 9q34 in Ashkenazi jews. *Am J Hum Genet* 1992;50:619-628.
- 55 Sirugo G, Keats B, Fujita R, Duclos F, Purohit K, Koenig M, Mandel JL: Friedreich ataxia in Louisiana Acadians. Demonstration of a founder effect by analysis of microsatellite-generated extended haplotypes. *Am J Hum Genet* 1992;50:559-566.