

## Hypokalemic Periodic Paralysis and the Dihydropyridine Receptor (CACNL1A3): Genotype/Phenotype Correlations for Two Predominant Mutations and Evidence for the Absence of a Founder Effect in 16 Caucasian Families

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### Summary

Hypokalemic periodic paralysis (hypoPP) is an autosomal dominant disorder belonging to a group of muscle diseases involving the abnormal function of ion channels. This group of muscle diseases also comprises hyperkalemic periodic paralysis and paramyotonia congenita, both sodium-channel diseases, and myotonia congenita, a chloride-channel disorder. HypoPP is characterized by acute attacks of muscle weakness concomitant with a fall in blood potassium levels. We recently localized the hypoPP locus (hypoPP1) to chromosome 1q31-32, in an interval where the  $\alpha 1$  subunit of the dihydropyridine receptor calcium channel (CACNL1A3) also maps. Subsequently, deleterious mutations in the voltage-sensor segment S4 were found, establishing the dihydropyridine receptor CACNL1A3 as the causative gene for hypoPP. In this paper, we report the study of 16 hypoPP families of Caucasian origin. We found only two mutations—Arg528His and Arg1239His—that cosegregated with hypoPP, each in half of the families. Analysis of the clinical characteristics of both groups of families demonstrated that incomplete penetrance is a distinctive feature of the Arg528His mutation. Using dinucleotide repeats contained within or close to the dihydropyridine receptor gene, in conjunction with evidence of a de novo Arg1239His mutation, we show that a founder effect is unlikely to account for the two predominant mutations.

### Introduction

Periodic paralyzes and nondystrophic myotonia constitute a particular group of muscle diseases known to implicate an abnormal function of ion channels: the “muscle ion-channel diseases.” Acute and reversible attacks of muscle weakness are due to depolarization of the sarcolemmal membrane and are the hallmarks of both hypokalemic and hyperkalemic periodic paralysis. Myotonia is caused by hyperexcitability of the sarcolemmal membrane and characterizes both paramyotonia congenita and myotonia congenita. All these muscle disorders are hereditary conditions of autosomal dominant inheritance, except myotonia congenita, which is transmitted with both autosomal recessive and dominant inheritance. A candidate-gene approach has revealed that hyperkalemic periodic paralysis and paramyotonia congenita are caused by allelic mutations of the muscle sodium-channel SCN4A (Fontaine et al. 1990; Ptacek et al. 1991, 1992; Rojas et al. 1991; McClatchey et al. 1992b), as are their clinical variants normokalemic periodic paralysis and sodium-channel myotonia (Heine et al. 1993; Plassart et al. 1994b; Ptacek et al. 1994b). Interspecies conservation of syntetic loci determined by analogy with a murine model of human myotonia permitted identification of the genetic anomalies causing myotonia congenita: allelic mutations of the muscle chloride-channel CLCN1, resulting in a null allele in the recessive form of the disease and in a dominant negative effect of the mutated allele in myotonia congenita with autosomal dominant inheritance (Koch et al. 1992; George et al. 1993).

The most recent member to be added to the muscle ion-channel diseases is hypokalemic periodic paralysis (hypoPP; MIM 170400 [McKusick 1990]). In this disease, acute attacks of muscle weakness are accompanied by a fall in blood potassium levels. Precipitating factors include both the ingestion of a meal rich in carbohydrates and rest after

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exercise. The onset of the disease is usually in the 2d decade, and penetrance is incomplete in women. In the 4th or 5th decade of life, a vacuolar myopathy appears, which causes permanent muscle weakness. Recently, by a combined candidate-gene and genomewide search, the hypoPP locus was localized to chromosome 1q31-32 and termed "hypoPP1" (Fontaine et al. 1994). In our initial study, hypoPP1 was colocalized with the calcium-channel CACNL1A3, also known as the  $\alpha 1$  subunit of the dihydropyridine receptor, rendering this gene a plausible location for the hypoPP1 gene defect (Fontaine et al. 1994).

The dihydropyridine receptor belongs to the family of "voltage-gated ion channels" and shares with potassium, sodium, and calcium channels a common structure consisting of six transmembrane segments organized in domains (Catterall 1993). The  $\alpha 1$  subunit of the dihydropyridine receptor is composed of four domains (Tanabe et al. 1987). Deleterious mutations have been demonstrated in the voltage-sensor segment S4 of the dihydropyridine receptor, establishing CACNL1A3 as the causative gene for hypoPP1 (Jurkat-Rott et al. 1994; Ptacek et al. 1994a). In this study of 16 hypoPP families of Caucasian origin, we found only two different mutations responsible for the disease. We report the genotype/phenotype correlations for both mutations, as well as the haplotypes segregating with both mutations analyzed by flanking markers of the hypoPP1 locus and intragenic dinucleotide repeats contained within the dihydropyridine receptor gene.

## Patients and Methods

### Patients

Sixteen Caucasian families displaying hypoPP linked to the hypoPP1 locus on chromosome 1q31-32 were studied. Families 9, 15, and 1 already have been described by Fontaine et al. (1994), as families A, B, and C, respectively; families 5 and 6 have been referred to as families 15 and 16 by Jurkat-Rott et al. (1994). Family 7 also has been analyzed elsewhere (Links et al. 1994).

### DNA Analysis

Blood samples were collected from all consenting family members and Caucasian controls. The protocol of the study was approved by the ethics committee of La Salpêtrière Hospital in 1993 and conformed to the Helsinki Convention. DNA extraction was performed by proteinase K lysis, phenol-chloroform extraction, and salt precipitation as described elsewhere (Gusella 1986). The following dinucleotide repeats were typed in all families and controls: CACNL1A3 (Gregg et al. 1993) and D1S1723 and D1S1726 (Jurkat-Rott et al. 1994). Genotyping was performed by the PCR-blotting technique described by Hazan et al. (1992). The dinucleotide-repeat alleles were numbered according to decreasing size on the same gel, to allow comparison between families (table 1).

### Mutations of the $\alpha 1$ Subunit of the Dihydropyridine-Sensitive Calcium-Channel CACNL1A3

Two mutations of the dihydropyridine receptor were found in our 16 families: the Arg528His and the Arg1239His mutations (Jurkat-Rott et al. 1994; Ptacek et al. 1994a). The first one causes the loss of a *BbvI* restriction site, and the latter causes the appearance of a *NlaIII* restriction site, allowing rapid screening by PCR and restriction-enzyme digestion as shown in figure 1. The sequencing of the mutation was performed for at least one affected individual per family.

### Genetic Analysis

The frequencies of dinucleotide-repeat alleles were determined by typing 82 unrelated and unaffected Caucasian individuals married to members of the studied families. The expected frequencies of the dinucleotide-repeat alleles and of the haplotypes of the studied families were calculated from the frequencies observed in controls. Expected and observed allele and haplotype frequencies in the affected population were compared using the  $\chi^2$  test (with the Yates correction when necessary). The relative risk of disease conferred by each allele was calculated with Woolf's (1955) formula.

## Results

### Arg528His and Arg1239His Mutations of the Dihydropyridine Receptor as the Predominant Mutations Causing hypoPP

By restriction-enzyme and sequence analysis, the Arg528His and the Arg1239His mutations were each found in 8 of 16 Caucasian families linked to chromosome 1. The mutations cosegregated with hypoPP in each family (fig. 1). A Japanese family with hypoPP also carried the Arg528His mutation (data not shown).

We observed a *de novo* Arg1239His mutation in family 10 (fig. 1B). Individual 10-3, an affected 36-year-old man, transmitted the disease to his 10-year-old son (10-5) but not to his daughter (10-6). His parents, 10-1 and 10-2, were both 72 years old and normal according to both medical history and clinical examination. As shown in figure 1B, 10-3 and 10-5 displayed the restriction pattern of the Arg1239His mutation, whereas in 10-1, 10-2, and 10-6 the restriction pattern was normal. By reconstruction of the haplotypes for D1S1726, CACNL1A3, and D1S1723, the affected individual 10-3 was shown to have inherited the diseased haplotype from his mother, 10-2 (data not shown). Nonpaternity was ruled out by typing 10 dinucleotide repeats (data not shown). The mutation, therefore, arose *de novo* in individual 10-3, from a maternally inherited chromosome. We did not find evidence of a *de novo* Arg528His mutation in the families that we studied.

### Incomplete Penetrance in Women as a Distinctive Feature of the Arg528His Mutation

Are there clinical particularities associated with each mutation? In order to answer this question, we divided the 16

**Table 1****Haplotypes Segregating with CACNL1A3 Mutations in 16 Caucasian Families with hypoPP**

GROUP AND FAMILY	HAPLOTYPE			NUCLEOTIDE	MUTATION	DOMAIN
	D1S1726	CACNL1A3	D1S1723			
A:						
1 .....	4	6	5	G1583A	Arg528His	IIS4
2 .....	3	8	2	G1583A	Arg528His	IIS4
3 .....	4	4	4	G1583A	Arg528His	IIS4
4 .....	4	4	6	G1583A	Arg528His	IIS4
5 .....	3	6	6	G1583A	Arg528His	IIS4
6 .....	4	1	4	G1583A	Arg528His	IIS4
7 .....	7	8	6	G1583A	Arg528His	IIS4
8 .....	7	2	6	G1583A	Arg528His	IIS4
B:						
9 .....	7	8	6	G3716A	Arg1239His	IVS4
10 .....	4	8	6	G3716A	Arg1239His	IVS4
11 .....	4	4	5	G3716A	Arg1239His	IVS4
12 .....	4	2	6	G3716A	Arg1239His	IVS4
13 .....	3	6	5	G3716A	Arg1239His	IVS4
14 .....	7	8	4	G3716A	Arg1239His	IVS4
15 .....	4	8	4	G3716A	Arg1239His	IVS4
16 .....	4	8	8	G3716A	Arg1239His	IVS4

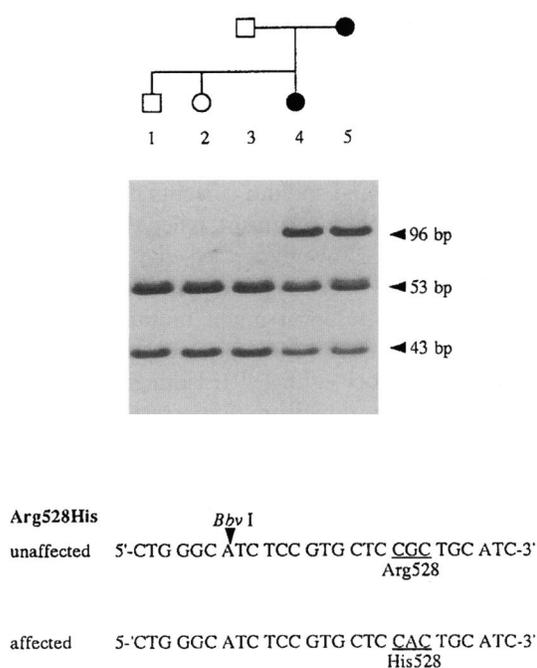
families into two groups, A and B, corresponding, respectively, to families 1–8 with the Arg528His mutation and families 9–16 with the Arg1239His mutation. As already described for hypoPP, we found male predominance (male:female sex ratio 1.23:1), which was not significantly different in groups A and B (data not shown). Interestingly, incomplete penetrance in women was observed only in group A. The estimated penetrance in females was 84% and 100% in groups A and B, respectively ( $\chi^2 = 7.37$ ;  $P < .01$ ). An example of incomplete penetrance is shown in family 6, where an unaffected woman (I-3) transmitted the disease to her children (fig. 2). One of her symptom-free daughters (II-6) transmitted hypoPP to her children (fig. 2). The Arg528His mutation was identified in these two women and in the asymptomatic daughter of the second woman (III-7) (fig. 2). In family 6, none of the females carrying the mutation suffered from hypoPP. No case of incomplete penetrance in males could be demonstrated. The mean age at onset was similar in both groups ( $14.8 \pm 1.3$  years and  $12.7 \pm 2.68$  years in groups A and B, respectively), as were the precipitating factors. The proportion of patients presenting clinical symptoms of myopathy was also similar in groups A and B (28.3% and 28.6% of the patients, respectively;  $\chi^2 = .002$ ; NS).

**No Evidence for a Founder Effect**

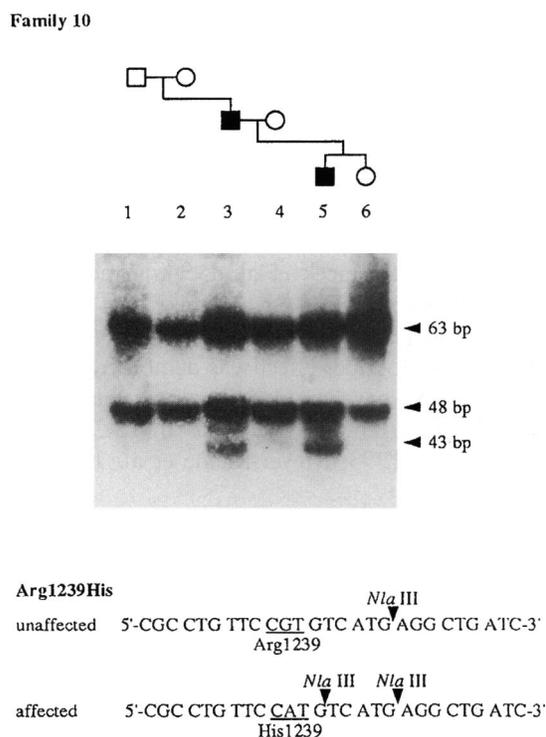
We typed CACNL1A3 and the flanking markers of the hypoPP1 locus, D1S1726 and D1S1723, in the 16 families and in unrelated individuals of Caucasian origin. The frequencies of CACNL1A3 alleles in the control population

were as follows: allele 1, .024; allele 2, .354; allele 3, .049; allele 4, .037; allele 5, .024; allele 6, .207; allele 7, .012; and allele 8, .293. D1S1726 and D1S1723 are separated by a genetic distance of 0 cM, as determined in the eight CEPH families used to construct the Génethon map (Gyapay et al. 1994; Jurkat-Rott et al. 1994). The physical distance between D1S1726 and D1S1723 is unknown. The haplotype 5-8 for D1S1726-CACNL1A3 is in linkage disequilibrium in the control population (frequency observed, 4.7%; frequency expected, 1%;  $\chi^2 = 5.6$ ;  $P < .05$ ), an observation consistent with the close genetic linkage of the two markers. CACNL1A3 allele 4 was significantly more frequent in hypoPP patients (frequency of allele 4, 18.75%; relative risk, 2.72;  $\chi^2 = 6.4$ ;  $P < .05$ ). An increase in the frequency of allele 4 of marker D1S1726 was found in hypoPP families (frequency of allele 4, 56%), compared with controls (frequency of allele 4, 25%) (relative risk, 2.05;  $\chi^2 = 6.7$ ;  $P = .01$ ). The frequency of allele 6 of marker D1S1723 was increased in the 16 families, compared with controls (frequency 43.8%, vs. 17.9% in controls; relative risk, 2.1;  $\chi^2 = 5.61$ ;  $P < .05$ ). However, for each marker, we did not observe a significant difference in the frequencies of these particular alleles in groups A and B. The haplotypes segregating with the disease for the flanking markers D1S1726 and D1S1723 of the hypoPP1 locus and for CACNL1A3 were reconstructed in the 16 families (table 1). Haplotypes segregating with the mutations were different. There was no haplotype preferentially associated with either mutation. In conclusion, our data do not support a founder effect in hypoPP.

A



B



**Figure 1** Rapid screening of dihydropyridine receptor, CACNL1A3, mutations causing hypoPP using PCR amplification of genomic DNA and restriction enzyme analysis: identification of a de novo Arg1239His mutation. The wild-type and the mutant dihydropyridine receptor gene sequences are shown for the Arg528His (A) and the Arg1239His (B) mutations; the restriction-enzyme sites that are modified

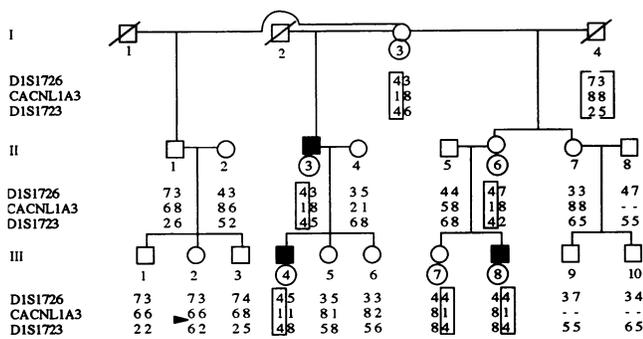
## Discussion

Eight of 16 Caucasian families with hypoPP displayed the Arg528His mutation, and the other 8 displayed the Arg1239His mutation, of CACNL1A3 (Jurkat-Rott et al. 1994; Ptacek et al. 1994a). The Arg1239His and the Arg528His mutations were originally reported in 10 and 9 families, respectively, but no information was given on the haplotypes segregating with either mutation (Jurkat-Rott et al. 1994; Ptacek et al. 1994a). A rarer mutation has also been described, by Ptacek et al. (1994a), at position 1239—the substitution of an arginine by a glycine—which was not found in our families. In addition to the families presented in the present paper, three other hypoPP families of Caucasian origin were studied. One of these families, also linked to chromosome 1q31-32, does not show any of the known mutations of the  $\alpha 1$  subunit of the dihydropyridine receptor (data not shown), suggesting that other mutations of the calcium channel remain to be discovered. The other two are not linked to the hypoPP1 locus. One is of French ancestry (Plassart et al. 1994a), and the other is of Portuguese origin (data not shown), establishing genetic heterogeneity in hypoPP (Plassart et al. 1994a). Taken together, these results indicate that the hypoPP1/CACNL1A3 locus on chromosome 1 is a major locus for hypoPP and that Arg528His and Arg1239His are predominant mutations, at least in the Caucasian population. Interestingly, a Japanese family with hypoPP also displays the Arg528His mutation, suggesting that it might also cause hypoPP in families with other ethnic backgrounds. The two mutations can easily be detected by PCR amplification of genomic DNA and restriction-enzyme analysis, enabling molecular diagnosis.

How do these data compare with those for other muscle ion-channel diseases? The Thr704Met and the Met1592-Val SCN4A mutations account for ~75% and ~25%, respectively, of hyperkalemic periodic paralysis families (Feero et al. 1993; Ptacek et al. 1993). In the French population, the Thr704Met mutation was the only SCN4A mutation observed in families with hyperkalemic periodic paral-

by the mutations are indicated—the Arg528His mutation abolishes a *Bbv*I site, and the Arg1239His mutation creates a new *Nla*III site. A, Pedigree of a family with the Arg528His mutation and the corresponding polyacrylamide gel stained by ethidium bromide, showing PCR-amplified genomic DNA fragments after digestion with *Bbv*I. PCR amplification and digestion in normal individuals (1–3) produce two bands (53 bp and 43 bp); PCR products of heterozygous affected individuals (4 and 5) contain a third, undigested band of 96 bp, resulting from the disappearance of a *Bbv*I site. B, Pedigree of family 10 and the corresponding Southern blot obtained by transfer of a polyacrylamide gel, showing PCR-amplified genomic DNA fragments after digestion with *Nla*III. PCR products of normal individuals (10-1, 10-2, 10-4, and 10-6) have two bands, of 63 bp and 48 bp, whereas PCR products of heterozygous affected patients (10-3 and 10-5) have a third band, of 43 bp, resulting from a new *Nla*III site in the 48-bp fragment.

Family 6



**Figure 2** Family with the Arg528His mutation showing incomplete penetrance in women. Clinically affected and unaffected individuals in family 6 are represented by blackened and unblackened symbols, respectively. Genotypes are indicated for markers D1S1726, CACNL1A3, and D1S1723. The haplotype segregating with hypoPP is boxed. Haplotypes between brackets are inferred. Recombination events are indicated by arrowheads. The identification numbers of individuals carrying the Arg528His mutation are circled. In this family, three women (I-3, II-6, and III-7) carry the mutation, but none are clinically affected.

ysis, despite the absence of a founder effect demonstrated by the analysis of intragenic dinucleotide repeats contained within SCN4A (Plassart et al. 1994b). The diversity of SCN4A mutations already described is more pronounced for paramyotonia congenita than for hyperkalemic periodic paralysis (Ptacek et al. 1993). Several SCN4A mutations occur more frequently than others, however: for example, the Thr1313Met mutation was observed in five of six families in the French population. In the German population, both the Arg1448Cys and the Arg1448His mutations of the sodium-channel SCN4A were found (Meyer-Kleine et al. 1994). A founder effect was demonstrated only for families originating in the Ravensberg area (Meyer-Kleine et al. 1994). The only study published so far with a large number of families with autosomal recessive myotonia congenita found the same mutation in the chloride-channel CLCN1 in only 15% of the families studied (Koch et al. 1993). Because of the lack of studies with large numbers of families affected by myotonia congenita, it is still impossible to draw definitive conclusions regarding the predominance of a particular CLCN1 mutation. It is interesting to note that, in the case of malignant-hyperthermia syndrome, genetic linkage to and a mutation in the ryanodine receptor RYR1 were published several years before a particular mutation was found that accounted for a significant number of cases (Quane et al. 1994).

Are there clinical characteristics preferentially associated with either mutation in hypoPP? We did not find a significant difference regarding the age at onset, the number of acute attacks, the precipitating factors, and the proportion of patients presenting a vacuolar myopathy. Interestingly, incomplete penetrance for women was found only in the families with the Arg528His mutation. As illustrated by

family 6, this observation is important for genetic counseling. These data also raise a fundamental question: Why do women carrying the Arg528His mutation not express the disease? Dihydropyridine receptors are located in the tubular system, which might be under hormonal control. A regulation of this type was recently demonstrated in a mammalian muscle-cell line expressing androgen receptors, where voltage-gated sodium currents decreased, through a posttranscriptional mechanism, when the cells were treated by androgens or overexpressed the androgen receptor (Tabb et al. 1994). Alternatively, the other subunits of the dihydropyridine receptor might modulate the phenotypic expression of the  $\alpha 1$  subunit, as already demonstrated for the  $\beta$  subunit (Lory et al. 1991; Isom et al. 1994). It is also possible that the dihydropyridine receptor might interact with other proteins, such as the ryanodine receptor, to induce a proper excitation-contraction coupling. These questions should be addressed by ongoing in vitro expression studies of the mutated dihydropyridine receptor. The role of a calcium channel in the occurrence of the vacuolar myopathy should also be clarified by these expression studies.

Finally, does a founder effect explain the origin of the two major mutations in hypoPP? When haplotypes were reconstructed for D1S1723, CACNL1A3, and D1S1726, no common haplotypes were found in patients sharing the same mutation. Mutations in dinucleotide repeats consist of an addition or deletion of a single base pair, at a rate estimated to be  $1/10^3$  (Weissenbach et al. 1992; Weber and Wong 1993). The dinucleotide-repeat mutation rate is therefore insufficient, by itself, to explain the variety of haplotypes shown by hypoPP patients. Moreover, both the demonstration of a de novo Arg1239His mutation, as already reported (Ptacek et al. 1994a), and the finding of a Arg528His mutation in a family of a different ethnic background (Japanese) do not support a founder effect. Similarly, no founder effect was demonstrated in hyperkalemic periodic paralysis and paramyotonia congenita, despite the recurrence of some of the SCN4A mutations (Wang et al. 1993; Meyer-Kleine et al. 1994; Plassart et al. 1994b).

Why do recurrent mutations occur within the same gene? From the studies of the sodium-channel SCN4A, we know that both conservative and nonconservative nucleotide changes may occur in the coding sequence of the gene (George et al. 1992; McClatchey et al. 1992a; Wang et al. 1992, 1993; Heine et al. 1993). Some of these nucleotide changes may not have biological significance and may be considered as benign polymorphisms, particularly when they are conservative in term of amino acids. The role of nonconservative nucleotide changes that do not segregate with the disease has not been investigated, however, by in vitro expression of mutated channels (Cannon et al. 1993; Cummins et al. 1993; Chahine et al. 1994). Since the sequence of ion channels is well conserved throughout evolu-

tion, nonconservative nucleotide changes may modulate the (abnormal) properties of the mutated channel, contributing to the well-known intrafamilial clinical variability in muscle ion-channel diseases. The predominance of certain mutations might be due to an observation bias caused by the phenotype observed, i.e., hypoPP in this case. Consequently, other mutations of the  $\alpha 1$  subunit of the dihydropyridine receptor might be associated with an as yet unlinked phenotype, as with hyperkalemic periodic paralysis and paramyotonia congenita and the sodium-channel gene SCN4A. It should be recalled that linkage of the dihydropyridine receptor to hypoPP was unexpected (Fontaine et al. 1994). Both of the predominant mutations in hypoPP result in an amino acid change of an arginine in the voltage sensor of the calcium channel, which belongs to a ring of positive charges shared by all the members of the voltage-gated ion-channel family (potassium, sodium, and calcium channels) that might play a crucial role in the voltage-dependent calcium flux and excitation-contraction coupling.

In conclusion, our study of 16 families with hypoPP demonstrates the predominance of two mutations in CACNL1A3: the Arg528His and the Arg1239His mutations. The phenotype of the families displaying the two mutations was similar except for incomplete penetrance, which was only observed in females with the Arg528His mutation. Mutations affecting the same amino acids might account for the recurrence of these two mutations—rather than a founder effect, which was ruled out by haplotyping the families.

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