Screening of the *Filamin C* Gene in a Large Cohort of Hypertrophic Cardiomyopathy Patients

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Background—Recent exome sequencing studies identified filamin C (*FLNC*) as a candidate gene for hypertrophic cardiomyopathy (HCM). Our aim was to determine the rate of *FLNC* candidate variants in a large cohort of HCM patients who were also sequenced for the main sarcomere genes.

Methods and Results—A total of 448 HCM patients were next generation–sequenced (semiconductor chip technology) for the *MYH7, MYBPC3*, *TNNT2*, *TNNI3*, *ACTC1*, *TNNC1*, *MYL2*, *MYL3*, *TPM1*, and *FLNC* genes. We also sequenced 450 healthy controls from the same population. Based on the reported population frequencies, bioinformatic criteria, and familial segregation, we identified 20 *FLNC* candidate variants (13 new; 1 nonsense; and 19 missense) in 22 patients. Compared with the patients, only 1 of the control's missense variants was nonreported (P=0.007; Fisher exact probability test). Based on the familial segregation and the reported functional studies, 6 of the candidate variants (in 7 patients) were finally classified as likely pathogenic, 10 as variants of uncertain significance, and 4 as likely benign.

Conclusions—We provide a compelling evidence of the involvement of *FLNC* in the development of HCM. Most of the *FLNC* variants were associated with mild forms of HCM and a reduced penetrance, with few affected in the families to confirm the segregation. Our work, together with others who found *FLNC* variants among patients with dilated and restrictive cardiomyopathies, pointed to this gene as an important cause of structural cardiomyopathies.

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Key Words: cardiomyopathy, hypertrophic ■ filamin C ■ genetics, diagnostics ■ genetics, human ■ next-generation sequencing

Hypertrophic cardiomyopathy (HCM) is the most common genetic myocardial disease. In approximately half of the patients, the disease is linked to variants in well characterized sarcomere protein genes, with *MYH7* (cardiac β-myosin heavy chain) and *MYBPC3* (cardiac myosin-binding protein C) accounting for most of the variants.[1,2] Next-generation sequencing (NGS) techniques facilitate the screening of the HCM genes in large cohorts of patients.[3] In spite of this advance, causative variants are not identified in a significant number of patients who may, thus, harbor pathogenic variants in new HCM genes. Using an exome sequencing approach, our group identified the filamin C (*FLNC*) as a candidate HCM gene.[4] A putative *FLNC* variant (c.C4824G, p.A1539T) segregated with the disease in a family with several HCM-affected individuals, otherwise negative for the known HCM genes. Moreover, a complete *FLNC* sequencing in 92 HCM cases identified 7 additional variants, which segregated with the disease in their families. Heart tissue studies in some of these patients showed marked sarcomeric abnormalities in cardiac muscle, and functional analysis in cell cultures revealed that expression of the *FLNC* variants resulted in the formation of large *FLNC* aggregates.[4] More recently, variants in *FLNC* have been found in dilated cardiomyopathy and restrictive cardiomyopathy (RCM) patients, suggesting an important role of *FLNC* in the development of cardiomyopathies.[5,6]

See Clinical Perspective

FLNC encodes filamin C, a protein involved in muscle function by interacting with proteins of the sarcolemma and the Z disc.[7,8] Although this protein is expressed in skeletal and heart muscle, *FLNC* variants had been associated to myofibrillar myopathy, with some of these patients also showing cardiomyopathy.[9–12] Interestingly, none of our HCM patients showed symptoms of myopathy, and the analysis of skeletal muscle biopsies from 2 of them showed a normal muscle fiber histology and biochemistry.[4] These findings suggest that *FLNC* variants are not restricted to skeletal muscle disorders, but can also generate cardiac muscle abnormalities without skeletal myopathy.

Our aim was to determine the mutational spectrum of *FLNC* in a large cohort of HCM patients to establish the

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genetic contribution of this gene to HCM and the correlate of *FLNC* variants with the phenotype.

Methods

Patient and Control Cohorts

This research was approved by the Ethical Committee of Hospital Universitario Central Asturias, and all the participants signed an informed consent. A total of 448 HCM nonrelated index cases were recruited through the Hospital Universitario Central Asturias-Reference Unit for Familial Cardiomyopathies in the period 2001 to 2015. HCM was diagnosed based on clinical symptoms and a left ventricular septum ≥15 mm in the absence of any other condition that could explain the hypertrophy. Clinical evaluation included medical history, physical examination, 12-lead EKG, M-mode, 2-dimensional, and Doppler echocardiography. Some patients have also records of cardiac magnetic resonance, pacemaker reports, 24-hour Holter monitoring EKG, or exercise test. The sudden cardiac death risk score was calculated according to the 2014 Guidelines in high risk of sudden cardiac death.[13] Patients with at least 1 known relative who had been also diagnosed with HCM were defined as familial cases. In patients who carried candidate variants, we performed echocardiography to all the relatives who agreed to participate to determine the left ventricular septum.

We sequenced the *FLNC* gene in a total of 450 individuals of the Renal Health in Asturias (RENASTUR) cohort. They were Whites aged 60 to 85 years and without symptom of HCM. The main characteristics of this cohort was reported elsewhere.[14,15]

Next-Generation Sequencing

All the patients were sequenced for the *MYH7*, *MYBPC3*, *TNNT2*, *TNNI3*, *ACTC1*, *TNNC1*, *MYL2*, *MYL3*, *TPM1*, and *FLNC* genes using NGS with a custom AmpliSeq gene panel and the Ion Torrent *Personal Genome Machine* (PGM) semiconductor chip technology (Thermo Fisher Scientific). The AmpliSeq panel was designated online (Ion AmpliSeq Designer v4; https://www.ampliseq.com) to cover the coding exons plus at least 5 intron flanking nucleotides of the 10 genes. We compared several primer design options and ordered the one that gave the maximum target sequence coverage. Primer pairs to amplify a total of 255 fragments that covered 98.2% of the target sequence were provided by the manufacturer in only 2 tubes (Table I in the Data Supplement).

The NGS procedure was previously validated and described in detail as supplementary file.[3,16] Briefly, DNA from each patient was obtained and adjusted to a final concentration of 10 ng/μL. DNA pools containing 10 μL of the corresponding DNAs were prepared. The number of samples used to create the pools was decided taking into account the load capacity of the chip, the total length of the target sequences (≈46 Kb), the dilution of a unique rare allele inside the pool, and the number of reads per amplicon necessary to achieve a minimum coverage of 50×. Each pool was amplified with the Ion AmpliSeq Library Kit 2.0 in conjunction with Ion AmpliSeq Custom Primer Pool protocols according to the manufacturer's procedures (Thermo Fisher Scientific) and following the next steps: polymerase chain reaction (PCR) in 2 tubes, partial digestion of the primers with FuPa Reagent, ligation of the bar code adapters, purification by Agencourt AMPure XP Reagent, PCR with the adapters using Platinum PCR SuperMix High Fidelity enzyme (Invitrogen), purification by Agencourt AMPure XP Reagent, quantification of the sample (Agilent Bioanalyser Tapestation 2200 and Qubit 2.0 Fluorometer), and dilution of the sample to a final concentration of 8 pmol/L.

After amplification, emulsion PCR and enrichment were performed using Ion OneTouch 2 instrument and Ion ES instrument following the manufacturer's instructions (Thermo Fisher Scientific). We performed an emulsion PCR using the Ion PGM template Hi-Q OT2 Kit in the Ion OneTouch 2 instrument (Thermo Fisher Scientific). Template-positive spheres were recovered using Dynabeads MyOne Streptavidin C1 beads in the ES instrument (Thermo Fisher Scientific).

The sequencing was performed in the Ion Torrent Personal Machine Sequencer (PGM) using the Ion PGM Hi-Q sequencing Kit, with 318-v2 semiconductor chips. We used a 500-flow runs, which support a template read length of ≈200 bp. The raw PGM data were processed with the Torrent Suite v5 software (Thermo Fisher Scientific) to generate sequence reads filtered by the pipeline software quality controls. Reads assembling and variant identification were performed using both Variant Caller v5 software and Ion Reporter v5 software, using FastQ files containing sequence reads and the Ion AmpliSeq Designer BED file software to map the amplicons. We used the Integrative Genome Viewer (Broad Institute) for the analysis of depth coverage, sequence quality, and variants identification. Variants were identified with the somatic sample default algorithm.

The raw data were aligned against the reference GRCh38 sequence. Because *Exome Aggregation Consotium* (ExAC) variants are numbered according to the GRCh37 (hg19) sequence, to facilitate the interpretation of the genetic variation by other research groups, we referred the nucleotide position relative to the GRCh37. In reference to the coding sequence, the nucleotides were numbered according to the Emsembl (www.ensembl.org) *FLNC* transcript, ENST00000325888 (NM_001458).

For each candidate variant identified in the pools, the corresponding DNAs were individually amplified and sequenced with BigDye chemistry in an ABI3130 equipment (Life Technologies) to identify the carriers.

Variants Classification

Nucleotide variants that result in frameshift changes or likely affected mRNA splicing (changes in the intron splicing consensus nucleotides) were classified as probably damaging. Nonsense missense amino acid changes either not present among the controls or in less than 3 exomes in the *Exome Sequencing Project* (ESP) database were also classified as candidate variants for segregation studies; otherwise, *FLNC* variants were considered as polymorphism. The potential effect of these variants was measured by in silico predictors Poly-phen2[17] and SIFT[18] and meta-predictors Condel[19] and CADD.[20] Candidate variants were initially classified as follows: those with a positive segregation in at least 3 HCM affected in the family were classified as likely pathogenic; those with a positive segregation in only 2 patients but disease-associated by functional studies were also classified as likely pathogenic; otherwise, were classified as of uncertain significance (variant of uncertain effect) or likely benign variants (Figure 1). The variants were also classified according to the American College of Medical Genetics and Genomics criteria for variant classification in nonvalidated genes, as was the case of *FLNC* in HCM.[21]

Results

FLNC Variants in HCM Patients and Controls

A total of 169 of the 448 patients (38%) were carriers of variants in the main HCM-related genes (*MYH7*, *MYBPC3*, *TNNT2*, *TNNI3*, *ACTC1*, *TNNC1*, *MYL2*, *MYL3*, *TPM1*; Table II in the Data Supplement). In reference to *FLNC*, 46 of the 48 exons (96%) gave optimal reads in the NGS, and only exons 3 and 29 were below a 50× coverage (Figure I in the Data Supplement). Because these sequence failures were replicated in all the PGM runs, we concluded that the absence of nucleotide reads was likely because of some characteristic that made them refractory to amplification (such as a high GC nucleotide content), and exons 3 and 29 were, thus, Sanger sequenced in all patients. Nine of the rare *FLNC* variants had been previously characterized, and all these nucleotide changes were also identified in the NGS (Figure II in the Data Supplement). We, thus, concluded that our semiconductor chip NGS approach was highly effective in the screening of *FLNC*.

Among the HCM patients, we found a total of 37 missense and 1 nonsense variant (Table 1). We performed the NGS with

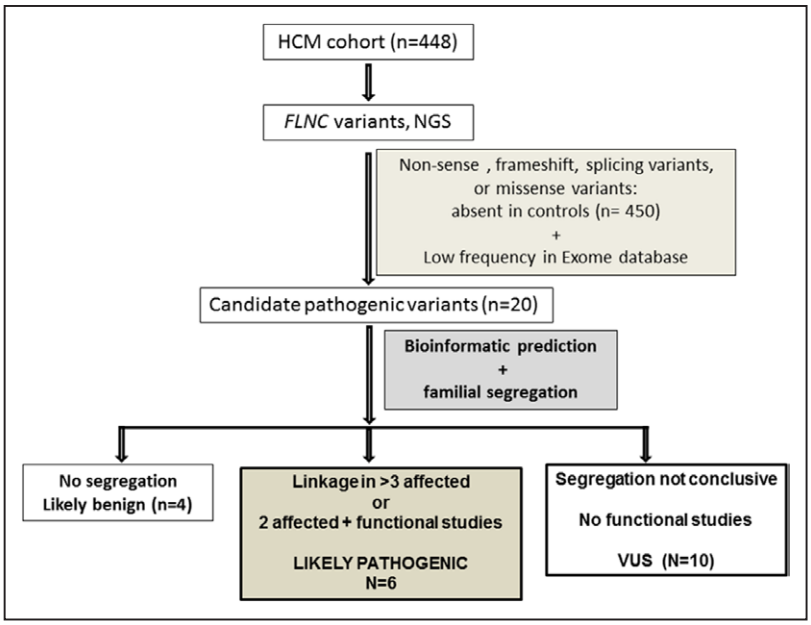

Figure 1. Flow chart showing the criteria for the selection of filamin C gene (*FLNC*) candidate variants in the hypertrophic cardiomyopathy (HCM) patients. NGS indicates next-generation sequencing; and VUS, variant of uncertain effect.

the same gene panel in 450 healthy elderly individuals, and we found a total of 22 missense variants (Table 2). We compared the frequency of variants found only in controls (n=9; 2%) versus only in patients (n=25; 5.6%), and the difference was significant; *P*=0.005. Only 1 of the control missense variants (c.5813C>T, p.P1938L) was not reported in both ESP and ExAC databases compared with 13 of the variants in the patients. Thus, the frequency of carriers of nonreported missense *FLNC* variants was 0.2% in the controls (1/450) versus 3.1% (14/448) in the patients (*P*=0.007; Fisher exact probability test). We could perform an echocardiography in the controls with 9 rare variants (including the p.P1938L carrier), and all of them presented septa in the normal range, which was consistent with the absence of HCM symptoms and nonpathogenic effect.

FLNC Variants Classification

A total of 20 *FLNC* nucleotide changes in 22 patients passed our basic criteria for candidate variants and were further subjected to familial screening (Figure 2). To our knowledge, none of the 20 *FLNC* variants have been found in patients with myopathy.

They were missense (n=19) or nonsense (n=1, p.E108X), absent in our control cohort, or reported at low frequency in the exome project database (Table 1). Among the 20 variants, 13 were new, 5 were rare nucleotide changes only in the ExAC, and only 2 in both ESP and ExAC databases. A total of 6 of the candidate variants were finally classified as likely pathogenic based on the familial segregation or positive functional studies: p.V123A, p.A1539T (2 patients), p.R2133H, p.R2140Q, p.P2298S, p.H2315N. Ten and 4 variants were classified as variant of uncertain effect and likely benign, respectively.

Four of the *FLNC* carriers also had a variant in the *MYH7* or *MYBPC3* genes. Thus, the frequency of *FLNC* rare variants was higher among noncarriers of a variant in the 9 sarcomere genes (18/279=6.4% versus 4/169=2.3%; *P*=0.03, Fisher exact probability test).

Family Studies

We could perform family studies in 15 of the 20 *FLNC* candidate variant carriers (Figure 3). Among these families, 4 were also positive for sarcomere variants. At least a second HCM affected was available for the genetic testing in 11 of the sarcomere-negative patients, and all them were also *FLNC* variant carriers (Table III in the Data Supplement). In these families, we identified a total of 36 candidate variant carriers, and 27 of them had hypertrophic left ventricular septa (excluding the individuals with borderline septum sizes; Table 3; Table IV in the Data Supplement). Thus, the *FLNC* candidate variants would lead to incomplete penetrance. Besides, 4 of the 6 nonaffected *FLNC* carriers were <40 years old, and we cannot exclude a late development of HCM in these individuals. Noteworthy, none of the HCM-affected relatives were negative for the corresponding *FLNC* variant.

Regarding the 4 carriers of an *FLNC* plus *MYBPC3* or *MYH7* variants, only *MYH7* p.R143Q in patient 479 was a bona fide pathogenic variant. Two children of this index case were screened for this and the corresponding *FLNC* variant (p.L1690F), resulting both negative for the *MYH7* variant, and a 6-year-old boy had the *FLNC* variant but a normal echocardiogram (Figure 3). Thus, the pathogenicity of this *FLNC* variant could not be confirmed by family studies and was, thus, classified as of uncertain effect. We could perform family studies in the index case with *MYBPC3* p.G1248R+*FLNC* p.E1408D. Despite the fact that several relatives were carriers of one or both variants, only the index case manifested HCM, and this questioned the pathogenicity of the 2 variants; *FLNC* p.E1408D was, thus, classified as a likely benign variant. The third index case was *MYH7* p.R1689H+*FLNC* p.N1843S, and no family members were available for the clinical and genetic study. Because of this limitation and the bioinformatic prediction, we classified this *FLNC* variant as likely nonpathogenic. The latter case was *MYBPC3* p.E258K+*FLNC* p.R2045Q. This *MYBPC3* variant was previously linked to HCM in several cohorts but present also in 3 individuals of the ExAC database. We could study 2

Table 1. Summary of the *FLNC* Variants Found in the 448 HCM Patients, Indicating the Pathogenic Effect According to Several In Silico Predictors and Meta-Predictors, and the Rare Variant Frequency in the ESP and ExAC Databases

Position	Coding	Effect	Exon	Protein Domain	SIFT	Polyphen-2	Condel	CADD	ESP Allele Frequency	ExAC Allele Frequency	In Control Cohort	Final Classification
128471013	c.322G>T	p.E108X*	1	ABD	…	…	…	38	No	No	No	VUS
128475395	c.368>C	p.V123A*	2	ABD	Damaging	Probably Damaging	Deleterious	26.9	No	No	No	Likely pathogenic
128477710	c.870C>A	p.N290K*	5	Ig-like 1	Damaging	Probably Damaging	Deleterious	33	No	No	No	VUS
128480139	c.1474A>G	p.K492E	9	Ig-like 3	Damaging	Benign	Deleterious	23	3/12 376	29/120 588	No	Polymorphism
128480184	c.1519G>A	p.G507R	9	Ig-like 3	Damaging	Probably Damaging	Deleterious	34	12/12 532	108/121 058	No	Polymorphism
128480629	c.1577G>A	p.R526Q	10	Ig-like 3	Damaging	Benign	Neutral	16.1	18/12 766	281/121 172	Yes	Polymorphism
128481578	c.2078A>C	p.D693A	13	Ig-like 5	Damaging	Possibly damaging	Deleterious	28.9	50/12 480	447/120 525	Yes	Polymorphism
128482291	c.2128G>A	p.D710N	14	Ig-like 5	Damaging	Benign	Neutral	24.1	3/12 642	32/116 320	Yes	Polymorphism
128482965	c.2507C>A	p.P836Q	16	Ig-like 6	Damaging	Probably damaging	Deleterious	25.4	24/12 712	70/119 636	Yes	Polymorphism
128482959	c.2501C>T	p.T834M	16	Ig-like 6	Tolerated	Benign	Deleterious	23.5	15/12 750	1081/121 092	No	Polymorphism
128484220	c.3092C>T	p.P1031L	20	Ig-like 8	Tolerated	Benign	Deleterious	19.8	No	8/120 960	No	Likely benign
128484823	c.3304C>T	p.P1102S	21	Ig-like 9	Damaging	Probably damaging	Deleterious	27.5	1/12 632	11/120 618	Yes	Polymorphism
128485240	c.3721C>T	p.R1241C	21	Ig-like 10	Damaging	Benign	Deleterious	27	86/12 852	756/121 212	Yes	Polymorphism
128485259	c.3740C>T	p.A1247V	21	Ig-like 11	Tolerated	Possibly damaging	Deleterious	23.8	No	7/119 154	Yes	Polymorphism
128486412	c.4022G>A	p.R1341Q	23	Ig-like 11	Tolerated	Benign	Deleterious	27.5	15/12 692	113/121 092	No	Polymorphism
128486895	c.4224G>C	p.E1408D	24	Ig-like 12	Tolerated	Probably Damaging	Deleterious	23.2	No	No	No	Likely benign
128488649	c.4616G>A	p.A1539T*	27	Ig-like 14	Damaging	Probably Damaging	Deleterious	32	No	No	No	Likely pathogenic
128488734	c.4700G>A	p.R1567Q	27	Ig-like 14	Tolerated	Possibly damaging	Deleterious	25	818/12 594	9212/121 038	Yes	Polymorphism
128488904	c.4795A>G	p.T1599A	28	Ig-like 14	Tolerated	Benign	Deleterious	8.5	No	1/121 146	No	VUS
128489475	c.5042C>T	p.T1681M	30	Ig-like 15	Damaging	Probably Damaging	Deleterious	27.7	No	4/120 642	No	VUS
128489501	c.5068C>T	p.L1690F	30	Ig-like 15	Damaging	Possibly Damaging	Deleterious	27.5	No	No	No	VUS
128490986	c.5528A>G	p.N1843S	33	Ig-like 16	Tolerated	Benign	Neutral	15.9	No	No	No	Likely benign
128491324	c.5578C>T	p.R1860C	34	Ig-like 17	Damaging	Possibly damaging	Neutral	34	57/12 410	646/120 992	Yes	Polymorphism
128491390	c.5644A>G	p.I1882V	34	Ig-like 17	Tolerated	Benign	Neutral	10.3	22/12 806	116/121 228	Yes	Polymorphism
128493011	c.6134G>A	p.R2045Q	37	Ig-like 19	Damaging	Benign	Deleterious	26.1	No	6/119892	No	Likely benign
128493052	c.6175G>A	p.V2059M	37	Ig-like 19	Tolerated	Benign	Neutral	23.9	9/12 556	135/121 022	Yes	Polymorphism
128493805	c.6398G>A	p.R2133H*	39	Interdomain	Damaging	Probably Damaging	Deleterious	34	No	No	No	Likely pathogenic
128493826	c.6419G>A	p.R2140Q	39	Interdomain	Damaging	Probably Damaging	Deleterious	34	1/12 726	2/121 150	No	Likely pathogenic

(*Continued*)

Table 1. Continued

Position	Coding	Effect	Exon	Protein Domain	SIFT	Polyphen-2	Condel	CADD	ESP Allele Frequency	ExAC Allele Frequency	In Control Cohort	Final Classification
128493858	c.6451G>A	p.G2151S*	39	Interdomain	Damaging	Probably Damaging	Deleterious	33	No	No	No	VUS
128494547	c.6808G>A	p.E2270K	41	Ig-like 20	Tolerated	Benign	Neutral	23.9	8/12 864	79/83 320	Yes	Polymorphism
128494631	c.6892C>T	p.P2298S	41	Ig-like 20	Damaging	Probably Damaging	Neutral	28.3	No	No	No	Likely pathogenic
128494640	c.6901G>C	p.P2301A	41	Ig-like 20	Damaging	Probably Damaging	Deleterious	26	No	No	No	VUS
128494682	c.943C>A	p.H2315N*	41	Ig-like 21	Damaging	Benign	Neutral	23.6	No	No	No	Likely pathogenic
128494691	c.6952C>T	p.R2318W	41	Ig-like 21	Damaging	Probably Damaging	Deleterious	30	No	3/121 184	No	VUS
128494727	c.6988G>A	p.G2330S	41	Ig-like 21	Tolerated	Probably Damaging	Deleterious	25.8	No	89/112 846	No	Polymorphism
128494922	c.7091G>A	p.R2364H	42	Ig-like 21	Tolerated	Benign	Neutral	25.7	25/12 412	234/120 948	Yes	Polymorphism
128494954	c.7123G>T	p.V2375F	42	Ig-like 21	Damaging	Probably Damaging	Deleterious	29.2	No	No	No	VUS
128496609	c.7750C>T	p.A2430V*	44	Ig-like 22	Tolerated	Possibly Damaging	Deleterious	11.6	2/12 626	11/121 122	No	VUS

Coding sequence nucleotides were numbered relative to the ENST00000325888.12 transcript. HCM indicates hypertrophic cardiomyopathy; *FLNC*, filamin C gene; Ig, immunoglobulin; and VUS, variant of uncertain effect.
*Cases with *FLNC* candidate variants in a previous sequencing of 93 patients.

relatives, 1 affected and *MYBPC3* carrier and 1 unaffected *FLNC* carrier. Thus, *FLNC* p.R2045Q was classified as likely benign.

Characteristics of Patients With *FLNC* Candidate Variants

Based on population frequencies, bioinformatic prediction, functional studies, and the familial segregation, a total of 6 variants were considered as likely pathogenic, 10 as of uncertain significance, and 4 as likely benign (Table 1). However, considering the American College of Medical Genetics and Genomics criteria for variant classification, only p.V123A and p.A1539T remained as likely pathogenic, the other variants being classified as variant of uncertain effect.

The main characteristics of the HCM index cases with *FLNC* candidate variants are summarized in Table 3. Most of the patients had mild symptoms, and this nonsevere clinical course would result in a reduced penetrance, which was in agreement with the lack of a large family history of HCM in most of the patients. Only 5 patients had a known family history of the disease (at least 1 known HCM-relative) at the time of their inclusion in the study. The mean onset age was 38 years (±12, range 20–64), and as presenting symptoms, 8 patients had dyspnea, 3 had syncope, and 3 had chest pain. The mean left ventricular wall thickness was 18.6 mm (±5, range 15–35). None of the patients had suffered resuscitated cardiac arrest, and 2 patients had received an ICD implanted for primary prevention, although only 1 of them had a high sudden cardiac death risk according to the 2014 Guidelines in high risk of sudden cardiac death. A patient with an initial diagnosis of HCM evolved to RCM and heart failure with transplant requirement. We compared the main characteristics of the patients with sarcomere and *FLNC* possibly/likely pathogenic variants, and none of the variables differed (1-way analysis of variance or Fisher exact probability tests) between the 2 groups (Table 4).

In Table 5, we summarized the mutational screening of the 9 sarcomere and the *FLNC* genes in our cohort of HCM patients. Interestingly, 4 of the 6 variants classified as likely pathogenic mapped in exons 39 and 41, near to the C-terminal domain, a fact that points to an important role for this *FLNC* region in the development of cardiac hypertrophy ($P<0.001$; χ^2 test; Figure 2).

Discussion

We performed a screening of *FLNC* in a cohort of 448 HCM patients and 450 healthy controls. In a previous analysis of 93 patients, we found 7 unique nonsense or missense variants, nonreported in the human genome variation databases. Here, we found a total of 38 missense/nonsense variants, and 20 were initially selected as candidate variants for family studies screening based on their absence or low frequency among controls. Four of the *FLNC* candidate variants were in patients who also carried an *MYBPC3* or *MYH7* variant.

The candidate variants were analyzed with several online tools. The in silico predictors can be used to measure the potential pathogenicity of missense variants, although the sensitivity and specificity is low, and this limits its usefulness as clinical tools.[22] Ultimately, the effect of these variants on disease risk should be confirmed by family segregation, which requires a minimum number of affected individuals to obtain a significant logarithm of odds (LOD) score. The highest LOD value

Table 2. *FLNC* **Missense Variants Identified in the Healthy Controls (RENASTUR Cohort), Indicating the Pathogenic Effect According to Several In Silico Predictors and Meta-Predictors, and the Rare Variant Frequency in the ESP and ExAC Databases**

Position	Coding	Effect	Exon	Protein Domain	Carriers	SIFT	Polyphen-2	CONDEL	CADD	ESP Allele Frequency	ExAC Allele Frequency	In HCM Cohort
128478381	c.1108A>G	p.M370V	7	Ig-like 2	1	Tolerated	Benign	Neutral	17	1/12706	7/120674	No
128478707	c.1261C>T	p.R421W	8	Ig-like 2	1	Damaging	Possibly damaging	Deleterious	33	No	3/117864	No
128478827	c.1381C>T	p.R461C	8	Ig-like 2	1	Damaging	Possibly damaging	Neutral	34	No	12/118654	No
128480620	c.1568T>C	p.V523A	10	Ig-like 3	1	Damaging	Possibly damaging	Deleterious	24	1/12766	23/121172	No
128480629	c.1577G>A	p.R526Q	10	Ig-like 3	4	Damaging	Benign	Neutral	16.1	18/12766	281/121172	Yes
128481562	c.2062G>A	p.A688T	13	Ig-like 5	2	Tolerated	Benign	Deleterious	21.2	No	5/120774	No
128481578	c.2078A>C	p.D693A	13	Ig-like 5	5	Damaging	Possibly damaging	Deleterious	28.9	50/12480	447/120525	Yes
128482291	c.2128G>A	p.D710N	14	Ig-like 5	8	Damaging	Benign	Neutral	24.1	3/12642	32/116320	Yes
128482965	c.2507C>A	p.P836Q	16	Ig-like 6	2	Damaging	Probably damaging	Deleterious	25.4	24/12712	70/119636	Yes
128484823	c.3304C>T	p.P1102S	21	Ig-like 9	2	Damaging	Probably damaging	Deleterious	27.5	1/12632	11/120618	Yes
128485240	c.3721C>T	p.R1241C	21	Ig-like 10	4	Damaging	Benign	Deleterious	27	86/12852	756/121212	Yes
128485259	c.3740C>T	p.A1247V	21	Ig-like 11	1	Tolerated	Possibly damaging	Deleterious	23.8	No	7/119154	Yes
128486052	c.3799C>T	p.R1267W	22	Ig-like 11	1	Damaging	Probably damaging	Deleterious	35	1/12618	3/118732	No
128486125	c.3872G>A	p.R1291H	22	Ig-like 11	1	Tolerated	Benign	Deleterious	23.1	2/12444	11/118840	No
128488734	c.4700G>A	p.R1567Q	27	Ig-like 14	38	Tolerated	Possibly damaging	Deleterious	25	818/12594	9212/121038	Yes
128489475	c.5042C>G	p.T1681R	30	Ig-like 15	1	Damaging	Possibly damaging	Deleterious	20.1	19/12924	69/120642	No
128491324	c.5578C>T	p.R1860C	34	Ig-like 17	12	Damaging	Possibly damaging	Neutral	34	57/12410	646/120992	Yes
128491390	c.5644A>G	p.I1882V	34	Ig-like 17	3	Tolerated	Benign	Neutral	10.3	22/12806	116/121228	Yes
128491653	c.5813C>T	p.P1938L	35	Ig-like 17	1	Damaging	Probably damaging	Deleterious	25.8	No	No	No
128493052	c.6175G>A	p.V2059M	37	Ig-like 19	6	Tolerated	Benign	Neutral	23.9	9/12556	135/121022	Yes
128494547	c.6808G>A	p.E2270K	41	Ig-like 20	2	Tolerated	Benign	Neutral	23.9	8/12864	79/83320	Yes
128494922	c.7091G>A	p.R2364H	42	Ig-like 21	3	Tolerated	Benign	Neutral	25.7	25/12412	234/120948	Yes

HCM indicates hypertrophic cardiomyopathy; *FLNC*, filamin C gene; and Ig, immunoglobulin.

corresponded to 2 families with 4 affected individuals each. After including the bioinformatic prediction, reported functional studies, and the familial data, we found a total of 6 likely pathogenic variants. Ten variants were finally classified as of uncertain significance and 4 as likely benign. Our threshold to define the likely pathogenicity was either the cosegregation in at least 3 HCM affected or a positive segregation in 2 HCM-affected plus functional studies that showed a pathogenic effect. These do not fully address the American College of Medical Genetics and Genomics requirements to classify a variant as definitely pathogenic.[21] These criteria have been established for well-characterized genes with large amount of data to conclude their role in the disease, which it is not yet the case of *FLNC* in HCM.[21] Under the American College of Medical Genetics and Genomics rules, only p.A1539T and p.V123A fulfill the criteria to be classified as likely pathogenic. The missense changes, p.R2133H, p.R2140Q, p.P2298S, and p.H2315N, did not accomplish these criteria, but either previous studies suggested a functional effect or had a positive segregation of at least 3 HCM affected and were, thus, considered as likely pathogenic.

In our cohort, the main limitation to classify the candidate variants was the small size of the families, with segregation across few meiosis to derive significant linkage values. In the absence of a clear familial segregation of the candidate variant with the disease, we cannot rule out that some of the rare amino acid changes are actually neutral, not related with the risk of developing HCM, and were, thus, classified as variant of uncertain effect.[23,24] This was likely a consequence of the

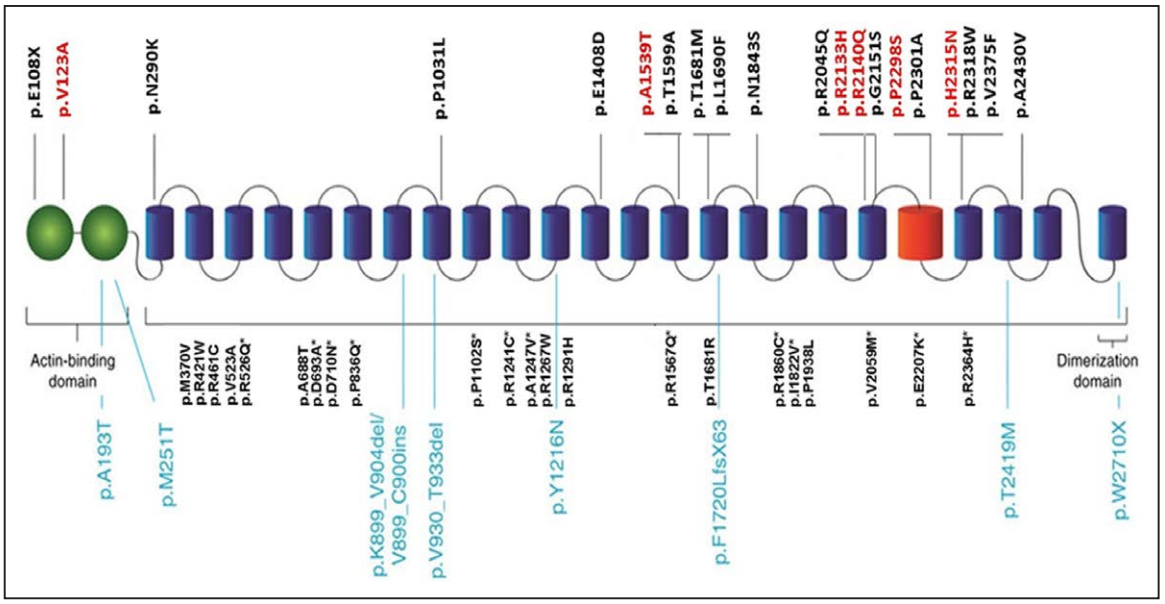

Figure 2. Map of the filamin C protein domains with the position of the candidate variants. **Above**, The 20 candidate variants identified by NGS: red, likely pathogenic variants; and black, VUS or likely benign. **Below**, Blue, myopathy-associated variants; and black, variants found in controls. *Control variants also found in the patients. NGS indicates next-generation sequencing; and VUS, variant of uncertain effect.

mild phenotype associated with most of the candidate variants, which could result in a reduced penetrance and a lack of large families to confirm the segregation. This is also a characteristic of some of the rare variants in well-characterized HCM genes, which were initially classified as disease-associated variants based on their absence in control cohorts of limited size and by bioinformatic predictors, but are now present in the exome databases.[25–28]

In HCM, the age-dependent penetrance that characterizes most of the candidate variants makes difficult their confirmation by familial linkage analysis. For instance, the *MYBPC3* p.G263X is the most common sarcomere mutation in our population. A total of 11 patients (2%) were carriers of this variant, compared with none of the controls. In spite of its clear pathogenic value, this nonsense change was associated with mild forms of HCM, with some carriers remaining

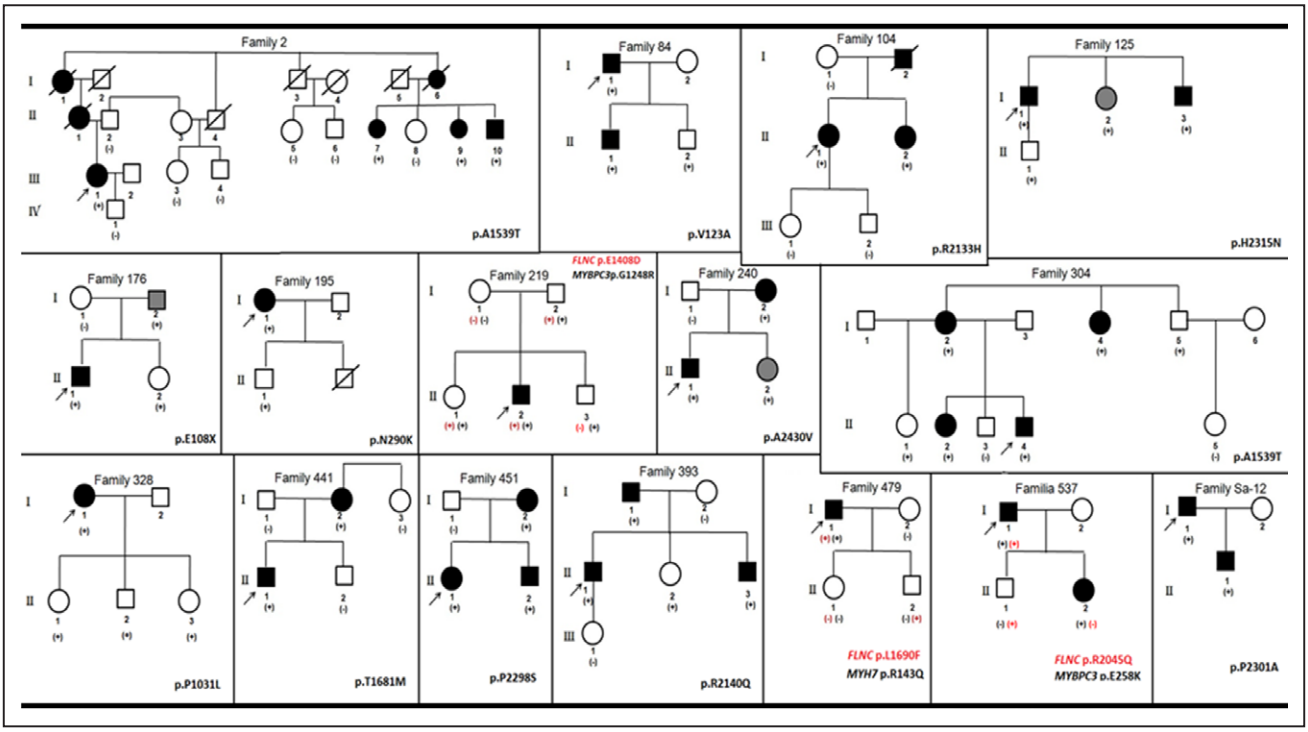

Figure 3. Pedigrees of the families with at least 2 hypertrophic cardiomyopathy (HCM) affected. Black filled symbol, HCM patients; gray, patients with borderline septa sizes. +Carriers of the candidate filamin C gene (*FLNC*) variant (–noncarriers).

Table 3. Main Clinical Features of the HCM Index Cases Carriers of the *FLNC* Likely Pathogenic/Uncertain Significance (VSU) Variants

Patient ID	Protein Change	ACMG Class	Other Variant	Sex	Age Onset	Family History HCM/SCD*	Carriers (Affected)	Dyspnea	SCD	CP	Syncope	HF	LVOTO	EF Range	EKG-H	AF	Holter	LVWT, mm	CMR Fibrosis	SCD Risk
176	E108X	VSU	NO	Male	21	0/0	3 (1)	0	0	0	0	0	0	N	1	0	0	15	0	low
84	V123A	Likely pathogenic	NO	Male	46	0/0	3 (2)	I/IV	0	1	0	0	1	N	1	1	0	20	…	low
195	N290K	VSU	NO	Female	64	0/1	2 (1)	I/IV	0	0	0	0	1	N	1	0	0	18	0	low
2	A1539T	Likely pathogenic	NO	Female	31	1/1	4 (4)	II/IV	0	0	0	0	1	N	1	1	NSVT	35	1	High
304	A1539T	Likely pathogenic	NO	Male	30	1/0	6 (4)	II/IV	0	0	0	0	0	N	1	0	0	15	…	low
441	T1681M	VSU	NO	Male	36	0/0	2 (2)	0	0	0	1	0	0	N	1	0	0	18	1	low
479	L1690F	VSU	MYH7 R143Q	Male	40	0/0	1 (1)	0	0	0	0	0	0	N	1	0	0	20	0	low
104	R2133H	Likely pathogenic	NO	Female	37	1/0	2 (2)	0	0	0	1	0	0	N	1	0	NSVT	18	…	Intenr
393	R2140Q	Likely pathogenic	NO	Male	44	0/0	4 (3)	0	0	0	1	0	0	N	1	0	0	18	…	low
242	G2151S	VSU	NO	Female	20	0/0	1 (1)	II/IV	0	0	0	1	0	N	1	0	0	TxR	…	…
451	P2298S	Likely pathogenic	NO	Female	26	0/0	3 (3)	0	0	0	0	0	0	N	1	0	0	16	1	low
Sa12	P2301A	VSU	NO	Male	36	1/1	2 (2)	III/IV	0	0	0	0	0	N	1	1	0	17	…	low
125	H2315N	Likely pathogenic	NO	Male	51	1/0	4 (2)	0	0	1	0	0	0	N	1	0	0	15	…	low
549	V2375F	VSU	NO	Male	50	0/0	1 (1)	I/IV	0	0	0	0	0	N	1	0	NSVT	17	…	low
240	A2430V	VSU	NO	Male	29	0/0	3 (2)	0	0	1	0	0	0	N	1	0	NSVT	19	…	Intenr
Sa8	A2430V	VSU	NO	Female	51	0/0	1 (1)	II/IV	0	0	0	0	0	N	1	0	0	18	…	Low

0=no, 1=yes. ACMG Class indicates American Collegue of Medical Genetics Classification; AF, atrial fibrillation; CMR, cardiac magnetic resonance; CP, chest pain; EF range, ejection fraction range (N=normal); EKG-H, electrocardiogram; *FLNC*, filamin C gene; HCM, hypertrophic cardiomyopathy; HF, heart failure; LVOTO, left ventricular outflow track obstruction; LVWT, left ventricular wall thickness (TxR=transplanted); and SCD, sudden cardiac death. SCD risk indicates new SCD risk prediction model from new 2014 Guidelines.

*History of SCD in first degree relatives.

Table 4. Main Characteristics of the HCM Index Patients According to the Presence of Candidate Variants in *FLNC* Versus the Main Sarcomere Genes and Patients Without Identified Candidate Variants

HCM Patients (n)	Sex (Male %)	Mean Inclusion Age	Mean Onset Age	HCM Family History, %	SCD Family History, %	Mean LVWT
Total (n=448)	45%	58±16	48±17	32	9	20.1±5
No variant (n=279)	46%	59±17	51±17	23	7	19.7±5
Sarcomere positive (n=169)	43%	54±16	44±16	47	12	20.9±6
FLNC candidate variants (n=10)	60%	49±16	36±9	50	20	18.7±6

None of the variables showed significant difference (P>0.05) between the groups (1-way ANOVA or Fisher exact tests). HCM indicates hypertrophic cardiomyopathy; *FLNC*, filamin C gene; LVWT, left ventricular wall thickness; and SCD, sudden cardiac death.

asymptomatic at elderly age.[29] As a consequence, we did not identify a second affected relative in 30% of the *MYBPC3* p.G263X index cases, who were, thus, apparently sporadic. In the case of *FLNC*, none of the candidate variants was found in >2 patients, and because of the benign course of HCM in most of the carriers, the private variants (found in only 1 patient) might be difficult to classify based on familial segregation.

Despite the uncertainty that characterizes most of the *FLNC* candidate variants, several data support a role for this gene in HCM. First, although in some of the families, there were carriers of the candidate variant who did not manifest the disease, none of the affected were negative for the corresponding candidate variant. Second, the frequency of nonreported missense changes was higher in the HCM compared with that in the healthy population cohorts. Only 1 of our control missense variants was not reported in the exome databases, compared with 13 of the HCM variants. Third, the frequency of *FLNC* carriers was lower among patients with a variant in any of the 9 well-characterized sarcomere genes compared with patients without variants in these genes (2.3% versus 6.4%; P=0.03). If *FLNC* variants were not associated with HCM, it would be expected a similar frequency of rare variants between the sarcomere-positive and -negative groups.

Table 5. Summary of the HCM Candidate Variants Found in the 10 Genes

Gene	Carriers of Likely Pathogenic	Percent in Total Patients	Percent in Candidate Variant Carriers
MYBPC3	100	22	56
MYH7	48	11	27
ACTC1	3	1	2
MYL2	4	1	2
MYL3	1	<1	1
TNNI3	8	2	5
TPM1	3	1	2
TNNT2	8	2	5
TNNC1	0	0	0
FLNC	7	2	4

HCM indicates hypertrophic cardiomyopathy; and *FLNC*, filamin C gene.

Variants in *FLNC* were previously reported in patients with myofibrillar myopathy, with some cases also showing cardiomyopathy.[9–12] Interestingly, none of the variants associated to myopathy overlapped with those found in our patients, who otherwise showed HCM without signs of myopathy. This suggests that the site of the amino acid change may determine in part the clinical manifestation, either myopathy or a pure cardiomyopathy without skeletal muscle disease. Similar results have been reported for other cardiomyopathy genes, such as *DES*, *LDB3*, *BAG3*, and *FHL1*, in which the variants resulted in pure or mixed heart and muscle affection.[30–35] Moreover, recent studies have linked *FLNC* candidate variants with structural cardiomyopathies in the absence of myofibrillary myopathy.[5,36,37]

Interestingly, 4 of the 6 *FLNC* likely pathogenic variants mapped to 2 exons, 39 and 41. On the other hand, the only rare missense variant that mapped to this region in the controls was classified as a polymorphism. These exons contain 41 and 90 amino acids, respectively, that represent 3.3% of the protein sequence. It, thus, seems that amino acid changes at the last immunoglobulin-like rod domains of *FLNC* are important for the development of HCM and other cardiomyopathies, while none of the myofibrillar myopathy–associated variants are in this protein region. Moreover, we reported that an HCM variant that mapped to this region (p.R2133H) formed *FLNC* aggregates.[4] In addition, Brodehl et al[5] performed exome sequencing in 2 families with several RCM patients and found 2 missense *FLNC* variants (p.S1624L and p.I2160F) that segregated with the disease and demonstrated the presence of cytoplasmic aggregates in heart tissue from RCM patients with p.S1624L. They also found that p.I2160F (localized in exon 39) showed fully familial segregation with the disease but did not form aggregates, suggesting that not all the *FLNC* variants exhibit the same pathomechanism. In this regard, mechanisms of protein aggregation, gain of function, and haploinsufficiency have been reported for the *FLNC* variants associated with myofibrillar myopathy.[12] Indeed, comprehensive functional studies are needed to undercover the *FLNC* mechanism that leads to the development of HCM and other cardiomyopathies, beyond the protein aggregation studies already reported.

In conclusion, we report the first large-scale screening of *FLNC* in HCM. Based on population, bioinformatic, functional, and familial segregation studies, we conclude that *FLNC* variants are likely associated with the development of HCM. The candidate variants were associated with mild forms of HCM and a reduced penetrance, making difficult their final classification as pathogenic and limiting their clinical usefulness.

Our work, together with others who found *FLNC* mutations among patients with dilated cardiomyopathy and RCM, points to *FLNC* as an important genetic determinant of structural cardiomyopathies.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Hypertrophic cardiomyopathy (HCM) is a genetic heterogeneous disease, with a few sarcomeric genes explaining most of the mutation-positive cases/families. However, a significant percentage of patients are negative for mutations in the known HCM genes and could, thus, have causative variants in not yet identified genes. Recent studies supported an important role for the filamin C gene (*FLNC*) in HCM and other cardiomyopathies, but the rate of *FLNC*-positive cases remains to be determined. Here we performed a next-generation sequencing of *FLNC* and the main sarcomere genes in 448 HCM patients. We identified 20 candidate variants in 22 patients, but only 6 were positively tested in the families and classified as likely pathogenic variants. Although we provide a compelling genetic evidence of the involvement of *FLNC* in HCM, the final classification of gene variants often relies in the familial cosegregation studies. Because this requirement is not always achieved, many of the variants are classified as of uncertain effect. This limits the usefulness of these candidate variants as tools for the genetic counseling and is applicable not only to FLNC but also to the well-characterized sarcomere genes. Our work highlights the importance of FLNC in HCM and the need to add this gene to the genetic screening, but also remarks the importance of family studies to obtain conclusions about the pathogenicity of candidate variants and the difficulty of reaching conclusions among isolated cases without a clear family history of the disease.