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Supplementary material

A Novel Founder Mutation in MYBPC3: Phenotypic Comparison With the Most Prevalent MYBPC3 Mutation in Spain

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SUPPLEMENTARY MATERIAL

Expanded Methods

Study Population

The index cases were diagnosed with hypertrophic cardiomyopathy (HCM) at the Inherited Cardiac Diseases Clinic of the Hospital Virgen de la Arrixaca University Hospital (Murcia, Spain). Hypertrophic cardiomyopathy was diagnosed by the presence of a hypertrophied left ventricle (maximum left ventricular wall thickness ≥ 15 mm in adult index patients or ≥ 13 mm in adult relatives) in the absence of any other cardiac or systemic disease able to cause left ventricular hypertrophy.[1,2] Disease penetrance was determined by echocardiography and/or electrocardiogram criteria.[3]

A pedigree was drawn for each patient and first-degree relatives were screened using the same protocol. Blood samples were taken for genetic analysis after participants gave written informed consent. The evaluation of probands and their relatives included medical history, physical examination, 12-lead electrocardiogram, M-mode, 2-dimensional and Doppler echocardiography, and, in affected individuals, an ambulatory 24-hour Holter electrocardiogram, an exercise test, and cardiovascular magnetic resonance.

Definitions: atrial fibrillation was considered when arrhythmia was present prior to the first evaluation or during follow-up in any of its forms (paroxysmal, persistent or permanent). Abnormal blood pressure response was considered when systolic blood pressure failed to increase at least 25 mmHg from baseline to maximum or peak exercise in the absence of the effect of medications. Any drop in systolic blood pressure during the treadmill test of more than 10 mmHg was also considered abnormal. Petersen's criteria for cardiac magnetic resonance images and Jenni´s for echocardiography were considered for the definition of left ventricular noncompaction.[4,5]

This study complies with the Declaration of Helsinki; it was approved by the local Ethics Committee.

Comparative Study

The phenotype of the 2 most prevalent mutations of the *MYBPC3* gene, which cause protein truncation (p.Pro108Alafs*9 and c.2308+1G>A) was compared. We described the clinical characteristics of 61 HCM carriers of

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c.2308+1G>A belonging to 21 families. Eighteen of them were previously published[6] and all were diagnosed at the same clinic.

Genetic Study

The complete *MYBPC3* gene was sequenced in the 13 index cases. All of them had a new identical mutation in the *MYBPC3* gene (p.Pro108Alafs*9/ P108Afs*9/ A107fsX116/ g.3095-3096insGCTGGCCCCTGCC, Reference sequence NG_007667.1, NM_000256.3). Next, the probands' relatives were offered genetic testing.

DNA was extracted from peripheral blood samples using standard protocols. The *MYBPC3* gene is located on the short arm of chromosome 11 close to the centromere from nt 47 352 957 to nt 47 374 253 according to the Ensembl database. Seven polymorphic markers that flank the *MYBPC3* gene were selected to investigate the presence of a common ancestor in carriers of *MYBPC3* p.Pro108Alafs*9 (*MYBPC3*-CA, D11S4109, D11S1784, D11S1326, D11S1763, D11S4165 and D11S1765).

RNA Isolation, cDNA Synthesis, and *MYBPC3* cDNA Amplifications

Total cellular RNA was isolated from whole peripheral blood collected into PAXgene blood collection tubes using Maxwell low elution volume (LEV) simply RNA kit (PROMEGA). cDNA synthesis was performed using the First Strand cDNA synthesis Kit for reverse transcription-polymerase chain reaction (RT-PCR) (AMW) (Roche). The cDNA products were amplified using 2 pairs of primers: forward 5'-AAGATTGACTTCGTACCCAGGC-3', reverse 5'-TCTTCTTGCGCTCCAGGATGT-3' and forward 5'-CCTCAAGGTCATAGAGGCAGA -3', reverse 5'-AGCAGTGAGCTGAAGTCCA-3' to amplify the aberrant cDNAs result from IVS23+1 G>A and Pro108Alafs*9 respectively. Polymerase chain reactions were performed in a final volume of 25 µL containing 2 µL of cDNA and using a touchdown PCR protocol between 62-60°C. Due to low *MYBPC3* expression in lymphocytes, the PCR were replicated to assess that all generated transcripts were amplified. The sizes of normal and mutated cDNA-PCR fragments were assessed, followed by size-fractionation on agarose gels. Subsequently, they were purified and sequenced on a DNA 3500XL Genetic Analyzer (Applied Biosystems).

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Puromycin Analysis

Lymphocytes from whole peripheral blood of a patient carrying c.2308+1 G>A and a control were short-cultured in RPMI 1640 + GlutaMAX medium (10% FBS, 1% penicillin-streptomycin) (Gibco) and phytohemagglutinin (Sigma). After 6-9 days, 2 subcultures were derived: one was treated with 250 µg/mL puromycin (Sigma) for 5 hours, while the other was left untreated. RNA was extracted as described above.

Statistical Analysis

A dedicated database was created for the study (based on Access 2000, Microsoft). The SPSS for PC statistical program (version 15.0) was used for the analysis. Continuous variables are presented as mean ± standard deviation. The 2-tailed Student *t* test and chi-square test were used to compare group data where appropriate. The Kaplan-Meier method was used for time-to-diagnosis analysis and time-to-event analysis.

A total of 140 individuals were included in the penetrance study. All 54 carriers of p.Pro108Alafs*9 (39 affected, 2 possibly affected and 13 unaffected) and 86 carriers of c.2308+1G>A (61 affected and 25 unaffected) were considered (Figure A-B of the supplementary material).

A total of 166 individuals were included in the sudden death (SD) survival analysis, including 147 carrier patients from the cohort and 19 historical SD cases; 6 cases from the latter had demonstrated HCM and 13 close relatives (n = 8 first-degree relatives, n = 5 second-degree relatives) did not (SD occurred before the date of inclusion and there was no available clinical or postmortem information). The age of SD cases with demonstration of HCM and those with no available information was similar (43.2 ± 0.8 vs 47.7 ± 18.2 years old, *P* = .2). In the cohort, there were 2 resuscitated cardiac arrests and 6 appropriate implantable cardioverter-defibrillator discharges, which were computed as SD equivalent for survival estimates. The age at each event or age at last follow-up were used for survival analysis. (Figure C-D of the supplementary material). A Kaplan-Meier chart was produced and Cox regression analysis was used for comparisons in the Figure of the supplementary material. The correlation between late gadolinium enhancement and left ventricular mass in carriers of 2 mutations was evaluated using Pearson's correlation test. A *P* value < .05 was considered statistically significant.

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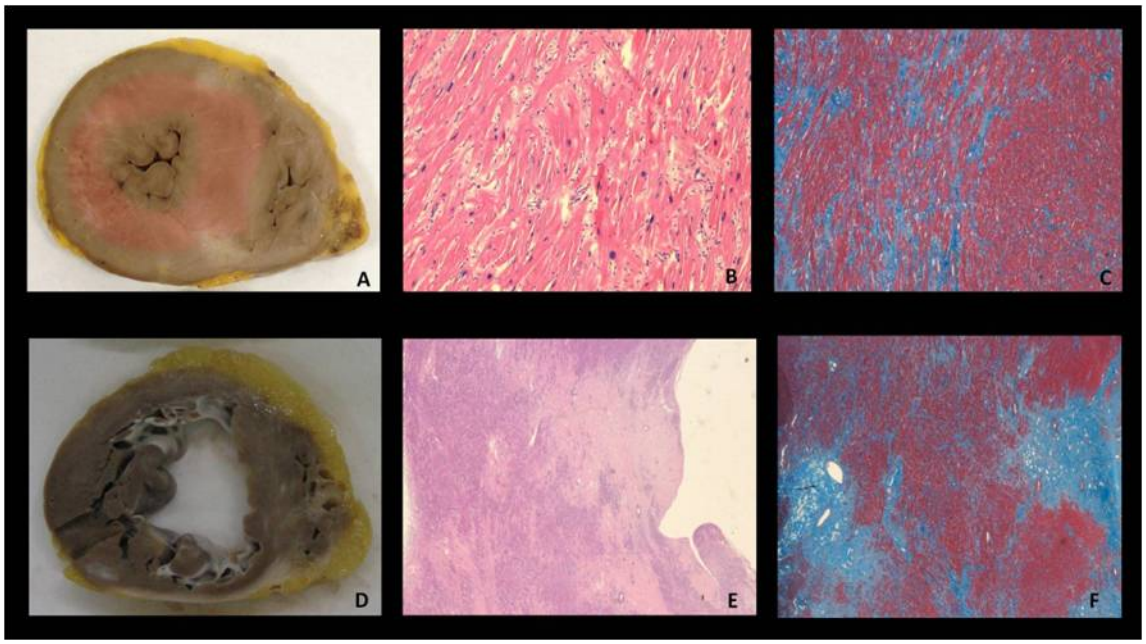
Figure of the supplementary material. Explanted hearts from carriers of the 2 mutations were available for histological study: A, B, C from p.Pro108Alafs*9 and D, E, F from c.2308+1G>A.



A: Macroscopic image from sections of ventricles where concentric hypertrophy with a small left ventricle cavity can be observed; B: Microscopically, ventricular hypertrophy and the characteristic disarray can be seen; C: Masson's trichrome stain shows a typical interstitial fibrosis with regular distribution; D: Macroscopic image from sections of ventricles where hypertrophy with signs of dilatation can be seen; E: a microscopic panoramic view with patchy fibrosis and F: Masson's trichrome stain shows the irregular fibrosis area.