A new polymorphism in human calmodulin III gene promoter is a potential modifier gene for familial hypertrophic cardiomyopathy

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Aims
Familial hypertrophic cardiomyopathy (FHC) is caused by mutations in genes encoding sarcomeric proteins. Incomplete penetrance suggests the existence of modifier genes. Calmodulin (CaM) could be of importance given the key role of Ca²⁺ for cardiac contractile function and growth. Any variant that affects CaM expression and/or function may impact on FHC clinical expression.

Methods and results
We screened the promoter region of human calmodulin III gene (CALM3) and identified a new −34T>A polymorphism with a T-allele frequency of 0.70. The distribution of CALM3 genotypes differed in 180 unrelated FHC patients carrying a known FHC mutation compared with 134 controls, with higher TT-genotype frequency (0.73 vs. 0.51) and lower frequencies of AT- (0.24 vs. 0.37) and AA genotypes (0.03 vs. 0.11; \( P = 0.0005 \)). To study whether the −34T>A polymorphism could play a modifier role, patients’ relatives including both affected and healthy carriers were added. Affected carriers had a 0.56 times higher odds of carrying a T allele than healthy carriers (\( P = 0.053 \)). We then investigated whether the −34T>A polymorphism affects the promoter activity using luciferase reporter vectors containing either CALM3-T or CALM3-A promoters. The activity of CALM3-T was lower than CALM3-A in HEK293 cells (1.00 ± 0.19 vs. 2.31 ± 0.13, \( P = 0.00001 \)) and in cardiomyocytes (0.96 ± 0.10 vs. 1.33 ± 0.08, \( P = 0.00727 \)).

Conclusion
These data suggest that the −34T>A CALM3 polymorphism is a modifier gene for FHC, potentially by affecting expression level of CALM3 and therefore Ca²⁺-handling and development of hypertrophy.

Keywords
Calcium • Cardiomyopathy • Hypertrophy • Genetics • Myocardium

Introduction
Hypertrophic cardiomyopathy is defined by the presence of increased ventricular wall thickness or mass in the absence of loading conditions (hypertension, valve disease) sufficient to cause the observed abnormality.¹ More than 70% of cases are familial (Familial hypertrophic cardiomyopathy—FHC). The major features of the disease are asymmetric left ventricular hypertrophy (LVH),
myocyte disarray, and interstitial myocardial fibrosis.\textsuperscript{2,3} The pure form of FHC is inherited in an autosomal dominant pattern and is associated with mutations in at least 13 genes encoding sarcomeric proteins (for review, see reference 4). Out of them, MYBPC3\textsuperscript{5,6} and MYH7\textsuperscript{7} encoding cardiac myosin-binding protein C (cMyBP-C) or β-myosin heavy chain, respectively, are the most frequently mutated genes.\textsuperscript{8}

One of the major features of FHC is a wide phenotypic heterogeneity among affected subjects, which is characterized by variable degree or distribution of hypertrophy and prognosis. Part of this can be explained by locus heterogeneity.\textsuperscript{4,9–12} Moreover, genetic studies have revealed the presence of clinically healthy individuals carrying the mutant allele, which is, in first-degree relatives, associated with a typical phenotype of the disease.\textsuperscript{13,14} This variable expressivity suggests the existence of modifier genes or polymorphisms, which modulate the phenotypic expression of the disease. Obvious candidate modifier genes encode proteins implicated in cardiac growth and hypertrophy. Several components of the renin–angiotensin–aldosterone system (RAAS) have been analysed in patients with FHC.\textsuperscript{15} An association has been found between the D allele of the angiotensin I-converting enzyme (ACE) polymorphism and cardiac hypertrophy in FHC patients carrying an MYH7 R403 codon mutation.\textsuperscript{16} The AT1 receptor A>C1166 and AT2 receptor A>C3123 polymorphisms contribute to cardiac hypertrophy in specific groups of patients with FHC.\textsuperscript{17,18} Finally, several genetic polymorphisms of the RAAS influence penetrance and degree of cardiac hypertrophy in gene carriers from one family with FHC caused by an MYBPC3 mutation.\textsuperscript{19}

Proteins involved in hypertrophic pathways or mediators of Ca\textsuperscript{2+} signalling in cardiomyocytes are promising candidates as modifier genes.\textsuperscript{20,21} Many of the actions of Ca\textsuperscript{2+} are mediated through its interaction with calmodulin (CaM), which serves as an intracellular sensor for Ca\textsuperscript{2+} homeostasis. Thus, any genetic variants that directly affect CaM gene expression and/or function would be expected to impact on the intracellular Ca\textsuperscript{2+} concentration. In humans, CaM is encoded by a multigene family consisting of three members, CALM1, CALM2, and CALM3, which are located on chromosomes 14q24–q31, 2p21.1–p21.3, and 19q13.2–q13.3.\textsuperscript{22} In the present study, we screened for mutations in the promoter region of the human CaM III gene (CALM3), identified a new T>A polymorphism at position −34 (−34T>A), which was differently distributed between FHC patients and controls and between affected and healthy carriers of an FHC mutation. Moreover, we found that the CALM3 promoter activity varied with the polymorphism in both HEK293 cells and neonatal rat cardiac myocytes (NRCMs). We provide evidence that the new −34T>A CALM3 polymorphism is a potential modifier gene for FHC in patients carrying a mutation in either the MYH7 or MYBPC3 gene.

**Methods**

The EUROGENE Heart Failure Project is a European consortium aimed at the evaluation of genetic factors in familial and sporadic forms of dilated and hypertrophic cardiomyopathies.

**Patients**

After informed consent was obtained by applying the guidelines of the Comité d’Éthique du Centre Hospitalier Universitaire de la Pitié-Salpêtrière (Paris), all subjects underwent clinical and cardiovascular examination including a 12-lead electrocardiogram (ECG) and M-mode, two dimensional, and Doppler echocardiography at the time of genotyping. The diagnosis of FHC was based on ECG and echocardiography as described previously; briefly, major echo diagnostic criteria were defined by a left ventricular end-diastolic maximal wall thickness (LVWT) >15 mm for index cases; for relatives, an LVWT >13 mm or the presence of major ECG abnormalities (LVH assessed by a Romhilt–Estes score ≥4 and/or Q waves >0.04 s or >1/3 R wave and/or significant ST-T changes). Left ventricular end-diastolic maximal wall thickness measurements were obtained at different locations (anterior and posterior septum, lateral and posterior wall) from the parasternal short-axis view, at both the mitral valve and papillary muscle levels, and at the parasternal long-axis view. Consecutive patients who fulfilled the inclusion criteria were screened to participate in the study from different tertiary centres in Europe (see Appendix). It could be a patient referred for the first time, a patient with a known diagnosis of FHC during a routine follow-up, or a relative screened through a familial survey. Control individuals (134, 72% spouses of the probands and 28% individuals without cardiac diseases), FHC index cases (180), and relatives (249) recruited from the EUROGENE Heart Failure Project were included in the study (Figure 1). Familial hypertrophic cardiomyopathy relatives were divided into two groups. The first was composed of affected carriers (112 individuals). The second was composed of healthy carriers (137 individuals). All individuals were Caucasian, and the age did not differ between index cases and controls and between affected and healthy carriers (Table 1). Both index cases and relatives were screened for mutations for the major FHC disease genes, and only those carrying a mutation in MYH7 or MYBPC3 genes were included in this study (Figure 1). Seven index cases and 8 relatives carry two mutations (13 compound heterozygotes, 2 homozygotes).

**Genetic analyses**

Genomic DNA was extracted from blood samples as described.\textsuperscript{23} The promoter region of the human CALM3 gene was determined on the basis of the genomic sequence (Genbank accession number
XS2606).24 Amplification was performed using the following primers: CALM3 F-5'-GCGGCGAGGAAAGTAGTGC-3' and CALM3 R-5'-GCCTCCGGCGCTCCACACT-3' on genomic DNA in a standard cycling programme composed of 36 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, with the GC-Rich PCR-System (Roche Diagnostics). The 279 bp polymerase chain reaction (PCR) fragment was then analysed by single-strand conformation polymorphism (SSCP); it was heat-denatured for 5 min in a formamide buffer at 95°C, kept on ice, loaded onto a 10% polyacrylamide gel, and then run at 8 mA per gel at 7°C and 20°C in the Mighty Small Gel Electrophoresis Unit (Hoeffer). The bands were visualized after silver staining of the gels (Pharmacia). Polymerase chain reaction products were sequenced using the BigDye™ Terminator (Applied Biosystems). The reaction products were then electrophoresed on an automated laser fluorescent DNA sequencer 377 (Applied Biosystems). The bands were visualized after silver staining of the samples separately (FHC and controls, and cell cultures); statistical analyses were performed using SAS 9.1.3 (Cary, NC, USA). The familial correlation was modelled assuming independent correlation for the working correlation matrix; a cumulative logit link with multinomial distribution was applied with a type 3 analysis (SAS PROC GENMOD, REPEATED option). Generalized estimating equations are a standard approach to the analysis of correlated data such as family data and can be intuitively thought of as similar to proportional odds logistic regression models in this case, except that they account for the correlation among pedigrees. The Bonferroni–Holm procedure was applied to account for the inflation of type I error due to multiple comparisons. P-values were adjusted for multiplicity for each of the two samples separately (FHC and controls, and cell cultures); P < 0.05 was considered significant. All tests performed were two-sided. Statistical analyses were performed using SAS 9.1.3 (Cary, NC, USA).

### Results

#### Identification of a −34T>A polymorphism in human CALM3 gene

A total of 134 control individuals were studied. A PCR fragment of the human CALM3 promoter region was analysed by SSCP sequencing or MALDI-TOF mass spectrometry (Figure 2). This shows three different patterns in the control population by SSCP (Figure 2B) and MALDI-TOF (Figure 2D). Sequence analysis revealed a T>A polymorphism at position −34 of the human CALM3 gene (−34T>A), which gave rise to three different genotypes, TT, AT, and AA (Figure 2C). The frequency of T allele was 0.70 in 134 control individuals. The distribution is compatible with the Hardy–Weinberg equilibrium.
Figure 2 Identification of a −34T>A polymorphism in the promoter region of human CALM3 gene. (A) Nucleotide sequence of the polymerase chain reaction fragment of human CALM3 promoter. Forward and reverse primer sequences used in polymerase chain reaction amplification are shown in blue. Green sequence represents the Ca²⁺ sensor region (AGGGA). The T>A polymorphism located at position −34 is indicated. (B) Single-strand conformation polymorphism patterns of control individuals performed in a 10% polyacrylamide gel run at 8 mA and 7°C. The different genotypes are indicated. (C) Reverse sequencing using the reverse primer shows the different genotypes, TT, AT, and AA (this corresponds to the antisense sequence). (D) Representative iPLEX spectra between ~4900 and 5500 Da of randomly chosen study samples exhibiting every genotype and the negative control. The very left signal (red dashed line) indicates the unextended iPLEX primer. The signal is expected at 5052 Da. The two possible signals on the right portion of the spectrum represent the expected allele identifying extension products (in yellow). The left signal at 5323 Da identifies the T allele; the right signal at 5379 Da identifies the A allele. If the expected signal is not observed, this label is displayed in red.

**Association of the −34T>A CALM3 gene polymorphism with hypertrophic cardiomyopathy**

A total of 180 unrelated FHC index cases with a known disease-causing mutation (data not shown) were analysed. The frequency of T allele was significantly higher in FHC patients when compared with controls (0.85 vs. 0.70, P < 0.0001, data not shown). Table 2 shows that the distribution of CALM3 genotypes significantly differed in FHC patients when compared with controls (P = 0.0005) with a higher frequency of TT genotypes (0.73 vs. 0.51) and a lower frequency of both AT (0.24 vs. 0.37) and AA genotypes (0.03 vs. 0.11). By separating FHC patients in two subgroups,
one carrying an MYBPC3 mutation and the other an MYH7 mutation, similar results were observed ($P = 0.0063$).

All patients’ relatives carrying a mutation in MYBPC3 or MYH7 were then analysed and divided into two groups. The first was composed of clinically affected carriers (112 individuals). The second was composed of clinically healthy carriers (137 individuals). The age did not differ between the two groups (Table 1). Affected carriers had a 0.56 times higher odds of carrying a T allele compared with healthy carriers, although this finding was not significant ($P = 0.053$, Table 3). In contrast, the odds for carrying a T allele were not different between index cases and either affected or healthy carriers (Table 4).

### Table 2 Distribution of the 34T>A CALM3 polymorphism genotypes in controls and index cases with familial hypertrophic cardiomyopathy

<table>
<thead>
<tr>
<th></th>
<th>$n$</th>
<th>TT</th>
<th>AT</th>
<th>AA</th>
<th>Adjusted $P$-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>134</td>
<td>69 (0.51)</td>
<td>50 (0.37)</td>
<td>15 (0.11)</td>
<td></td>
</tr>
<tr>
<td>FHC, index cases</td>
<td>180</td>
<td>131 (0.73)</td>
<td>43 (0.24)</td>
<td>6 (0.03)</td>
<td>0.0005</td>
</tr>
<tr>
<td>FHC, MYBPC3 index cases</td>
<td>105</td>
<td>76 (0.72)</td>
<td>25 (0.24)</td>
<td>4 (0.04)</td>
<td>0.0063</td>
</tr>
<tr>
<td>FHC, MYH7 index cases</td>
<td>75</td>
<td>55 (0.73)</td>
<td>18 (0.24)</td>
<td>2 (0.03)</td>
<td>0.0063</td>
</tr>
</tbody>
</table>

$n$ corresponds to the number of individuals. FHC patients were compared with control individuals.

### Table 3 34T>A CALM3 polymorphism genotypes in familial hypertrophic cardiomyopathy-affected and healthy carriers

<table>
<thead>
<tr>
<th></th>
<th>$n$</th>
<th>TT</th>
<th>AT</th>
<th>AA</th>
<th>Odds ratio</th>
<th>95% Confidence level</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy carriers</td>
<td>137</td>
<td>95 (0.69)</td>
<td>36 (0.26)</td>
<td>6 (0.05)</td>
<td>0.56</td>
<td>0.31–1.01</td>
<td>0.053</td>
</tr>
<tr>
<td>Affected carriers</td>
<td>112</td>
<td>89 (0.79)</td>
<td>23 (0.21)</td>
<td>0 (0)</td>
<td>0.70</td>
<td>0.39–1.13</td>
<td>0.14</td>
</tr>
</tbody>
</table>

$n$ corresponds to the number of individuals. Carriers correspond to relatives (affected and healthy) of the index cases with a mutation in either MYH7 or MYBPC3 gene. Affected carriers were compared with healthy carriers.

### Table 4 34T>A CALM3 polymorphism genotypes in familial hypertrophic cardiomyopathy index cases and in affected and healthy relatives

<table>
<thead>
<tr>
<th></th>
<th>$n$</th>
<th>TT</th>
<th>AT</th>
<th>AA</th>
<th>Odds ratio</th>
<th>95% Confidence level</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHC, index cases</td>
<td>180</td>
<td>131 (0.73)</td>
<td>43 (0.24)</td>
<td>6 (0.03)</td>
<td>0.70</td>
<td>0.39–1.13</td>
<td>0.14</td>
</tr>
<tr>
<td>Affected carriers</td>
<td>112</td>
<td>89 (0.79)</td>
<td>23 (0.21)</td>
<td>0 (0)</td>
<td>0.70</td>
<td>0.39–1.13</td>
<td>0.14</td>
</tr>
<tr>
<td>Healthy carriers</td>
<td>137</td>
<td>95 (0.69)</td>
<td>36 (0.26)</td>
<td>6 (0.05)</td>
<td>1.19</td>
<td>0.75–1.90</td>
<td>0.46</td>
</tr>
</tbody>
</table>

$n$ corresponds to the number of individuals. Carriers correspond to relatives (affected and healthy) of the index cases with a mutation in either MYH7 or MYBPC3 gene. Affected and healthy carriers were compared with FHC index cases.

Calm3 promoter activity is modulated by the 34T>A polymorphism

We then investigated whether the 34T>A polymorphism impacts on CALM3 promoter activity after the transfection of HEK293 cells and NRCM with pGL2-luciferase reporter vectors containing either CALM3-T or CALM3-A promoters (Figure 3). In both cell types, the vector containing the CALM3-T promoter activity gave rise to at least four-fold higher luciferase activity than the positive control consisting of the minimal SV40 promoter, indicating that the 5' flanking region of the CALM3 gene refers significant promoter activity as shown previously in another cell type.25 Importantly, the CALM3-T promoter activity was only 43 and 72% of that of the CALM3-A activity in HEK293 cells ($1.00 \pm 0.19$ vs. $2.31 \pm 0.13$, $P = 0.00001$) and in NRCM ($0.96 \pm 0.10$ vs. $1.33 \pm 0.08$, $P = 0.00727$), respectively.

### Discussion

In the present study, we describe a new 34T>A polymorphism in the 5' flanking region of human CALM3 gene, show that the distribution of the CALM3 genotypes varied between FHC patients and controls, and provide evidence that CALM3 promoter activity depends on the polymorphism in both HEK293 cells and NRCM.

The distribution of CALM3 genotypes in FHC patients was significantly different when compared with control individuals and did not depend on the underlying FHC mutation. This is different to what was previously observed with the I/D ACE polymorphism, for which a correlation between the DD genotype and FHC was found only in individuals carrying the Arg403 MYH7 mutation.16
More importantly, the distribution of CALM3 genotypes was different between affected and healthy carriers. Indeed, TT frequency was higher in affected carriers when compared with healthy carriers. This suggests that the $-34T>A$ polymorphism is a potential modifier gene for FHC.

Calmodulin is a ubiquitous, highly conserved $Ca^{2+}$ sensor of 17 kDa involved in the regulation of a wide variety of cellular events. In humans, CaM is transcribed from three genes that encode identical amino acids, although the coding sequences of the three genes differ markedly in the nucleotide composition. Although CALM1 and CALM2 genes contain a classical regulatory element, a TATA box, the 5′-flanking region of CALM3 has no TATA box but a typical feature of a house-keeping gene with a high GC content. The three CaM genes give rise to five different transcripts that are present in most of the tissues. In the rat heart, four isoforms are present, but no complete study has been obtained in the human heart. The few studies performed in humans showed that CALM3 gene is five-fold more actively transcribed than the other two genes in proliferating teratoma cells, proliferating activated T lymphocytes and several lymphoblastoid cell lines. The protein level of CaM was shown to decline several-fold in close temporal association with the declining population of proliferating rat cardiomyocytes. These data suggest that the CALM3 gene may be specifically and differently regulated during cardiac cell proliferation and/or cardiac hypertrophy.

The CALM3 gene polymorphism is located close to a conserved regulatory element, AGGGA, previously noted in promoters of other $Ca^{2+}$-binding protein genes of higher vertebrates. Particularly, this element was found in promoters of the three human CaM genes, chicken CaM gene, human and rat parvalbumin genes, and in mouse and chicken myosin light chain II/III. It has been shown that this element is repeated and is a $Ca^{2+}$-responsive element that regulates the expression of CALM2 and calbindin D28 genes in mouse Purkinje cells. Because the CALM3 polymorphism is located close to this element, it could result in differential regulation of the transcription of the CALM3 gene.

In the present paper, we show that the CALM3 promoter containing the T allele exhibits lower activity compared with the promoter with the A allele in both HEK293 cells and NRCM. If these data were translatable to the human heart, patients with the T allele would be expected to have lower CALM3 mRNA levels. Unfortunately, this question cannot be directly addressed due to the lack of myocardial tissues of these patients. But it is interesting to note that reduced CALM3 mRNA levels have been observed in myocardial samples from patients with end-stage heart failure. Interestingly, recent data indicate that CaM modifies the phenotype of Drasophila overexpressing human cMyBP-C. How a decrease in the amount of CaM could affect the development of hypertrophy and/or dysfunction of the heart remains elusive at present. But given the essential role of CaM for virtually all aspects of electromechanical coupling, growth and energy metabolism (for reviews, see references 43–45), any changes in one of the central $Ca^{2+}$-binding proteins are very likely to have multiple and important consequences. Studies are needed that specifically address this question, for example by knock-in the polymorphism in the CALM3 gene in mice.

In conclusion, the present study provides evidence that the $-34T>A$ CALM3 polymorphism is a potential candidate to modulate the development of FHC. Further analyses are needed to correlate the CALM3 genotypes and CaM expression in myocardial tissue of patients with FHC.

Acknowledgements

We would like to thank our family members for their collaboration.

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![Figure 3](image-url) Analysis of human CALM3 promoter activity after transfections of HEK 293 cells and neonatal rat cardiac myocytes. Experiments were performed as described in the Methods section. (A) Luciferase assays performed after the transfection of the different plasmids in HEK 293 cells. (B) Luciferase assays performed after the transfection of the different plasmids in neonatal rat cardiac myocytes. $(-)$ negative control; $(+)$ positive control; (A) CALM3-A promoter containing the A allele; (T) CALM3-T promoter containing the T allele. Number of experiments is indicated in bars.
Conflict of interest: none declared.

Appendix

The members of the EUROGENE Heart Failure project were as follows:

Coordinator: Richard Isnard (Paris, France).
France: Michel Komajda, Philippe Charron, Marie-Claude Aumont (Paris), Olivier Dubourg (Boulogne).
Germany: Christian Hengstenberg (Regensburg), Anette Richter, Bernhard Maisch (Marburg), Thomas Wichter (Münster).
Italy: Alessandra Serio, Eloisa Arbustini, Luigi Tavazzi (Pavia).
Portugal: Dulce Brito, Hugo Madeira (Lisbon).
Spain: Ana Diaz, Enrique Galve (Barcelona).
Sweden: Stellan Morner, Anders Waldenstrom (Umeå).
UK: John G.F. Cleland (Kingston-upon-Hull).

References


CARDIOVASCULAR FLASHLIGHT

**Cardiac metastasis of a gastric adenocarcinoma**

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A 76-year-old male was presented for artificial hip operation due to extensive arthrosis and reduced walking distance caused by pain. As the patient reports to suffer occasionally from palpitations on admission, further cardiologic examination is planned before the operation. On conventional transthoracic echocardiography, a mass of unknown origin is seen in the left atrium. Multi-slice thoracic computed tomography revealed a mass adjacent to the heart of unknown origin with a diameter of 60 mm (Panel A). Thus, the patient is referred for further evaluation by cardiac magnetic resonance (CMR) imaging.

A CMR examination using a 1.5 T whole-body scanner was performed. On steady-state free-precession imaging, a large (64 x 58 x 62 mm³) inhomogeneous mass in the atrioventricular groove between left atrium and left ventricle could be visualized. The mass infiltrated atrium and ventricle, and compromised their function as seen on cine images. Transversal T2-weighted imaging (Panel B) showed mainly isodense signal intensity of the tumour. Ten minutes after application of 0.2 mmol/kg body-weight gadolinium-based contrast agent (Magnevist, Schering, Germany), a gradient-echo inversion recovery sequence (late gadolinium enhancement) was performed (Panels C and D). The mass revealed irregular contrast uptake. Areas of high signal intensity signify large extracellular distribution of the mass that is partly due to necrosis. In areas of low signal intensity, high intensity of cellular distribution or lack of contrast diffusion due to destruction or extrusion of vessels could be presumed. Hence, a central necrotic tumour would be most probable. Furthermore, the infiltration of the mass into the myocardium could be demonstrated by the late gadolinium-enhancement images.

A gastric adenocarcinoma was diagnosed following tumour staging after CMR diagnosis. The cardiac mass was most likely identified to be a metastasis of the gastric cancer. Being confronted with the situation, the patient refused further treatment and was dismissed on his own request.

The combination of cross-sectional imaging including CMR in this case has been shown to be helpful for further cardiac differential diagnosis. Besides information about ventricular and atrial volumes, a cardiac tumour could be verified providing additional tissue information by CMR and triggering further diagnosis and potential therapy in this particular patient. In this case, the acquisition of late gadolinium-enhancement images provided additional information about the infiltration of the tumour into the left atrium and ventricle. Thus, CMR should be considered in cardiovascular diagnosis in line with other diagnostic tests for abnormal masses because of its high diagnostic value which has direct implication for patient management.