A Missense Mutation in the CHRM2 Gene Is Associated With Familial Dilated Cardiomyopathy

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Abstract—Circulating autoantibodies against the M2-muscarinic acetylcholine receptor (CHRM2) have been detected in patients with dilated cardiomyopathy (DCM). However, it has yet to be determined whether the pathogenesis of familial DCM may be linked to the genetic variability of the CHRM2 gene. The coding regions of the CHRM2 gene were examined by direct DNA sequencing. Plasma concentrations of autoantibodies against CHRM2 were determined by ELISA in 7 unrelated DCM families. Linkage analysis demonstrated cosegregation of autoantibodies against CHRM2 were determined by D7S509 and D7S495 that flank the CHRM2 gene, with the familial form of DCM. A novel missense mutation (C722G) replacing cysteine with tryptophane (Cys176Trp) was identified in the CHRM2 gene in all affected members but was absent in unaffected members. Additionally, 139 sporadic DCM patients and 450 normal volunteers were screened for the same mutation, but none were identified. Among the 12 affected members with familial DCM, 5 patients had died suddenly and 7 experienced ventricular arrhythmia, atrioventricular conduction block, and heart failure. All mutation carriers were positive for autoantibodies against CHRM2. Survival analysis disclosed that prognosis in patients who were mutation carriers with familial DCM was poorer than that seen in patients who were noncarriers with sporadic DCM (P < 0.05). We have identified a novel missense mutation (C722G) in the CHRM2 gene associated with familial DCM. We also show that this variant correlates with the presence of autoantibodies against CHRM2. Patients with C722G mutation have more progressive disease, characterized by sudden death, arrhythmia, and heart failure. (Circ Res. 2008;102:0-0.)

Key Words: autoimmune ■ genetic ■ heart failure ■ sudden death

Muscarinic acetylcholine receptors (CHRM) belong to the G protein–coupled receptor family and contain 5 subtypes (M1 to M5). The M2 receptor (CHRM2) is primarily expressed in the heart (human and other mammalian species), and its activation results in negative chronotropic and inotropic effects by inhibiting adenylyl cyclase, decreasing intracellular cAMP,1,2 and reducing L-type Ca2+ current.3 It has been reported that the density of cardiac CHRM2 is significantly higher in patients with CHF as compared to normal controls.4

Autoimmune-mediated myocardial damage has been generally accepted to be the major mechanism causing dilated cardiomyopathy (DCM).5 Circulating autoantibodies against CHRM2 (anti-CHRM2) are present in 38% to 48% of patients with DCM.6 Previous studies from our group and others have demonstrated that anti-CHRM2 display “agonistic activity” against their target receptor and result in myocardial injury and cardiac dysfunction.7 Another study found that anti-CHRM2 alters the sinus node function, suggesting that this electrophysiological action may be responsible for arrhythmias in both patients with DCM and idiopathic atrial fibrillation.8

Moreover, we have previously demonstrated that anti-CHRM2–positive sera obtained from DCM patients have similar effects as those exerted by the CHRM2 agonist carbachol. Specifically, in cardiac myocytes isolated from guinea pigs, anti-CHRM2–positive sera decreases peak intensity and density of L-type Ca2+ and exerts significant negative inotropic effects. These effects are completely blocked by the CHRM2 antagonist, atropine.9 Collectively, these clinical and experimental evidence suggest that overproduction of anti-CHRM2 may play an important role in the development of DCM. However, the mechanisms responsible for this pathological autoantibody production remain unknown.

It has been reported that 20% to 40% of DCM cases are familial, with a predominance of autosomal-dominant inheritance pattern.10–18 A few novel loci have been mapped on the chromosome 7, including 7q22.3-31.19 and 7q12.1-7q21.20

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and these loci have been correlated with familial DCM. Previous linkage studies have shown that the markers D7S471 and D7S501 cosegregate with the disease-containing mutation that causes DCM. Collectively, evidence to date indicates that genetic factors play an important role in the etiology of DCM. However, the mechanism by which genetic variations may result in cardiac damage remains to be determined.

Autoantibodies have been shown to correlate with genetic mutations, and the presence of autoantibodies and autoimmunity have been reported to correlate with specific variations in DCM. Indeed, currently available clinical results suggest that overproduction of autoimmune antibody against CHRM2 may be related to genetic variations on chromosome 7. However, there is a lack of direct evidence to support this notion.

The purpose of this study is to determine whether genetic variations may underlie the familial form of DCM. Because production of anti-CHRM2 has been shown to result in cardiac injury, and genetic variations on chromosome 7 have been linked to DCM, we hypothesized that variations in the encoding regions of the CHRM2 gene may provide a link between genetic variation, autoimmune production, and cardiac injury in patients with familial DCM.

**Patients and Methods**

**Clinical Evaluation and Study Cases**

A diagnosis of DCM was made in accordance with the criteria established by the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the Classification of Cardiomyopathy. The familial DCM patients and all sporadic DCM patients were evaluated by collecting a detailed clinical history, physical examination, 12-lead ECG, chest radiography, and transthoracic echocardiography (M-mode, 3D, and Doppler). The diagnostic criteria for DCM were defined as an ejection fraction <50% and/or left ventricular shortening fraction <28% by echocardiography analysis, regional fractional shortening on M-mode analysis, or both in the presence of a left ventricular internal diastolic diameter ≥2.7 cm2/m of body surface area. The disease status of deceased individuals was based on a review of medical records. The study protocol was approved by the Ethics Committees of the Beijing Chao Yang Hospital and conformed to the Declaration of Helsinki. All participants gave written informed consent.

All patients in this study were of Han (Chinese) descent from the Northern part of China. Secondary causes of DCM, including coronary heart disease, myocarditis, hypertension, and valvular heart disease, were carefully excluded from the present study. Diagnostic criteria for familial DCM is as described above.

**Mutation Detection**

Blood samples were collected into tubes containing EDTA to allow for DNA extraction. DNA was extracted subsequently according to the standard procedure. Using the sequence of CHRM2 from GenBank (accession no. M16404), 6 sets of primers were designed to amplify the entire coding region of the gene according to its published genomic DNA sequence from nucleotide 195 to 1595 (see the expanded Materials and Methods section, available at http://circres.ahajournals.org). The PCR products were sequenced directly in an ABI3700 DNA sequencer (Applied Biosystems). The sequence alignment was performed by BLAST analysis through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). These sequences were also examined manually.

ELISA-Based Screening for Autoantibodies Against CHRM2

Autoantibody against M2-muscarinic acetylcholine receptor (anti-CHRM2) has been detected in the sera of patients with dilated cardiomyopathy and has received increased attention in recent years. CHRM2 contains 3 extracellular loops and 3 cytoplasmic loops. The second extracellular (E2) loop (residues 168 to 192, V-R-T-V-E-D-G-E-C-Y-I-Q-F -F-S-N-A-A-V-T-F-G-T-A-I-) acts as an autoimmune target. Synthetic peptides corresponding to the E2 loop were used as antigens in an ELISA-based screening for anti-CHRM2 in the sera of all subjects in the study. The shadow above demonstrates the recognized region of autoantibody. The ELISA immunoassay was performed according to the method described by Fu et al.

**Linkage Analysis**

Two-point linkage analysis was performed using the MLINK program of the linkage software package version 5.10 to explore whether familial DCM correlated with the variation of CHRM2. We modeled the disease as an AD inheritance with 100% penetrance, with a uniform distribution for the allele frequencies. Six short-tandem-repeat polymorphisms flanking the CHRM2 gene were used, as well as fluorescence-labeled forward primers (Table I in the online data supplement, linkage in family DCM to markers at 7q31-35 of CHRM2 gene). The PCR products were separated in an ABI3700 DNA sequencer (Applied Biosystems). Data collection and genotyping were conducted by ABI Prism Genemapper version 3.0 software.

**Data Analysis**

The influence of CHRM2 genotype on the occurrence of DCM was determined by analyzing contingency tables using Fisher’s exact test, with the results reported as odds ratios. Categorical data were presented as mean values±SEM or percentages. A positive score of anti-CHRM2 was defined as a ratio (sample OD—blank OD/negative control OD—blank OD) of ≥2.1. Survival analysis was estimated by the Kaplan–Meier method over a 3-year period. In all cases, a value of P<0.05 was considered as statistically significant. Analyses were performed with the GraphPad 4.0 software package (San Diego, Calif).

**Results**

**Clinical Data and Characteristics**

We screened the CHRM2 gene for DNA sequence variation in 7 unrelated DCM families, 139 unrelated patients with sporadic DCM, and 450 normal individuals by direct sequencing analysis. Twelve patients were identified who had the same missense mutation in the CHRM2 gene, and, interestingly, they all belong to the same DCM family. We found that different phenotypes showed different clinical characteristics. Specifically, the age of DCM onset was considerably younger in patients with the C722G allele as compared with sporadic DCM. In contrast, patients with sporadic DCM had worse left ventricular end diastolic diameter, systolic diameter, ejection fraction, and heart function when compared to mutation carriers in the DCM family (Table 1).

**Mutation Screening**

A missense mutation, C722G (Figure 1), which results in conversion of amino acid 176 (GenBank accession no. NM_001006630) from cysteine to tryptophane (Cys176Trp), was identified in 12 affected members in a DCM family. No mutation was identified in the CHRM2 gene in the unaffected family members, the other 6 unrelated DCM families, the 139 sporadic DCM cases, or 450 healthy controls. These results...
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demonstrate that the C722G substitution in the CHRM2 gene is a mutation associated with DCM, rather than rare polymorphism. In contrast, a mutation screen performed on several other previously characterized cardiovascular genes, such as the β1- and β2-adrenoceptors genes, the angiotensin II type 1 receptor gene and the angiotensin-converting enzyme gene, was negative.

These data indicate that the CHRM2 gene influences the onset age of DCM in patients who are C722G carriers of familial DCM. The patients with sporadic DCM had lower level of cardiac function than patients who were C722G carriers. However, the phenotype present in the family is notable for an autosomal dominant pattern of inheritance of the mixed DCM, which is notable for the presence of sudden death and severe arrhythmia (Table 2). These observations suggest that the C722G genotype may be an important prognostic indicator in the clinical evaluation of patients with familial DCM.

Pedigree and Linkage Analysis
The DCM family consists of 49 members spanning 6 generations (Figure 2). Of these individuals, 25 were available for blood collection and clinical evaluation. Review of the family history and medical records revealed that 5 affected individuals (III:1, III:3, III:5, IV:17 and V:10) had died suddenly or had diagnosed heart failure before the cohort could be identified as being predisposed to DCM. Twelve members carried the C722G mutation (Table 2), in which 5 members (IV:1, IV:11, IV:13, V:1, and V:12) died suddenly and developed CHF, whereas IV:3, IV:7, V:2, V:7, V:8, V:16, and V:11 experienced either arrhythmia or heart failure (supplemental Figures I through III, representative ECG findings and cardiac arrhythmias). All other members of the family were free of any symptoms associated with arrhythmia and heart failure.

Linkage analysis was performed to investigate the correlation of CHRM2 and familial DCM. Logarithm of the odds score of 4.62 and 4.43 (θ=0) was achieved at the D7S509 and D7S495 loci, respectively, that flank the CHRM2 gene and cosegregate with familial DCM. Taken together, these data provide strong evidence of linkage to the CHRM2 gene with the familial form of DCM.

Effect of CHRM2 Genotype on Anti-CHRM2
Interestingly, all of the C722G mutation carriers were positive for anti-CHRM2, a significantly higher rate than that seen in sporadic DCM (100% versus 45.4%, P<0.01). The rate of occurrence of this mutation was also significantly higher than what has been reported in the normal individuals (100% versus 9.1%, P<0.001). Likewise, the geometric mean titers were significantly higher than in nonmutation carriers with sporadic DCM, as well as the normal individuals (Figure 3). These results demonstrate that the mutation of CHRM2 gene is highly correlated to the presence of anti-CHRM2. We speculate that the immunoreactions induced by this missense mutation are an important causative factor but that there may be additional factors involved yet to be determined.

Survival Analysis
Kaplan–Meier survival analysis was performed using 3-year survival data (Figure 4). There were 5 deaths in 12 subjects with C722G mutation from the DCM family, in which 4 patients died suddenly and 1 patient succumbed to rapid atrial

Table 1. Clinical Characteristics of Different Phenotype

<table>
<thead>
<tr>
<th></th>
<th>Sporadic DCM (n=139) (WT)</th>
<th>Familial DCM (n=12) (C722G)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age*</td>
<td>43.7±1.1</td>
<td>29.4±3.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Age†</td>
<td>51.3±1.3</td>
<td>33.1±3.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>64/75</td>
<td>4/8</td>
<td>NS</td>
</tr>
<tr>
<td>HR (min)</td>
<td>97.7±1.3</td>
<td>88.2±1.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>101.7±1.1</td>
<td>103.8±1.6</td>
<td>NS</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>64.8±0.8</td>
<td>62.7±1.5</td>
<td>NS</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>68.9±0.5</td>
<td>62.7±2.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>56.9±0.6</td>
<td>51.7±3.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>33.8±0.8</td>
<td>40.1±2.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HF (NYHA)</td>
<td>3.2±0.1</td>
<td>2.3±0.3</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Data are means±SEM. *Clinical presentation; †age at time of experiment. HR indicates heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVEF, left ventricular ejection fraction; HF, heart failure.

Effect of CHRM2 Genotype on Anti-CHRM2

Interestingly, all of the C722G mutation carriers were positive for anti-CHRM2, a significantly higher rate than that seen in sporadic DCM (100% versus 45.4%, P<0.01). The rate of occurrence of this mutation was also significantly higher than what has been reported in the normal individuals (100% versus 9.1%, P<0.001). Likewise, the geometric mean titers were significantly higher than in nonmutation carriers with sporadic DCM, as well as the normal individuals (Figure 3). These results demonstrate that the mutation of CHRM2 gene is highly correlated to the presence of anti-CHRM2. We speculate that the immunoreactions induced by this missense mutation are an important causative factor but that there may be additional factors involved yet to be determined.

Survival Analysis
Kaplan–Meier survival analysis was performed using 3-year survival data (Figure 4). There were 5 deaths in 12 subjects with C722G mutation from the DCM family, in which 4 patients died suddenly and 1 patient succumbed to rapid atrial
fibrillation and heart failure. Ambulatory ECG monitoring showed that the mutation carriers experienced severe arrhythmia, ventricular tachycardia, rapid atrial fibrillation, or first or second degree atrioventricular conduction block (Table 2). These study results suggest that the mutation carriers have a significantly poorer prognosis than noncarriers (P<0.001, 5 deaths in 12 cases versus 27 deaths in 139 cases).

Analyses of Other DCM Cases
There were 10 patients with familial DCM from whom we obtained DNA samples. These patients were in the other 6 unrelated DCM families. We reviewed their medical records and observed that these patients have progressive disease characterized by progressive cardiac enlargement and end-stage heart failure, which resulted in 1 patient undergoing heart transplantation. This patient is alive and well at the time of publication. Only ECG records could be obtained, which showed arrhythmia in patients with sporadic DCM. However, because Holter data were unavailable, it is not sufficient to report these results.

Discussion

Evidence and Prognosis for a Disease-Associated Mutation
In the present study, we identified and reported a novel Cys176Trp conversion at amino acid site 176 of the CHRM2 gene in the affected members of a 6-generation DCM family. This family has progressive DCM manifested by sudden death, arrhythmias, and heart failure. The altered DNA sequence is considered a disease-associated mutation of familial DCM rather than a polymorphism for several reasons. Firstly, the C722G mutation is present in all affected family members but is absent in the unaffected family members, the 139 unrelated patients with sporadic DCM, or the 450 normal volunteers. Secondly, the CHRM2 gene

Table 2. Phenotype Data for the DCM Family With the C722G Mutation

<table>
<thead>
<tr>
<th>Pedigree</th>
<th>Sex/Onset</th>
<th>LVEDD (mm)</th>
<th>LVESD (mm)</th>
<th>LVSF/E (%)</th>
<th>Cardiac Phenotype</th>
<th>Holter/ECG Findings</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV:1</td>
<td>M/38</td>
<td>76.6</td>
<td>68.2</td>
<td>11.0/36.0</td>
<td>DCM</td>
<td>VT</td>
<td>Died while walking</td>
</tr>
<tr>
<td>IV:3</td>
<td>M/42</td>
<td>72.6</td>
<td>65.4</td>
<td>9.9/39.6</td>
<td>DCM</td>
<td>VT</td>
<td>CHF</td>
</tr>
<tr>
<td>IV:7</td>
<td>F/34</td>
<td>63.4</td>
<td>50.1</td>
<td>20.0/41.2</td>
<td>DCM</td>
<td>VT</td>
<td>CHF</td>
</tr>
<tr>
<td>V:1</td>
<td>M/19</td>
<td>58.9</td>
<td>46.1</td>
<td>21.8/40.6</td>
<td>DCM</td>
<td>VT</td>
<td>Died while running</td>
</tr>
<tr>
<td>V:2</td>
<td>M/17</td>
<td>52.3</td>
<td>43.6</td>
<td>16.7/35.6</td>
<td>DCM</td>
<td>AF</td>
<td>CHF</td>
</tr>
<tr>
<td>V:11</td>
<td>M/38</td>
<td>69.1</td>
<td>59.0</td>
<td>14.7/34.1</td>
<td>DCM</td>
<td>2°AVB</td>
<td>Died in sleep</td>
</tr>
<tr>
<td>V:13</td>
<td>F/40</td>
<td>65.0</td>
<td>54.0</td>
<td>16.9/38.4</td>
<td>DCM</td>
<td>2°AVB</td>
<td>Died while walking</td>
</tr>
<tr>
<td>V:7</td>
<td>M/18</td>
<td>59.2</td>
<td>48.1</td>
<td>18.8/32.6</td>
<td>DCM</td>
<td>VT</td>
<td>CHF</td>
</tr>
<tr>
<td>V:8</td>
<td>M/16</td>
<td>58.7</td>
<td>41.0</td>
<td>30.2/56.0</td>
<td>DCM</td>
<td>1°AVB</td>
<td>Nonsymptomatic</td>
</tr>
<tr>
<td>V:12</td>
<td>M/39</td>
<td>70.3</td>
<td>57.8</td>
<td>17.8/35.6</td>
<td>DCM</td>
<td>AF</td>
<td>Died from CHF</td>
</tr>
<tr>
<td>V:16</td>
<td>F/30</td>
<td>60.7</td>
<td>50.6</td>
<td>16.6/37.0</td>
<td>DCM</td>
<td>VT</td>
<td>CHF</td>
</tr>
<tr>
<td>V:1</td>
<td>F/22</td>
<td>58.9</td>
<td>48.0</td>
<td>18.5/43.4</td>
<td>DCM</td>
<td>AF</td>
<td>CHF</td>
</tr>
</tbody>
</table>

LVEDD indicates left ventricular end diastolic dimension; LVESD, left ventricular end-systolic dimension; LVSF/LVEF, left ventricular shortening fraction/ejection fraction; VT, ventricular tachycardia; AVB, atrioventricular conduction block; AF, atrial fibrillation.

Figure 2. Pedigree of the family DCM. Symbols represent the following traits: ■, affected males; ●, affected females; □, normal males; ○, normal females; slashed symbols, deceased individuals; arrow, proband; +, presence of mutation; −, absence of mutation; ✽, individuals who died suddenly or heart failure; ?, unknown clinical status and without DNA samples.
variant (C722G) is a DCM-associated mutation and the transition is shown to cosegregate with familial DCM, in which the mutation replaces a highly conserved amino acid. Thirdly, all of the C722G mutation carriers are positive for anti-CHRM2 and have the Cys176Trp substitution in the protein product. Fourthly, the age of onset of DCM in mutation carriers is significantly younger than in noncarriers. Lastly, it is of importance that the mortality in patients with the C722G mutation was significantly higher than the mortality rate observed in the noncarriers during the 3-year follow up. Because we cannot exclude the possibility that this DNA variant is in linkage disequilibrium with the DNA variant that is actually pathogenic, additional studies using transgenic animals are being designed to address the possible "causality" of the mutation.

The Cys176Trp Altering the Binding of Autoantibodies Against CHRM2

The CHRM2 gene is located at the 7q31-7q35. The transcribed mRNA product of the CHRM2 gene corresponds to nucleotides 195 to 1595 of the genomic sequence and codes for a 466 amino acid. Structural analysis indicates that CHRM2 contains 7 transmembrane domains, 3 extracellular loops (E1, E2 and E3), 3 intracellular loops (C1, C2 and C3), an extracellular amino terminus, and an intracellular carboxyl terminus.29–31

The second extracellular loop (E2) and the sequence of human muscarinic receptor subtypes (HM1 to HM4) share less homology (maximum 50%), but the E2 sequence of CHRM2 is conserved in various mammalian species, including Homo sapiens, Bos taurus, Mus musculus, Sus scrofa, Rattus norvegicus, and Pan troglodytes. Exposures to synthesized peptides based on the amino acid sequence of CHRM2 with the use of immune rabbits as antigens was observed to correlate with rise in the titer of autoantibodies against M2 receptors.32 It was predicted that the E2 sequence may serve as an antigen in an autoimmune reaction. The CHRM2 bears both an orthosteric and an allosteric binding site. Site-directed mutagenesis of the cysteine residue located in the E2 loop could lead to alternation of ligand binding. The missense mutation Cys176Trp identified in this study may alter the autoantibody binding to CHRM2,33 and studies are underway to confirm this.

Possible Pathogenesis of DCM in Mutation Carriers

The mutation in the E2 loop may function by altering the structure of either the orthosteric or allosteric binding site on cardiomyocytes. The substitution of cysteine destroyed the disulfide bond formation of the molecules, subsequently reducing the E2 loop flexibility and affecting the binding of autoantibodies against CHRM2 (anti-CHRM2).34 The autoantibodies, behaving like an agonist, could induce desensitization and/or downregulation of the receptors, leading to autonomic disturbances in DCM patients. Anti-CHRM2 can also behave as a positive allosteric modulator. We, therefore, presume that the mutation Cys176Trp alters allosteric binding sites such that agonists may not be able to bind to CHRM2 normally, therefore failing to protect the heart by boosting the parasympathetic system. However, the exact molecular mechanisms by which the C722G mutation contributes to familial DCM requires further investigation.

Possible Pathogenesis of Arrhythmia in Mutation Carriers

In this study, all of the C722G mutation carriers are positive for anti-CHRM2. Medei et al reported that the anti-CHRM2 have proarrhythmic effects.35 It was proved that electrophysiological effects of these autoantibodies can indicate altered activity of ion channels. Experimental evidence demonstrated that G protein–regulated inwardly rectifying K⁺ channels can operate as dynamic integrators of α-adrenergic and cholinergic signals in atrial myocytes. The autoantibodies combine allosteric binding sites. These channels are then activated by direct interactions with βγ subunits of the inhibitory G proteins and efficiently translate CHRM2 activation into membrane hyperpolarization.36 The CHRM2 activation shortens action potential duration and effective refractory period in myocardial cells, which may result in arrhythmia.37

Our previous study has shown that anti-CHRM2 decreases the stimulated L-type Ca²⁺ current and appears to have a negative inotropic effect in cardiac myocytes (supplemental

Figure 3. Relationship of CHMR2 genotype to the anti-CHMR2 titers. ***P<0.0001 vs NC; ###P<0.0001 vs NC or SWT. NC indicates normal control; FC722G, familial DCM; SWT, sporadic DCM.

Figure 4. The comparison of survival curves in carriers and non-carriers. Kaplan–Meier cumulative survivals data for death is in patients with the C722G mutation (dashed line) and patients with DCM but no C722G mutation (solid line). The relative risk is derived by log rank test (2.769) (χ²=4.863, P=0.027, 95% confidence interval of ratio 1.197 to 21.21).
Figure IV, the effects of anti-CHRM2 on L-type current is comparable to that of Carb). We therefore postulate that the alteration of CHRM2 gene may play an important role in the immune response associated with DCM, and may be related to slow arrhythmia or conduction block. However, the exact molecular mechanisms by which the C722G mutation contributes to arrhythmia in familial DCM remains to be further elucidated.

Recent studies have mapped inherited and dilated cardiomyopathy to different loci on chromosome 7. These subjects have similar phenotypic characteristics to that of familial DCM, and we have attributed to the CHRM2 gene, which resides on 7q31-7q35, including heart failure and sudden cardiac death occurring at relatively young ages. Thus, the present study suggests that the C722G mutation in the CHRM2 gene may be a novel disease mutation and may provide insights into novel pathophysiologic mechanism of cardiac dilation and arrhythmia beyond the familial form of the disease.

Limitations

There is an important limitation with regard to the results of the anti-CHRM2. We were unable to completely explain why 46% patients with sporadic DCM were positive for anti-CHRM2. Therefore, these results have to be considered as a conjecture that the immunoreactions induced by a gene mutation may be 1 of several important causative factors. We intend to further explore the biological mechanism of the presence of anti-CHRM2 in sporadic DCM patients, including the possibility that a different mutation may be present in the gene and or a different splice variant of the gene may account for these other cases.

Conclusions

Our study demonstrates that the CHRM2 gene plays an important role in the alteration of cardiac structure and function and suggests that the C722G mutation substitution in the CHRM2 gene may be a pathogenic mutation and not a benign polymorphism. In addition, we have observed that the patients who carry the C722G mutation have progressive disease characterized by sudden death, severe arrhythmia, and heart failure. To our knowledge, this is the first report linking a mutation in the CHRM2 gene to familial DCM and correlating this mutation with immunomechanic mechanisms.

Acknowledgments

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Disclosures

None.

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locus for an inherited cardiomyopathy maps to chromosome 7. 


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A Missense Mutation in the CHRM2 Gene Is Associated With Familial Dilated Cardiomyopathy

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Supplemental Materials & Methods

PCR reactions and sequence of oligonucleotide primers

Blood samples were collected into tubes containing EDTA, genomic DNA was extracted and served as the template for polymerase chain reaction (PCR)-based screening of the test populations. PCR was performed in 25-μL reaction solution in which the final concentrations were: forward primer 0.1 uM, reverse primer 0.1 uM, dNTP 0.05mM, MgCl₂ 1.5mM, genomic DNA 100ng, and Taq DNA polymerase 1U (Promega corporation, Madison, USA). The PCR products were purified and subsequently sequenced using an automated DNA sequencer (ABI 3730xl DNA Analyzer, Applied Biosystems, Foster City, Calif.). Sequence data were analyzed with the ABI PRISM ® Sequencing Analysis Software V 5.0 package. Nomenclature used in this report was based on the published gene coding sequence where the first nucleotide of the start codon is denoted as +195. The Squencher software package (Gene Codes Corporation, Ann Arbor, MI) was used to facilitate the detection of any variation of the nucleotide sequence of CHRM2 gene. In addition, individual sequences were carefully examined manually.

Six sets of primers were designed to amplify across the entire human CHRM2 gene according to its published genomic DNA sequence from nucleotide 195 to 1595 (Gene bank accession N0. M16404). The sequences of these primers were as follows: (5’→3’, forward, reverse)

1. TGCAGGTTTAAATGTTTATTTGCT, GTAGAGGGTGTAACAAGTTCA TGGA;
2. GGCCTGTGCT GACCTTATCATAG , CCCTACAATGAACTGCCAGAA GA;
3. AGTCAAGCGGACCACAATGGC, TTGTTTGGCTTCACTATCCTTC CT;
4. AGACCCCGTTTTCTCCAAGTCTG, GCCAATTCTGATGCATGTTTGCT;
5. GGCCATTCCAAAGATGAGAACTC, AGGTGTTAATGAGCACCATGACA;
6. AGCAGCCTGCAAAAAAGAAGCT, TCCCTTTTATTCTTCTCAAGCTCC. Of note, it is the observation that the DNA fragment located in the coding region from nucleotide 696-770
encodes the amino acids sequence (residues 168-192) that is used as the antigenic target in the ELISA screen for anti-CHRM2.

References
### Online Table I.  
Linkage in Family DCM to Markers at 7q31-35 of CHRM2 Gene

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*The maximal 2-point LOD score.
Representative ECG findings and cardiac arrhythmias

**Online Figure I.** The paroxysmal ventricular tachycardia of the IV:1 patient

**Online Figure II.** The second degree atrioventricular conduction block of the IV:11 patient

**Online Figure III.** The Atria fibrillation of the V:12 patient
Effects of anti-CHRM2 on I_{Ca,L} in isolated ventricular myocytes (IVM) from Guinea pigs by using standard whole-cell recording. I_{Ca,L} were elicited every six seconds by step-wise increase of voltage from a holding potential of –40 to +60 mV for 200 ms. Net inward I_{Ca,L} was defined as the difference between the peak inward current and the current 200 ms after the onset of the depolarizing pulse. Anti-CHRM2 acts as the CHRM2 agonist and induces robust modulations of I_{Ca,L}. The effects induced by anti-CHRM2 were similar to that of the CHRM2 agonist Carbchol (Carb). The CHRM2 antagonist Atropine (Atr) attenuated the effects of both anti-CHRM2 and Carb.

Panel A and C: Peak I_{Ca,L} and current-voltage relations of I_{Ca,L} in isolated ventricular myocytes (n=9). Iso (1.6 μM) significantly increased L-type Ca^{2+} current (Iso vs Con, P<0.01). Superfusion of IVM with Iso (1.6 μM) produced a significant elevation of peak I_{Ca,L} and currents.
density over basal $I_{\text{Ca,L}}$ (Iso vs Con, 18.83±1.14pA/pF vs 12.73±1.11pA/pF, P<0.01). Iso-induced elevation of peak $I_{\text{Ca,L}}$ and currents density was attenuated by Carb (14µM) (Iso vs Iso+Carb, 18.83±1.14pA/pF vs 10.72±1.06pA/pF, P<0.01). The CHRM2 antagonist, atropine (Atr) was able to prevent the effects of Carb on $I_{\text{Ca,L}}$ (Iso+ Carb vs Iso+ Carb +Atr, 10.72±1.06pA/pF vs 15.40±1.01pA/pF, P<0.01). These results verified that Carb inhibited $I_{\text{Ca,L}}$ activation via binding to CHRM2.

**Panel B and D:** Peak $I_{\text{Ca,L}}$ and current-voltage relations of $I_{\text{Ca,L}}$ in isolated ventricular myocytes (n=9). Anti-CHRM2 positive sera (10 µM) generated a decrease of peak $I_{\text{Ca,L}}$ and currents density increased by isoprenaline over basal $I_{\text{Ca,L}}$ (Iso vs Iso+Abs, 18.13±1.03pA/pF vs 5.54±0.81pA/pF, P<0.01). Furthermore, the decrease on $I_{\text{Ca,L}}$ was blocked by Atr (14µM)(Iso+Abs vs Iso+Abs+Atr, 5.54±0.81pA/pF vs 10.86±1.04pA/pF, P<0.01). These results suggested that anti-CHRM2 separated from CHF patients was able to affect $I_{\text{Ca,L}}$ similar to the CHRM2 agonist Carb.

**Abbreviations:**

CHRM2 (M$_2$-muscarinic acetylcholine receptor), anti-CHRM2 (autoantibodies against M$_2$-muscarinic acetylcholine receptor), DCM (dilated cardiomyopathy), $I_{\text{Ca,L}}$ (L-type Ca$^{2+}$ currents), IVM (isolated ventricular myocytes), Iso (Isoprenaline), Carb (Carbchol), Atr (Atropine), Con (Control), CHF (chronic heart failure).