

# Genetic Heterogeneity for Autosomal Dominant Familial Hypertrophic Cardiomyopathy in a Pakistani Family

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## ABSTRACT

**Objective:** To identify the gene causing inherited hypertrophic cardiomyopathy (HCM) in a Pakistani family.

**Study Design:** Cross-sectional, observational study.

**Place and Duration of Study:** Department of Cardiology, Shifa International Hospital and Biomedical and Genetic Engineering Laboratories, Islamabad, from 2005 to 2007.

**Methodology:** A large family of 17 individuals was included in this study. In the family 6 members were suffering from hypertrophic cardiomyopathy. Linkage analysis was carried out to map the disease-causing gene. Genomic DNA from each individual of the whole family was genotyped for microsatellite markers for all the known HCM loci followed by a whole genome search. Automated DNA sequencing was done for mutation identification in the candidate genes.

**Results:** Linkage analysis of 17 family members showed a maximum two point Lod score of 3.97 with marker D1S1660 at chromosome 1q 32.2. A disease region of 4.16cM was defined by proximal and distal cross-overs with markers GATA135F02 and D1S3715 respectively. This region contained the candidate genes *TNNI2* (cardiac troponin T) and *TNNI1* (troponin I 1). Direct sequencing of these genes for the whole family containing 17 members showed no disease-associated mutation in either of these genes.

**Conclusion:** Through linkage analysis, a disease locus for HCM family was mapped within a region of 4.16cM at chromosome 1q31.3-q32.1. So far no disease-associated mutation has been found in the candidate genes.

**Key words:** Hypertrophic cardiomyopathy. Troponin gene. Linkage analysis. Inheritance.

## INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is one of the commonly inherited myocardium associated cardiac disorders. It is characterized by left and/or right ventricular hypertrophy that is usually asymmetric and involves the interventricular septum. Typically, the left ventricular volume is normal or reduced with systolic gradients, arrhythmias and premature sudden deaths.<sup>1</sup> HCM is the most common cause of sudden death in the young people whereas it is a major cause of morbidity and mortality in the elders.<sup>2</sup>

In the USA, the prevalence of HCM is 0.2% in the general population indicating that HCM is a relatively common genetic disease with a widespread occurrence.<sup>3</sup> The first genetic linkage study of HCM was reported in a French-Canadian family.<sup>4</sup> Since then, major advances have been made in understanding the molecular basis of developing HCM. HCM is a heritable heterogeneous disease and to-date more than 20 causative genes have been described.<sup>5</sup> Mutations in the genes encoding

sarcomere proteins cause either HCM or dilated cardiomyopathy (DCM). At least 70% of HCM is caused by mutations in genes for protein components of the sarcomere, that include the cardiac myosin heavy chain (*MYH7*; 14q), cardiac troponin T (*TNNI2*; 1q32), tropomyosin skeletal muscle alpha (*TPM1*; 15q21), myosin-binding protein C (*MYBPC3*; 11p11.2), cardiac troponin I (*TNNI3*; 19q13), myosin light chain (*MYL2* and *MYL3*; 12q24 and 3p21), titin (*TTN*; 2q31), actin (*ACTC*; 15q14) and the gamma subunit of protein kinase A (*PRKAG2*; 7q36).<sup>6,7</sup>

To-date more than 450 different mutations have been reported within 13 sarcomere and myofilament-related genes.<sup>8</sup> Of these, 80 unique mutations have been reported in the *MYH7* gene-causing disease in approximately 40-50% cases of HCM. Around 20-30% HCM cases are due to the mutations in *MYBPC3* gene, a 137kD structural and regulatory protein of the sarcomere. The third major cause of HCM (approx.15%) is troponin T gene mutations.<sup>9</sup> Gene mutations for other proteins like alpha-tropomyosin and troponin I account for less than 5% of HCM cases. The severity and pattern of hypertrophy, age of disease onset and progression to heart failure are dependent on the precise gene mutation. For example studies have shown that subjects carrying a mutation in *MYH7* were affected at a significantly younger age than subjects carrying a mutation in *MYBPC3* gene.<sup>10</sup> *TNNI2* mutations are generally associated with a high incidence of sudden deaths despite only mild left ventricular hypertrophy (LVH). A lot more work still needs to be done to

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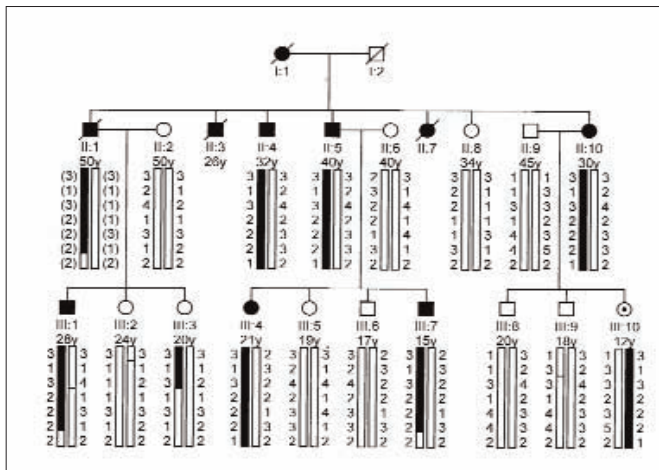
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Received June 25, 2010; accepted February 25, 2011.

fully understand the molecular bases of HCM with a special emphasis on cases with family histories.

This report describes the genetic analysis findings in a large, multi generation Pakistani family, suffering from autosomal dominant hypertrophic cardiomyopathy (Figure 1a). The objective of this study was to identify the disease-causing gene in this family.



**Figure 1a:** Pedigree of an autosomal dominant HCM family with genotypic data for the microsatellite markers analysed. Circles: females; squares: males; filled symbols: affected individuals; unfilled symbols: unaffected individuals; diagonal line through a symbol: deceased family member; filled circle in the symbol: asymptomatic carrier at risk; alleles in parentheses: inferred alleles.

### METHODOLOGY

This study was carried out at Biomedical Genetic Engineering Laboratories, Islamabad, in collaboration with the Department of Cardiology, Shifa International Hospital, Islamabad, from 2005 to 2007.

A large family of 17 individuals was included in this study. The family was selected on the bases of confirmed history of HCM among its members. In the family, 6 members were suffering from hypertrophic cardiomyopathy. The clinical diagnosis for hypertrophic cardiomyopathy was made using the diagnostic criteria described elsewhere.<sup>11</sup> To establish the disease status, all family members were clinically evaluated by physical examination, 12 lead electrocardiography (ECG) and echocardiography. Echocardiography was performed with 2.5 or 3.5 MHz transducers and images were stored on VHS video tape for the record. To view the parasternal short/long-axis and apical 4th and 2nd chamber, M-mode two dimensional echocardiography was done. Doppler ultrasonography was done to assess the direction, velocity, and turbulence of blood flow.

Since the family had a history of sudden cardiac death, the affected and normal individuals were collected and were analyzed by linkage analysis to identify the inheritance pattern of the disease-causing gene. Further, sequencing analysis was done to find the gene mutation. This study was supported by Wellcome Trust

grant to Dr. S. Qasim Mehdi. The study complied with the Declaration of Helsinki and was approved by the Institutional Review Board. With informed consent, 10 ml blood samples from all the available family members were collected in ACD vacutainers. Genomic DNA was extracted from whole blood using the standard phenol chloroform extraction procedure.

Linkage analysis was performed to identify the genetic locus responsible for the disease. For exclusion analysis genotyping was carried out using genomic DNA and microsatellite markers specific for known loci implicated with the disease-causing genes. PCR reactions were each performed in a 10 µl volume, containing 1.5 mM MgCl<sub>2</sub>, 0.6 µM of each primer, 200 µM dNTPs, 1 U *Taq* DNA polymerase and PCR buffer {16 mM (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>, 67 mM Tris-HCl (pH 8.8), and 0.01% of the non-ionic detergent Tween-20} (Bio-line, London, UK). Amplification was performed with an initial denaturation for 3 minutes at 95°C followed by 30 cycles of denaturation at 95°C for one minute, annealing at 55°C for one minute, extension at 72°C for one minute and a final extension at 72°C for 3 minutes. The PCR products were separated on 8-10% non-denaturing polyacrylamide gels. The gels were stained with ethidium bromide and photographed under UV illumination. Alleles were assigned and haplotypes were constructed of all family members. Genotypic data was used to calculate the Lod scores using the MLINK computer software program. (version 5.2, [ftp://linkage.rockefeller.edu/software/linkage/](http://linkage.rockefeller.edu/software/linkage/)) The phenotype was analyzed as an autosomal dominant trait with nearly complete penetrance (0.99) at a frequency of 0.0001 for the disease allele. Lod score greater than 3 was considered strong linkage.

A whole genome search was also carried out using commercially available microsatellite markers (Res. Genet. V:8) designed after every 10-15 cM.

Genes for cardiac troponin; *TNNI2* (191045) and *TNNI1* (191042) at 1q31.3-q32.1 were analyzed by direct sequencing. For direct dideoxy DNA sequencing, exon specific intronic forward and reverse primers were designed for both the candidate genes. Initially the PCR reactions were performed using total genomic DNA samples and exon specific gene primers. At a second step the amplified PCR products were sequenced using a commercially available ready reaction sequencing kit (Perkin Elmer-ABI, Warrington, UK), and the products were analyzed on an automated DNA sequencer (model 377; Perkin Elmer-ABI).

### RESULTS

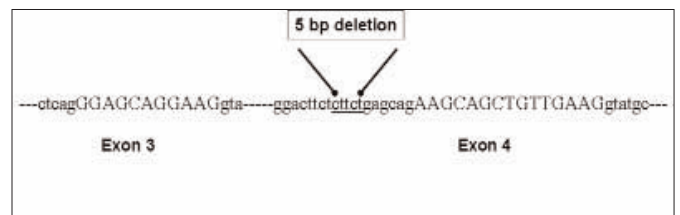
The HCM family (6 affected and 11 unaffected members) showed a dominant mode of inheritance of the disease phenotype (Figure 1a). Detailed clinical examinations of all the family members were carried out. The clinical presentations of some selected individuals

**Table I:** Clinical features of the family suffering from HCM.

Individual pedigree #	Age / Gender	Clinical history	2D Echo						ECG	
			LVEF	SH	PW	ESD	EDD	Other		
II:5	40 M	Palpitation, atypical chest pain	> 50%	18.8	12	6	39	SAM	LVH, abnormal ST segments	
II:10	30 F	Dyspnea, atypical chest discomfort	> 50%	34	18	18	28	SAM	LVH, abnormal ST segments	
III:1	28 M	Dyspnea, atypical chest pain	> 50%	19	13	24	40		Normal	
III:4	21 F	No complaint	> 50%	17	12	40	28		LVH, abnormal ST segments	
III:7	15 M	Dyspnea, atypical chest discomfort	> 50%	20	14	26	38		LVH, abnormal ST segments	
III:8	20 M	No complaint	> 50%	14	11	28	38		Q waves in II, III, aVF	
III:10	12 M	Normal	Normal 2D Echo							Normal

are summarized in Table I. The family history revealed a trend of sudden cardiac death. Individual number II:3 died at the age of 26 years without any medical history and his sister (II:7) who had been diagnosed as a case of HCM had died at the age of 43 years. Individual II:1 had a sudden death at the age of 50 years. Unfortunately no clinical details were available for the deceased family members. Individuals II:5, II:10, III:4 and III:7 fulfilled all the major echocardiography and ECG characteristic criteria for HCM and have similar phenotypes. Individual III:1 fulfilled only the echocardiography criteria for HCM, whereas his ECG was absolutely normal. Individual III:8 fulfilled the few major criteria of ECG and two minor echocardiography criteria, however, his genotype is normal. This indicates that this clinical presentation might be due to some other unknown reason. Another individual III:10 (aged 12 years) did not fulfill any criteria of ECG/echocardiography to fall within the category of HCM and had no complaint of illness at the time of clinical examination.

To identify the disease-causing gene for HCM, 17 available family members were genotyped for over 200 microsatellite markers. Initially for exclusion analysis known loci for autosomal dominant HCM were first screened using locus specific microsatellite markers. A hint of linkage with few crossovers was observed at chromosome 1q31. The gene for cardiac muscle troponin T (*TNNT2*) protein comes in this region and its gene mutations are known to be associated with hypertrophic cardiomyopathy. That convinced us to consider *TNNT2* a candidate gene for this family. Therefore, affected and non-affected members of this family were screened for mutations in *TNNT2* gene. Sequencing analysis of all 15 coding exons revealed no disease associated mutation. A five base pair splice site deletion (-cttct; rs59023175) was detected in intron 3 (Figure 1b). Due to the presence of the rs59023175 variant in the splice junction site, all family members were screened for its segregation with the disease



**Figure 1b:** A schematic presentation of a 5-bp deletion polymorphism in intron 3 of *TNNT2* gene.

phenotype. The analysis showed that 5bp-del was not segregating with the disease phenotype.

It was observed that the splice site SNP (rs59023175) was homozygous for 5bp-del in most of the family members. The majority of the affected and unaffected individuals had D/D genotype. Therefore, to calculate the frequency of the 5bp-del further analysis of 50 healthy individuals from randomly collected Pakistani population was carried out. Frequency of 5bp-del was found to be quite high (58%) among the normal individuals analysed, which explained the high frequency of SNP rs59023175 identified in the HCM family. Although *TNNT2* gene was a very strong candidate gene for the HCM but the results indicate the presence of another gene in the region that might be responsible for HCM phenotype in this family.

Therefore, to identify the locus for the novel disease-causing gene, a genome wide search was carried out using a set of polymorphic markers spanning the entire human genome at ~15-20cM intervals (Research Genetics; version-8). Significant exclusion was obtained for all markers except the locus at chromosome 1q31. Linkage was observed with microsatellite marker D1S1660. More flanking markers were then tested for linkage and to define the disease region by establishing the proximal and distal crossovers. Haplotypes for these markers are shown in Figure 1a. Two point Lod scores between HCM and the markers in this region are summarized in Table II. A maximum two-point Lod score

of 3.97 was obtained for marker D1S1660 with no crossover ( $\theta = 0.0$ ). In addition positive Lod scores of 3.14, 3.01, 2.10 and 1.68 were obtained for markers GATA135F02, D1S3715, D1S413 and D1S408 respectively. Recombination events involving markers GATA135F02 and D1S3715 (proximal and distal crossovers respectively) define the boundaries of the disease region. The proximal crossover was observed in individual III:3 and more recent distal crossover was seen in individual III:7. Since genotype of II:1 was inferred we assumed that subject III:1 inherited this crossover from his affected father. These crossovers localized the novel disease gene within a region of approximately 4.16 cM on chromosome 1q31.

Further searches on the genetic database within 1q31.3-q32.1 region identified another possible candidate gene, troponin I, slow-twitch skeletal muscle isoform (*TNNI1*). The gene is physically present within the HCM disease region and is expressed in the heart muscles. Because it could be the probable disease causing gene, the *TNNI1* gene was also screened for mutations in this family. No disease associated mutation was found in this gene as well.

## DISCUSSION

This study describes a novel gene locus for hypertrophic cardiomyopathy. The results show that the novel disease-causing gene for this family that was suffering from autosomal dominant familial hypertrophic cardiomyopathy was present at chromosome 1q32.2. Haplotype analysis of individual III:10 indicated that she was carrying a disease chromosome which she inherited from her mother and was an asymptomatic carrier. It can be assumed that due to the age related penetrance, she has not developed any symptoms yet but she might be at a risk to develop the disease later. HCM is an autosomal dominant disorder for which more than 20 gene/mutation(s) have already been reported. The most frequently found mutations are in *MYBPC3* and *MYH7* genes,<sup>13</sup> followed by *TNNT2*. However, these reports were based on a small number of patients. *TNNT2* gene mutations were found in only 1.6% Spanish patients.<sup>14</sup> While, the HCM patients of the Chengdu population from China have no *TNNT2* mutation associated with the disease.<sup>15</sup> Mutations in *MYH7* are expected in case of severe hypertrophy whereas *TNNT2* mutations are associated with a mild degree of hypertrophy.<sup>16</sup> On the other hand, no phenotypic differences have been observed in some studies between carriers of the various mutations. Genetic screenings of the patients for prominent sarcomere genes have been recommended by various groups for genetic counselling to stratify the family members at risk and for better clinical management in HCM families. It has been suggested that caution is required in using the genetic information since most of these studies are based on a small number

of populations.<sup>17</sup> The locus and allelic heterogeneity for HCM has further complicated the investigational strategies. In this situation the identification of more disease associated genes in various other populations might be more helpful for a better understanding of the disease and its underlyingly causes.

With the increasing importance of extensive structural polymorphisms in the human genome due to their association with various diseases, an emphasis should also be focused on single nucleotide polymorphisms (SNPs) in the known sarcomeric genes. In a combined study on various world populations, a common *MYBPC3* variant (25bp del.) has been reported to be associated with cardiomyopathy only in south Asians.<sup>18</sup> Similarly in a recent study, human resistin (*RETN*) gene promoter polymorphism (-420 C > G) has been shown to be significantly associated with HCM.<sup>19</sup> In view of these observations the significance of the 5bp-del in *TNNT2* and disease associated SNPs of *MYBPC3* and *RETN* genes need to be investigated in a larger cohort of HCM patients from different populations. It will ultimately enable the cardiologists to get the genetic information for the detection of asymptomatic carriers or exclusion of the disease in unaffected individuals and other family members. Special attention should be given to the asymptomatic carriers; in such cases onset of the disease symptoms can be delayed or controlled by regular medical check ups and by adopting the improved healthy life style.

## CONCLUSION

Linkage mapping in a Pakistani pedigree identified a novel locus for HCM at chromosome 1q31.3-q32.1. All other loci and candidate gene(s) were excluded through linkage and sequencing analysis. There could be another undiscovered novel gene presently obscured in the nearby region that might be responsible for HCM.

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