MYBPC3 gene variations in hypertrophic cardiomyopathy patients in India

Reena R Tanjore PhD¹, Advithi Rangaraju MSc¹, PG Kerkar MD², Narsimhan Calambur MD³, Pratibha Nallari PhD¹

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BACKGROUND: Hypertrophic cardiomyopathy (HCM) is a complex cardiac muscular disorder, inherited as an autosomal dominant disease with variable penetrance. Cardiac myosin-binding protein C (MyBPC) is the predominant myosin-binding protein isoform in the heart muscle. One hundred forty-seven mutations have been detected in MYBPC3, accounting for 15% of all HCM cases.

OBJECTIVE: To screen exons 16, 18, 19, 22, 24, 28, 30, 31 and 34 in the MYBPC3 gene in Indian HCM patients.

METHODS: Sixty control and 95 HCM samples were collected from cardiology units of the CARE Hospital (Nampally, Banjara Hills, Secunderabad, India) for genomic DNA isolation followed by polymerase chain reaction and single-stranded conformational polymorphism analysis.

RESULTS: Screening of the exons revealed two variations – one novel frame shift mutation in exon 19 at the nucleotide position 11577^Δ11578 and one novel single nucleotide polymorphism (SNP) in codon 1093 of exon 31, coding for glycine with a C>T transition (GGC/GGT), in addition to the seven known SNPs mainly in the intronic region and one known missense mutation D770N in this population.

CONCLUSION: The novel frame shift mutation identified in exon 19, D570fs, with the insertion of an adenine residue in codon 570 coding for aspartate, results in a premature termination codon that produces a truncated protein lacking myosin- and titin-binding sites, explaining the role of the nonsense-mediated decay pathway. A novel SNP identified in codon 1093 of exon 31 was found to be a synonymous codon, which may have a regulatory effect at the translational level, attributing to affinity differences between codon-anticodon interactions. The screening of this gene may be relevant in the Indian context.

Key Words: Hypertrophic cardiomyopathy; MYBPC3; Nonsense-mediated decay pathway; Novel mutation

Hypertrophic cardiomyopathy (HCM) is a complex muscular disorder of the heart, inherited as an autosomal dominant disease with variable penetrance in at least 50% of cases. The prevalence of HCM has been reported to be one in 500 in a population of young adults (1,2). The clinical course is variable and unpredictable, ranging from a benign asymptomatic course to severe heart failure and sudden cardiac death. Although transmission of HCM is usually considered to be dominant, recessive mutations have been encountered in a few cases (3,4).

Mutations in 12 sarcomeric and cytoskeletal genes have been implicated in HCM, with more than 400 different gene mutations reported, accounting for approximately 50% to 70% of all HCM cases.

Cardiac myosin-binding protein C (MyBPC) is the predominant myosin-binding protein in the heart muscle (5), which participates in thick filament assembly by binding myosin and titin, contributing to sarcomere stability (6). Because it undergoes reversible phosphorylation by cyclic AMP-dependent protein kinase (5) and calcium/calmodulin-dependent protein kinase II (7), its role in the adrenergic regulation of cardiac contraction has been suggested. The MyBPC isoform is expressed exclusively in cardiac tissue (8) and is

Les variations du gène MYBPC3 chez les patients atteints de myocardiopathie hypertrophique en Inde

HISTORIQUE : La myocardiopathie hypertrophique (MCH) est un trouble complexe du muscle cardiaque, hérité sous forme de maladie autosomale dominante à pénétration variable. La protéine C cardiaque de liaison à la myosine (MyBPC) est le principal isoforme de la protéine de liaison à la myosine du muscle cardiaque. On a décelé 147 mutations dans le gène MYBPC3 ce qui représente 15 % de tous les cas de MCH.

OBJECTIF : Dépister les exons 16, 18, 19, 22, 24, 28, 30, 31 et 34 dans le gène MYBPC3 de patients indiens atteints de MCH.

MÉTHODOLOGIE : Les auteurs ont colligé 60 sujets témoins et 95 échantillons de MCH dans des unités de cardiologie de l'Hôpital CARE (de Nampally dans les montagnes de Banjara à Secunderabad, en Inde) afin d'isoler l'ADN génomique et de procéder à une réaction en chaîne de la polymérase et à une analyse polymorphique conformationnelle monocaténaire.

RÉSULTATS : Le dépistage des exons a révélé deux variations – une nouvelle mutation à trame décalée dans l'exon 19 à la position 11577^Δ11578 du nucléotide et un nouveau polymorphisme d'un nucléotide simple (PNS) dans le codon 1093 de l'exon 31, qui code la glycine avec une transition C>T (GGC/GGT), en plus des sept PNS connus surtout situés dans la région intronique et d'une mutation faux-sens D770N connue au sein de notre population.

CONCLUSION : La nouvelle mutation à trame décalée repérée dans l'exon 19, D570fs, avec l'insertion d'un résidu d'adénine dans le codon 570 codant pour l'Asp, provoque un codon de terminaison prématuré qui produit une protéine tronquée, sans liaison à la myosine et à la titine, ce qui explique le rôle de voie de désintégration à médiation de terminaison. Un nouveau PNS repéré dans le codon 1093 de l'exon 31 était un codon synonyme, qui pourrait avoir un effet régulateur au niveau traductionnel causé par les différences d'affinité entre interactions codon-anticodon. Le dépistage de ce gène peut être pertinent en Inde.

located in sarcomere 'A' bands, forming a series of seven to nine transverse bands spaced at 43 nm intervals; the gene is located on chromosome 11p11 and consists of 37 exons.

Approximately 147 mutations have been detected in MyBPC3, the gene that encodes for MyBPC, accounting for nearly 15% of all HCM cases. Hence, the screening of exons 16, 18, 19, 22, 24, 28, 30, 31 and 34 of this gene was considered in the present study in view of its functional domains and interactions with the other sarcomere proteins. We have identified two variations – one novel frame shift mutation in exon 19 at the nucleotide position 11577^Δ11578 and one single nucleotide polymorphism (SNP) in codon 1093 of exon 31, coding for glycine with a C>T transition (GGC/GGT), in addition to the seven known SNPs mainly in the intronic region, one missense mutation D770N and a previously reported 25-base pair deletion.

METHODS

HCM was diagnosed by physical examination, electrocardiogram, echocardiogram and magnetic resonance imaging. Sixty control samples were collected from healthy blood donors with no history of a

¹Department of Genetics, Osmania University, Jamai Osmania PO, Hyderabad, Andhra Pradesh; ²King Edward Memorial Hospital, Mumbai; ³CARE Hospital, Hyderabad, India

Correspondence and reprints: Dr Pratibha Nallari, Department of Genetics, Osmania University, Hyderabad 50007, Andhra Pradesh, India.

Telephone 91-98-85-48102, e-mail prathinallari@yahoo.com

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Figure 1) Pedigree of the index case with a mutation D570fs in exon 19 (arrowhead indicates proband). Squares represent male cases and circles represent female cases. y Years

heart disorder, and 95 HCM samples, along with those of 144 available family members, were collected from cardiology units of the CARE Hospital (Nampally, Banjara Hills, Secunderabad, India) for genomic DNA isolation and single-stranded conformational polymorphism (SSCP) analysis. Informed, written consent was obtained from all patients and family members, in accordance with the study protocol approved by the institutional ethics committee of the above-mentioned hospital.

Genomic DNA was extracted by a rapid nonenzymatic method, as described by Lahiri and Nurnberger (9). The isolated DNA was later amplified based on the primer sequences available online, as cited by Seidman (genetics.med.harvard.edu/~seidman/cg3/genes/MYBPC3_exons.html). Polymerase chain reaction (PCR) was carried out in 0.2 mL tubes. Each tube contained 100 ng of genomic DNA, 50 pmol each of forward and reverse primer, 0.5 U to 1 U of Taq DNA polymerase enzyme, 200 μ M of dNTP, 1 $\times$ PCR buffer and water to make up the final volume to 25 μ L. Amplification was carried out in an Eppendorf Master Gradient thermocycler (Eppendorf Scientific Inc, USA). Initial denaturation was carried out at 95°C for 3 min, followed by a denaturation step at 95°C for 30 s. The annealing temperature varied from 54°C to 65°C (based on the exon and DNA sample) for 30 s and an extension was carried out at 72°C for 1 min. A final extension step was performed at 72°C for 2 min at the end of the reaction.

The amplified DNA samples were subjected to SSCP analysis on 8% to 12% nondenaturing/native polyacrylamide gels. Based on the amplicon length and the mobility of the PCR products, an appropriate gel percentage was standardized for each exon. The gel was stained with silver nitrate, and samples showing abnormal bands or mobility shift were commercially sequenced using the 3730xl DNA Analyzer (Macrogen, Korea).

RESULTS

Mutations that have previously been reported in exon 16 include T457M, F412fs, G416S, Q425X, V437fs, L447fs and E451Q (FHC mutation database, 2005; Cardiogenomics mutation database, 2005). PCR-based SSCP analysis of exon 16 revealed an abnormal band pattern in one patient sample. The sequencing results revealed a variant in the intronic region 13 bases upstream of exon 16, with a G>A transition (patient's intronic variation thickness of 15 mm [IVS15] –13G>A).

The proband, 36 years of age, was heterozygous for the mutation and had obstructive HCM, whereas his clinically unaffected sibling, 27 years of age, did not harbour the mutation. The mutation was found to be close to the splice acceptor site analysis using NetGene splicer software (Center for Biological Sequence Analysis, Denmark). Because no change was observed in the splice scores, the mutation was unlikely to be the pathogenic cause of HCM. However, the genetic

variation observed in conjunction with other variations may contribute to the pathogenicity of HCM.

SSCP analysis of exon 19 revealed two types of band patterns (A and B), with mobility shift in two patient samples. Sequencing of type A identified a novel frame shift mutation D570fs, with the insertion of an adenine residue in the nucleotide position 11577[^]11578 of the MYBPC3 gene, whereas type B was normal. The proband harbouring the D570fs mutation had nonobstructive HCM, with an IVS thickening of 21 mm, and was diagnosed at the age of 36 years. The mother of the proband was clinically affected, and the sudden death of the maternal uncle indicates a positive family history (Figure 1). Clinical evaluation of the other family members did not reveal any symptoms of HCM.

The normal sequence of exon 19 is gtg ttc aaa tgt gag gtc tca GAT gag aat gtt cgg ggt gtg tgg. The mutated amplicon with the novel D570fs mutation showed an insertion of 'A' in codon 570, which led to a frame shift: gtg ttc aaa tgt gag gtc tca GAA Tga (termination codon) g aat gtt cgg ggt gtg tgg.

Screening of exon 22 revealed three types of band patterns (A, B and C). Of these, types A and B were observed in both patients and control samples, with predominance of type A, whereas type C was found only in two patient samples. On sequencing, two intronic variants in type B and C samples were observed, and type A was found to be the normal sequence with no polymorphisms. Type B samples were heterozygous (C>T) for an SNP, which was present 56 bases downstream of the exon (IVS22 +56C/T); the frequency of the SNP in HCM patients was similar to that of the control subjects. The type C individuals had a different SNP (G>A), which was present 118 bases downstream of the exon (IVS22 +118G/A). However, the frequency of this SNP is not known in the wild-type population; hence, its regulatory role in MYBPC3 gene expression and HCM remains unclear. Large sample studies of both patients and controls are necessary to confirm this.

Exon 24 of the MYBPC3 gene showed six types of band patterns, designated types A to F. Type A did not show any mutations or polymorphisms. Sequencing of samples showing the type B band pattern identified genetic variation (two SNPs), present 18 bases (C>G) and 35 bases (G/T) downstream of the exon. All samples were heterozygous for these two SNPs. Samples with the type C band pattern revealed a heterozygous SNP (C>G) 18 bases downstream of the exon. Samples with the type D band pattern showed an SNP (C>T) that was present five bases upstream of the exon and was heterozygous for all the samples. Type E was found in only one patient sample, in which sequencing revealed homozygosity (G>G) for the SNPs present 18 bases downstream of exon 24. Type F was found in one patient sample with familial HCM, in which sequencing revealed the known missense mutation Asp770Asn.

Screening of exon 31 showed three types of band patterns (A, B and C), which were observed in both patient and control samples. Subsequent sequencing identified two synonymous polymorphic variants: one in type B at codon 1093, coding for glycine with a C>T transition (GGC/GGT), and one in type C, an A>G transition in codon 1096 (GAA/GAG). The SNP in codon 1096 (Figure 2A) is a known polymorphism, whereas the 1093 SNP was a novel polymorphism (Figure 2B) identified in our samples, clearly revealing genetic variation, accounted for by geographic and ethnic variation. The frequency of the polymorphisms was compared in patients and controls, but no significant association was observed.

SSCP analysis of exon 34 (Figure 3) revealed abnormal bands in two probands and three control samples. Sequencing identified a 25-base pair deletion in intron 33 of the MYBPC3 gene in the nucleotide position 21347[^]21372. The available family members of the two probands were screened for HCM and the 25-base pair deletion.

In the first family, the proband 37 years of age had obstructive HCM with atrial fibrillation and was diagnosed with HCM at the age of 34 years. His IVS thickening was 26 mm and he had a left ventricular outflow tract (LVOT) gradient of 60 mmHg. The mother of the proband died suddenly at the age of 65 years, and his daughter, 12 years

Figure 2) A Chromatogram of exon 31 showing single nucleotide polymorphism (SNP) at codon 1096. **B** Chromatogram of exon 31 showing a novel SNP at codon 1093

of age, had mild hypertrophy and a grossly abnormal electrocardiogram. The proband had two siblings who were clinically unaffected. The 25-base pair deletion was present in the proband and in his affected daughter, but was absent in his unaffected siblings (Figure 4A).

In the second family, the proband was a 58-year-old woman who had been diagnosed with obstructive HCM and apical lateral hypertrophy at the age of 53 years. She had an IVS thickening of 28 mm, an apical lateral wall thickening of 16 mm and an LVOT gradient of 30 mmHg. The proband had two clinically affected sons and one clinically unaffected daughter; she also had a history of eight stillbirths and neonatal deaths, the causes of which were unknown. One of the affected sons had obstructive HCM, with an IVS thickening of 22 mm and an LVOT gradient of 21 mmHg, whereas the other son had asymmetrical septal hypertrophy without obstruction, with an IVS thickening of 19 mm. Unlike the proband, neither of the sons had apical lateral hypertrophy (Figure 4B).

The 25-base pair deletion was present in the proband and one of the affected sons, but was absent in the other clinically affected son. Both the proband and her son who harboured the 25-base pair deletion had obstructive HCM with a severe phenotype, unlike the other affected son without the deletion.

The mutated amplicon with the 25-base pair deletion 21347^21372del AGCCTGGATG GCTTCCCTCCCTCTC appeared as follows: GcaggccatggtactcactctgtgtccatgtttgttccagcctgtggcatagtcaggagactctgtgggacccccgagtagaacaacagatgtgtctccctgggtcctgtccagggtccctctCTTactctatttatag...ccaagatttctgtgcaagaatggcctgacctgggagaagacgccgcttcgcagatgttcagcaagcaggagtggtgactctggagattagaaagccctgcccctttgacgggggcatctatgtctgcaggccaccaacttacaggcgaggcacgggtgtgagtgccgctggaggtgcgag...gtgaggagccctcggggcccaggcctggagatgggaagagcggcg. However, mutation screening of exons 18, 28 and 30 did not reveal any mutations or polymorphisms, indicating a hotspot variation in this population.

DISCUSSION

The MYBPC3 gene is present on chromosome 11p11 and consists of 37 exons. Most of the HCM-causing mutations in the myosin-binding

Figure 3) Single-stranded conformational polymorphism gel of exon 34

Figure 4) A Pedigree of the first family screened for a 25-base pair deletion in exon 34 (arrowhead indicates proband). **B** Pedigree of the second family screened for a 25-base pair deletion in exon 34 (arrowhead indicates proband). Asterisks indicate the woman and one of the sons carrying the same 25-base pair mutation (note that the other affected son did not have the mutation). Squares represent male cases and circles represent female cases. y Years

protein have been reported in its myosin- and titin-binding carboxy terminal domain; hence, the screening of exons 16, 18, 19, 22, 24, 28, 30, 31 and 34 encoding the myosin- and titin-binding domain was considered. More than 400 different mutations have been identified, the majority of which are missense or frame shift mutations. Other than these, splice site mutations have also been reported (10-14). In general, each affected family has a unique mutation. Even among family members with the same mutation, disease expression seems to differ (2,15), suggesting the role of other factors such as sex, physical activity, nutrition, ethnic background, modifier gene effects and other genetic markers in its etiology (16-19).

To date, 147 mutations have been identified in the MYBPC3 gene, 22 of which are frame shift mutations (16 deletions; four insertions and two duplications), 22 missense mutations, 19 splice site mutations and eight nonsense mutations (FHC mutation database, 2005; Cardiogenomics mutation database, 2005). The majority of mutations result in the truncation of the MyBPC protein, with loss of the myosin- and titin-binding sites, whereas missense mutations preserve the myosin- and titin-binding sites. Studies have indicated that patients carrying MyBPC mutations have a later onset, lower penetrance, better prognosis and longer life expectancy (20-21). Because carriers of MyBPC mutation can be completely asymptomatic, it is possible that the mutation is more prevalent than previously thought.

Mutation screening of nine hotspot exons of the MYBPC3 gene revealed one novel frame shift mutation D570fs in exon 19, with the insertion of an adenine residue in codon 570 coding for aspartate. This causes a shift in the reading frame, resulting in a premature termination codon, which, in turn, produces a 570-amino acid long truncated myosin-binding protein lacking the titin- and myosin-binding sites. The transcripts containing the premature termination codon are degraded to prevent the translation of unnecessary or aberrant transcripts, which may explain the role of the nonsense mediated decay pathway/messenger RNA surveillance pathway. It may alter the inheritance pattern of the disease trait and eventually modify the ultimate phenotype. Failure of this pathway may result in the expression of large amounts of aberrant truncated proteins with potential dominant-negative or gain-of-function effects in the cells.

One known missense mutation D770N in the type F band pattern in exon 24 of one patient sample was identified that was previously reported by Van Driest et al (22). Because the mutation was absent in the family members, this individual might have been a compound heterozygote. This substitution occurs in the last base of the exon; therefore, it may result in the loss of the splice donor site in the protein codes for some novel amino acids, resulting in a truncated protein lacking the myosin- and titin-binding sites.

REFERENCES

1. Marian AJ, Mares A Jr, Kelly DP, et al. Sudden cardiac death in hypertrophic cardiomyopathy. Variability in phenotypic expression of beta-myosin heavy chain mutations. *Eur Heart J* 1995;16:368-76.
2. McKenna WJ, Behr ER. Hypertrophic cardiomyopathy: Management, risk stratification, and prevention of sudden death. *Heart* 2002;87:169-76.
3. Jäskeläinen P, Soranta M, Miettinen R, et al. The cardiac beta-myosin heavy chain gene is not the predominant gene for hypertrophic cardiomyopathy in the Finnish population. *J Am Coll Cardiol* 1998;32:1709-16.
4. Nishi H, Kimura A, Harada H, et al. Possible gene dose effect of a mutant cardiac beta-myosin heavy chain gene on the clinical expression of familial hypertrophic cardiomyopathy. *Biochem Biophys Res Commun* 1994; 200:549-56.
5. Gautel M, Zuffardi O, Freiburg A, Labeit S. Phosphorylation switches specific for the cardiac isoform of myosin binding protein-C: A modulator of cardiac contraction? *EMBO J* 1995;14:1952-60.
6. Schultheiss T, Lin ZX, Lu MH, et al. Differential distribution of subsets of myofibrillar proteins in cardiac nonstriated and striated myofibrils. *J Cell Biol* 1990;110:1159-72.
7. Schlender KK, Bean LJ. Phosphorylation of chicken cardiac C-protein by calcium/calmodulin-dependent protein kinase II. *J Biol Chem* 1991;266:2811-7.
8. Fougereousse F, Delezoide AL, Fisman MY, Schwartz K, Beckmann JS, Carrier L. Cardiac myosin binding protein C gene is specifically expressed in heart during murine and human development. *Circ Res* 1998;82:130-3.
9. Lahiri DK, Nurnberger Jr JI. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. *Nucleic Acids Res* 1991;19:5444.
10. Bonne G, Carrier L, Bercovici J, et al. Cardiac myosin binding protein-C gene splice acceptor site mutation is associated with familial hypertrophic cardiomyopathy. *Nat Genet* 1995;11:438-40.
11. Fung DC, Yu B, Littlejohn T, Trent RJ. An online locus-specific mutation database for familial hypertrophic cardiomyopathy. *Hum Mutat* 1999;14:326-32.
12. Richard P, Charron P, Carrier L, et al. Distribution of disease genes in 102 genotyped families with hypertrophic cardiomyopathy. *Circulation* 2001;104(Suppl)II-521.
13. Marian AJ, Roberts R. The molecular genetic basis for hypertrophic cardiomyopathy. *J Mol Cell Cardiol* 2001;33:655-70.
14. Keller DI, Carrier L, Schwartz K. Genetics of familial cardiomyopathies and arrhythmias. *Swiss Med Wkly* 2002;132:401-7.
15. Davies MJ. The cardiomyopathies: An overview. *Heart* 2000;83:469-74.
16. Watkins H, Rosenzweig A, Hwang DS, et al. Characteristics and prognostic implications of myosin missense mutations in familial hypertrophic cardiomyopathy. *N Engl J Med* 1992;326:1108-14.
17. McKenna WJ, Cocco F, Elliott PM. Genes and disease expression in hypertrophic cardiomyopathy. *Lancet* 1998;352:1162-3.
18. Arad M, Seidman JG, Seidman CE. Phenotypic diversity in hypertrophic cardiomyopathy. *Hum Mol Genet* 2002;11:2499-506.
19. Ortlepp JR, Vosberg HP, Reith S, et al. Genetic polymorphisms in the renin-angiotensin-aldosterone system associated with expression of left ventricular hypertrophy in hypertrophic cardiomyopathy: A study of five polymorphic genes in a family with a disease causing mutation in the myosin binding protein C gene. *Heart* 2002;87:270-5.
20. Charron P, Dubourg O, Desnos M, et al. Clinical features and prognostic implications of familial hypertrophic cardiomyopathy related to the cardiac myosin-binding protein C gene. *Circulation* 1998;97:2230-6.
21. Niimura H, Bachinski LL, Sangwatanaroj S, et al. Mutations in the gene for cardiac myosin-binding protein C and late-onset familial hypertrophic cardiomyopathy. *N Engl J Med* 1998;338:1248-57.
22. Van Driest SL, Vasile VC, Ommen SR, et al. Myosin binding protein C mutations and compound heterozygosity in hypertrophic cardiomyopathy. *J Am Coll Cardiol* 2004;44:1903-10.

The SNP in the type D band pattern acts as a splicing enhancer, contributing in spliceosome complex formation, thereby affecting the stoichiometry of the molecule.

A previously reported 25-base pair deletion in intron 33 was also identified in two patients. Furthermore, screening of the family members of the two probands revealed a segregation of the deletion with the disease in one HCM family, whereas no such segregation in the other family was observed, indicating the presence of other causative mutation in addition to this, suggesting that this deletion may act as a 'modifying gene' or a 'recessive mutation', which is per se not only rarely cause HCM, but may enhance the phenotype of a mutation responsible for the disease. This is supported by the results of a similar study by Jäskeläinen et al (3), who identified two missense mutations R719W and M349T in a proband with a severe HCM phenotype.

A novel SNP was identified in codon 1093 of exon 31, indicating genetic variation. The presence of a synonymous SNP may have a regulatory effect at the translational level, which can be attributed to affinity differences between codon-anticodon interactions.

CONCLUSION

In the present study, mutation screening of hotspot regions of the MYBPC3 gene revealed two variations – one novel frame shift mutation in exon 19 and one SNP codon 1093 of exon 31, in addition to the seven known SNPs mainly in the intronic region, one missense mutation D770N and a previously reported 25-base pair deletion. The frequency of MYBPC3 gene mutations is higher; therefore, the screening of this gene may be relevant in the Indian context.

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