

# Homozygosity for a Novel Splice Site Mutation in the Cardiac Myosin-Binding Protein C Gene Causes Severe Neonatal Hypertrophic Cardiomyopathy

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Received 29 May 2007; Accepted 1 July 2007

Hypertrophic cardiomyopathy is typically inherited in an autosomal dominant pattern and has a variable age of onset and prognosis. Mutations in the myosin-binding protein C (*MYBPC3*) gene are one of the most frequent genetic causes of the disease. Patients with *MYBPC3* mutations generally have a late onset and a relatively good prognosis. We report here more than 20 Old Order Amish children with severe neonatal hypertrophic cardiomyopathy caused by a novel homozygous splice site mutation in the *MYBPC3* gene. The affected children typically presented with signs and symptoms of congestive heart failure during the first 3 weeks of life. Echocardiography revealed hypertrophic non-obstructive cardiomyopathy. These children had a life span averaging 3–4 months. All patients died from heart failure before 1 year of age unless they received a heart transplant. A genome-wide mapping study was performed in three patients. The disease related gene was localized to a 4.6 Mb

region on chromosome 11p11.2-p11.12. This homozygous block contained *MYBPC3*, a previously identified cardiomyopathy related gene. We identified a novel homozygous mutation, c.3330 + 2T > G, in the splice-donor site of *MYBPC3* intron 30. The mutation resulted in skipping of the 140-bp exon 30, which led to a frame shift and premature stop codon in exon 31 (p.Asp1064GlyfsX38). We have found a substantial incidence of this phenotype in Old Order Amish communities. It is also concerning that many unidentified heterozygous individuals who are at risk for development of hypertrophic cardiomyopathy do not receive proper medical attention in the communities. © 2007 Wiley-Liss, Inc.

**Key words:** hypertrophic cardiomyopathy; *MYBPC3*; Old Order Amish; Mennonite

**How to cite this article:** Xin B, Puffenberger E, Tumbush J, Bockoven JR, Wang H. 2007. Homozygosity for a novel splice site mutation in the cardiac myosin-binding protein C gene causes severe neonatal hypertrophic cardiomyopathy. *Am J Med Genet Part A* 143A:2662–2667.

## INTRODUCTION

Hypertrophic cardiomyopathy, clinically defined as thickening of the myocardial wall in the absence of other causes for left ventricular hypertrophy, affects one of every 500 people [Maron et al., 1995; Zou et al., 2004]. The disease, generally being inherited in an autosomal dominant pattern, has a broad spectrum of clinical manifestations from a benign asymptomatic course to a malignant course with serious arrhythmias, heart failure, and sudden cardiac death. Genetic causes of hypertrophic cardiomyopathy are also fairly diverse with more than 400 mutations identified in at least nine genes encoding sarcomeric proteins [Ho and Seidman, 2006; Ashrafian and Watkins, 2007].

One of most common genetic causes for hypertrophic cardiomyopathy involves mutations in cardiac myosin-binding protein C (*MYBPC3*) gene [Charron et al., 1998; Niimura et al., 1998, 2002; Erdmann et al., 2001, 2003; Konno et al., 2003; Richard et al., 2003; Van Driest et al., 2004]. There are approximately

This article contains supplementary material, which may be viewed at the American Journal of Medical Genetics website at <http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html>.

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DOI 10.1002/ajmg.a.31981

150 mutations identified so far as listed in the website (<http://www.cardiogenomics.org>) developed by Genomics of Cardiovascular Development [2007], Adaptation, and Remodeling program since the first disease-causing mutations were found in 1995 [Bonne et al., 1995; Watkins et al., 1995]. Cardiac myosin-binding protein C is a sarcomeric protein belonging to the intracellular immunoglobulin superfamily [Einheber and Fischman, 1990]. By binding to the myosin heavy chain and cytoskeleton protein titin, Cardiac myosin-binding protein C contributes to the structural integrity of the sarcomere. The protein may also play a role in regulating cardiac contractility [Flashman et al., 2004]. In general, patients with hypertrophic cardiomyopathy caused by *MYBPC3* gene mutations seem to have a more favorable clinical profile, characterized by a late onset and a relatively good prognosis [Niimura et al., 1998]. The clinical expression of the mutations in the *MYBPC3* is often delayed until middle age or old age. Homozygous mutation in the *MYBPC3* gene causing severe hypertrophic cardiomyopathy has not been reported to our knowledge, although two cases of severe hypertrophic cardiomyopathy caused by compound heterozygous mutations have been found recently [Lekanne Deprez et al., 2006].

Here we describe a cohort of patients with severe hypertrophic cardiomyopathy presenting in the neonatal period, caused by homozygosity for a novel mutation in the *MYBPC3* gene.

## MATERIALS AND METHODS

### Subjects

The study was approved by DDC Clinic for Special Needs Children (DDC Clinic) Institutional Review Board. All 23 affected infants were Old Order Amish, with 20 of them from the Geauga County settlement in Ohio, one from the Holmes County settlement in Ohio, and two from a settlement in New York. The patients were clinically evaluated by at least one clinician in the list of authors and the diagnosis of hypertrophic cardiomyopathy was established based on family history, physical examination, and the characteristic clinical course of the disease. Confirmation was made by both electrocardiogram and two-dimensional echocardiography reviewed by pediatric cardiologists. DNA samples from three patients, their parents and siblings were acquired with informed consent. All three patients died before the study was completed. One of them expired before the study started, thus the DNA sample preserved by another research laboratory was transferred to us per parent's request.

### Genotyping and Mutation Detection

The DNA isolation, genotyping, linkage analysis and mutation detection were performed as described

previously [Puffenberger et al., 2004; Strauss et al., 2005, 2006]. Polymerase chain reaction (PCR) primers were designed to amplify each of the 34 protein-coding exons and their flanking intronic sequences of *MYBPC3*. Primer sequences are provided in Table 1 of online supplementary material, which is published as supporting information on the AJMG web site (see the online Table 1 at <http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html>).

### RNA Isolation and cDNA Amplification

Total RNA was isolated from whole blood using QIAamp RNA blood kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. The cDNA was synthesized and amplified using primers F3113 and R3479 located in exons 29 and 31. The primers were determined according to the mRNA sequence NM\_000256.

## RESULTS

### Clinical Phenotype

The children affected with hypertrophic cardiomyopathy were typically born after an uneventful pregnancy and delivery. They were usually full term with birth weight, length and occipitofrontal circumference all within normal ranges. There were no significant dysmorphic features noticed in any of those newborns at birth and afterwards. All patients passed the routine State Newborn Screening. A routine chromosomal analysis was performed on at least one newborn, which was reported as normal.

Approximately one third of the affected infants in this cohort presented with respiratory distress, an audible heart murmur or gallop rhythm soon after birth, which led to further evaluation before or soon after discharge from the hospital. The remaining two thirds of infants presented to the primary care physicians' office during the first 1–3 weeks of life with poor feeding, excessive sweating during feeding, lethargy, difficulty with breathing, irritability and intermittent perioral cyanosis. Abnormal findings from the initial physical examinations often included excessive sweating, poor perfusion with prolonged capillary refill time, tachypnea, sinus tachycardia, gallop rhythm and enlarged liver. Chest X rays showed cardiomegaly. Echocardiography revealed hypertrophic non-obstructive cardiomyopathy in the right or left ventricle or in both ventricles with ventricular dysfunction. Mild to moderate ventricular dilation was observed in some patients. Except for small ventricular defects discovered in several patients, a normal segmental anatomy without other significant structural heart defects was found in all the patients.

The heart failure initially found in all our patients was progressive despite treatment with

beta-blockers, diuretics and inotropes. All patients died from heart failure before 1 year of age unless they received a heart transplant. The life span of the affected children ranged from seven to 319 days (average 120 days with a median of 89 days for 20 infants with such information available to us). Two patients who successfully received heart transplants remained fairly healthy except for some minor transplant related issues.

### Genotyping and Mapping

We carried out a genome-wide mapping analysis using the Affymetrix GeneChip Mapping 10K SNP Arrays to identify the disease locus with three affected children from different families (Fig. 1). A large, shared block of homozygous SNPs was identified on chromosome 11p11.2-p11.12 in the three affected individuals (see the online Fig. S1 at <http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html>). The homozygous segment contains 12 contiguous SNPs and spans 4.6 Mb. Examination of the minimal shared region which was flanked by SNPs rs1401417 and rs1916207 in the affected individuals, revealed 96 known or predicted genes based on both the NCBI and Celera annotations, 37 of which are characterized. Among these genes, a known hypertrophic cardiomyopathy-related gene, *MYBPC3*, was selected for mutation analysis.

### Mutation Analysis

Genomic DNA sequence analysis of the *MYBPC3* gene in one affected patient revealed a novel homozygous mutation in the consensus splice donor site of intron 30, c.3330 + 2T > G (Fig. 2). Further sequencing analysis revealed that all three patients from the pedigrees (Fig. 1) were homozygous for the mutation, their parents were heterozygous, and no unaffected siblings (n = 8) were homozygous for the change (Fig. 2).

### RNA Analysis

To further investigate the consequence of this mutation at the transcript level, lymphocyte RNA from two heterozygous carriers was amplified by RT-PCR. Amplification of *MYBPC3* cDNA with primers F3113 and R3479 yielded two products: the expected 367-bp fragment and an abnormal shorter product (Fig. 3). Direct sequencing of the PCR products after gel extraction revealed skipping of the 140-bp exon 30 in the shorter fragment which led to a frame shift and premature stop codon in exon 31 (p.Asp1064GlyfsX38). As a control, amplification of lymphocyte RNA from two homozygous normal individuals only gave rise to the expected 367-bp product (Fig. 3).

### DISCUSSION

In this study, we performed whole-genome linkage analysis and mutational analysis in three patients who suffered from severe unexplained hypertrophic cardiomyopathy from three consanguineous families. We mapped the disease locus to a 4.6 Mb region on chromosome 11p11.2-p11.12. We further identified a novel homozygous mutation, c.3330 + 2T > G, in a putative splice-donor site of the *MYBPC3* gene within this region that is associated with this severe condition.

The c.3330 + 2T > G mutation in the *MYBPC3* gene has not been reported previously or been listed on the public database (<http://www.cardiogenomics.org>). Due to the unavailability of RNA or protein samples from cardiac tissue of the affected individuals at the current time, the consequence of the mutation was determined using lymphocyte RNA from heterozygous carriers of the c.3330 + 2T > G mutation. It was demonstrated that the mutated allele produces an aberrant transcript with skipping of the associated exon 30. Skipping of the 140-bp exon 30 led to a frame shift. The aberrant mRNA was predicted to encode a truncated protein

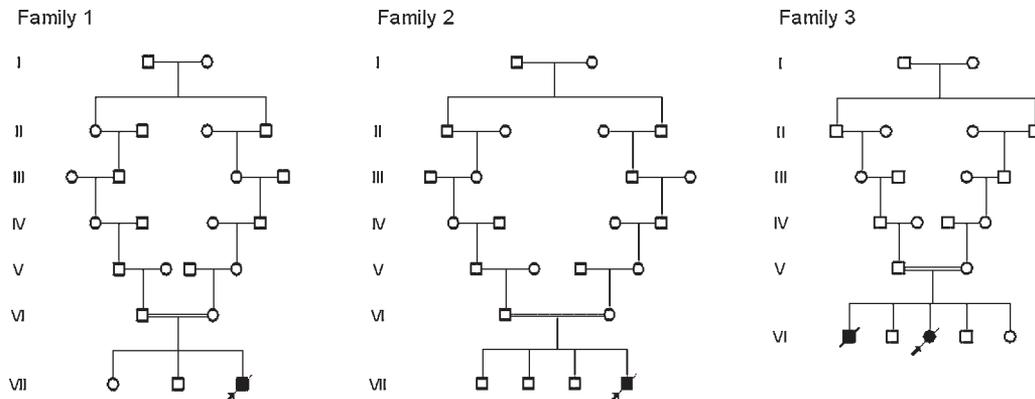


FIG. 1. Pedigrees of the three families used in the mapping study and mutational analysis of hypertrophic cardiomyopathy. The probands are indicated with an arrow in each family.

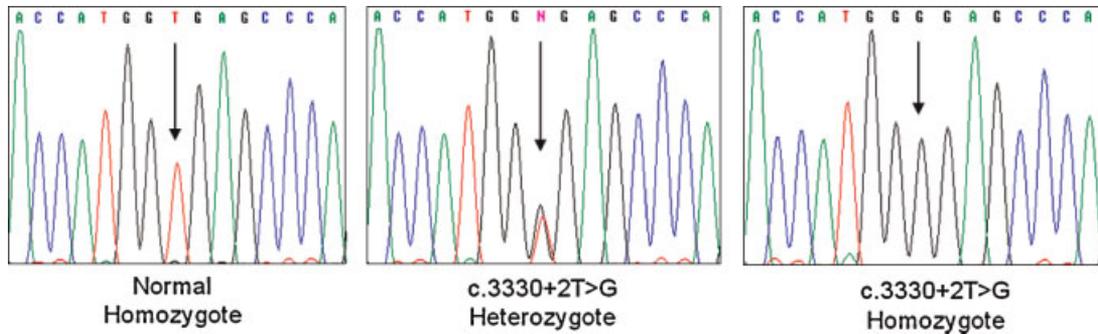


FIG. 2. Identification of c.3330 + 2T > G mutation in the *MYBPC3* gene. Partial sequence of the boundary region of exon and intron 30 is shown and the three genotypes with respect to c.3330 + 2T > G mutation are presented (arrows). [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

(p.Asp1064GlyfsX38). As a consequence, 211 amino acids of the conserved COOH terminus are deleted. We expect the same consequences of the splice donor site mutation in the myocardium.

Cardiac myosin-binding protein C is composed of 11 domains referred to as C0–C10 [Carrier et al., 1997]. Previous functional studies have demonstrated that the major myosin binding domain is located within the C10 consisting of the last 102 amino acids [Okagaki et al., 1993; Alyonycheva et al., 1997]. The c.3330 + 2T > G mutation is predicted to produce a truncated protein with complete missing of C10 domain, which is required for the incorporation of cardiac myosin-binding protein C into the A band, titin interaction and myosin binding [Freiburg and Gautel, 1996; Gilbert et al., 1996]. It is speculated that homozygosity of the mutation reported in the present study acts as null alleles and leads to complete loss of function of cardiac myosin-binding protein C, which may explain the severity of the disease phenotype in these patients. Indeed, all individuals affected with the disease present with signs and symptoms of heart failure during the neonatal period with an average life span of 3–4 months, and all patients die before 1 year of age unless they receive a heart transplant. A recent

report has described two lethal cases of neonatal hypertrophic cardiomyopathy caused by compound heterozygosity for *MYBPC3* mutations [Lekanne Deprez et al., 2006]. However, this is the first time, to our knowledge, that a homozygous mutation in the *MYBPC3* gene is reported causing a severe type of hypertrophic cardiomyopathy.

It is noted that majority of children (20) affected by this severe neonate hypertrophic cardiomyopathy in this study are from the Geauga settlement of Ohio and all of them were born during the last 16 years. Based on the number of Amish births in this settlement during this interval, we estimate that a birth incidence of the disease is approximately 1 in 350. By using the Hardy-Weinberg equilibrium and the incidence estimate, the heterozygous carrier frequency is calculated as approximately 10%. However, we are somewhat surprised with the prevalence of this severe type of hypertrophic cardiomyopathy beyond this settlement. During the study, we have been contacted by affected Amish families or health professionals from Delaware, Illinois, Indiana, Kentucky, Mississippi, New York, North Carolina and Pennsylvania with many similar cases as we reported here. The genotype of these affected children remains to be determined, but may be reasonably speculated as the same as reported here. In fact, we have been contacted by two Mennonite families from different states, and each of them has one deceased child with hypertrophic cardiomyopathy. Not unexpectedly, DNA analysis in one of the Mennonite couples with an affected infant has revealed that both parents carry the same single c.3330 + 2T > G mutation in the *MYBPC3* gene. Thus, we speculate that this infant suffered from the same type of hypertrophic cardiomyopathy as well, thus the disease also affects the Mennonite Community. It has been illustrated that Old Order Amish and Old Order Mennonite populations have unique genetic heritages despite a common religious and geographic history [Puffenberger, 2003]. The hypertrophic cardiomyopathy presented in this study might be one of a few diseases where

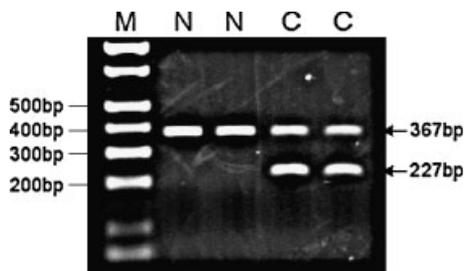


FIG. 3. Detection of the aberrant transcript as a consequence of c.3330 + 2T > G mutation. The *MYBPC3* cDNA was amplified by RT-PCR from lymphocyte RNA from homozygous normal individuals (N) and heterozygous carriers (C) using primers F3113 and R3479 located in exons 29 and 31. Samples from heterozygous carriers contain the normal 367-bp product and also a 227-bp product resulting from skipping of the 140-bp exon 30. M, DNA molecular marker.

identical mutations segregate in both populations, as is the case for Crigler-Najjar syndrome and propionic acidemia in the Amish and Mennonites of Lancaster County, PA. Higher prevalence and larger geographic distribution of severe hypertrophic cardiomyopathy might imply more distant common ancestors.

In the past, many genetic disorders identified in the Old Order Amish and Mennonite communities have been autosomal recessive diseases related to the founder effect [McKusick, 1978; Morton et al., 2003; Puffenberger, 2003], and the parents of probands generally do not have any signs or symptoms of the disease. Here, we are apparently dealing with disorder with a disease inherited in an autosomal dominant pattern with very severe phenotype in the homozygotes. Although incomplete penetrance often occurs in this autosomal dominant disorder, it remains concerning that many unidentified heterozygotes in the community are carrying a single c.3330+2T>G mutation and therefore might have, or have the potential to develop hypertrophic cardiomyopathy. Indeed, we have noticed many reports of cardiac symptoms, such as chest pain, fatigue and palpitation from probands' parents or relatives during the study. These individuals, presumably being heterozygotes of c.3330+2T>G mutation, have been one of our major concerns throughout the study. Notably, a previously reported mutation c.3330+5G>C, very similar to the mutation c.3330+2T>G found in this study, has been documented as a cause of hypertrophic cardiomyopathy in those heterozygous carriers [Watkins et al., 1995]. The consequence of being a heterozygous carrier of c.3330+2T>G mutation during a lifetime is still to be determined, but we expect that certain individuals, particularly those middle age and older, might be affected to some degree. At least three direct family members of the affected infants have died from sudden cardiac death at middle age to our knowledge. It is disconcerting that many individuals with alarming symptoms do not receive proper medical attention in this Amish community. While we are working on further understanding the pathology of both homozygosity and heterozygosity of this particular mutation, we believe that it is equally important to work with these individuals who are heterozygous carrier of c.3330+2T>G mutation to better define the clinical course of the disease. There is an urgent need to develop a practical strategy to deliver medical services to these individuals who often do not have health insurance.

#### ACKNOWLEDGMENTS

We thank the Amish and Mennonite families in this report for their support. This study would not have been possible without the invaluable assistance of

family members. We are indebted to many pediatric cardiologists who provided outstanding and compassionate care to the children affected by the disease, particularly Dr. Chandrakant R. Patel of Akron Children's Hospital, Dr. Kenneth Zahka of Rainbow Babies & Children's Hospital, and Dr. Gerard J. Boyle of Children's Hospital of Cleveland Clinic among others. We are grateful for the help of Dr. Andrew Crosby's laboratory at St. George's Hospital Medical School, London in the isolation of three DNA samples. We appreciate Mr. Joe Weaver for his genealogy consultation and Dr. Lawrence Greksa of Case Western Reserve University for his demographic consultation. The study was supported in part by The Elisabeth Severance Prentiss Foundation.

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