LETTER TO JMG

Two cases of severe neonatal hypertrophic cardiomyopathy caused by compound heterozygous mutations in the *MYBPC3* gene

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Background: Idiopathic (primary) hypertrophic cardiomyopathy (HCM) is mainly caused by mutations in genes encoding sarcomeric proteins. One of the most commonly mutated HCM genes is the myosin binding protein C (*MYBPC3*) gene. Mutations in this gene lead mainly to truncation of the protein which gives rise to a relatively mild phenotype. Pure HCM in neonates is rare and most of the time childhood HCM occurs in association with another underlying condition.

Objective: To study the presence of mutations in the *MYBPC3* gene in idiopathic childhood HCM.

Methods: *MYBPC3* coding region and splice junction variation were analysed by denaturing high performance liquid chromatography (DHPLC) and sequencing in DNA isolated from two neonates with severe unexplained HCM, who died within the first weeks of life.

Results: Truncating mutations were found in both alleles of the *MYBPC3* gene in both patients, suggesting there was no functional copy of the *MYBPC3* protein. Patient 1 carried the maternally inherited c.2373_2374insG mutation and the paternally inherited splice-donor site mutation c.1624+1G→A. Patient 2 carried the maternally inherited frameshift mutation c.3288delA (p.Glu1096fsX92) and the paternally inherited non-sense mutation c.2827C→T (p.Arg943X).

Conclusions: The findings indicate the need for mutation analysis of genes encoding sarcomeric proteins in childhood HCM and the possibility of compound heterozygosity.

I diopathic (primary) hypertrophic cardiomyopathy (HCM) is diagnosed clinically by the presence of left ventricular hypertrophy (often asymmetrical) in the absence of congenital heart disease, hypertension, valve lesions, infection, or other systemic illnesses. It often manifests with the insidious onset of congestive heart failure, chest pain, dyspnoea, or arrhythmias, or by sudden death. In infants a heart murmur is commonly present. HCM is characterised by impaired diastolic function with normal or even supranormal systolic function. Systolic function usually remains intact until late in the course of the disease. Pathological features consist of myocyte hypertrophy, myofibrillar disarray, and often increased amounts of interstitial fibrosis throughout the myocardium.[1][2] Approximately 10% of HCM cases in adults progress to left ventricular dilatation and the development of dilated cardiomyopathy with systolic and diastolic dysfunction.[2]

In adults HCM has an estimated prevalence of 1:500 in the general population and is caused mainly by mutations in

genes encoding sarcomeric proteins.[1–3] HCM, the most common cause of sudden cardiac death in young adults, is clinically heterogeneous, with inter- and intrafamilial variations in the degree and pattern of hypertrophy, penetrance, age of onset, and clinical course (ranging from a benign asymptomatic course to a malignant course with a high risk of heart failure, serious cardiac arrhythmias, and sudden death).[1] Mutations in myosin binding protein C (*MYBPC3*) are most common, generally leading to protein truncation and in most of the cases to a relatively mild phenotype with an adult age of onset. In the Netherlands, mutations in the *MYBPC3* gene account for approximately 40% of all HCM cases, in contrast to 20% in most other countries (personal experience of diagnostic testing by RLD).[4][5] This is because of the c.2373_2374insG founder mutation observed in about 25% of new index cases. A recent combined statistical analysis in five HCM study populations found no significant difference between sarcomeric genotypes, such as *MYBPC3*-HCM and *MYH7*-HCM, and phenotypic expression.[6]

Pure HCM is generally diagnosed during adult life and therefore presentation during infancy or childhood is very rare (annual incidence of about 0.47 per 100 000 children).[7][8] More often childhood HCM is seen in association with other underlying conditions such as Noonan's syndrome, metabolic disorders (Pompe's disease, fatty acid oxidation defects, mucopolysaccharidoses (Hurler's syndrome)), or endocrinological disorders (maternal diabetes, hyperthyroidism).[9][10]

In this paper we describe two neonates with severe hypertrophic cardiomyopathy caused by compound heterozygous truncating mutations in the *MYBPC3* gene.

METHODS

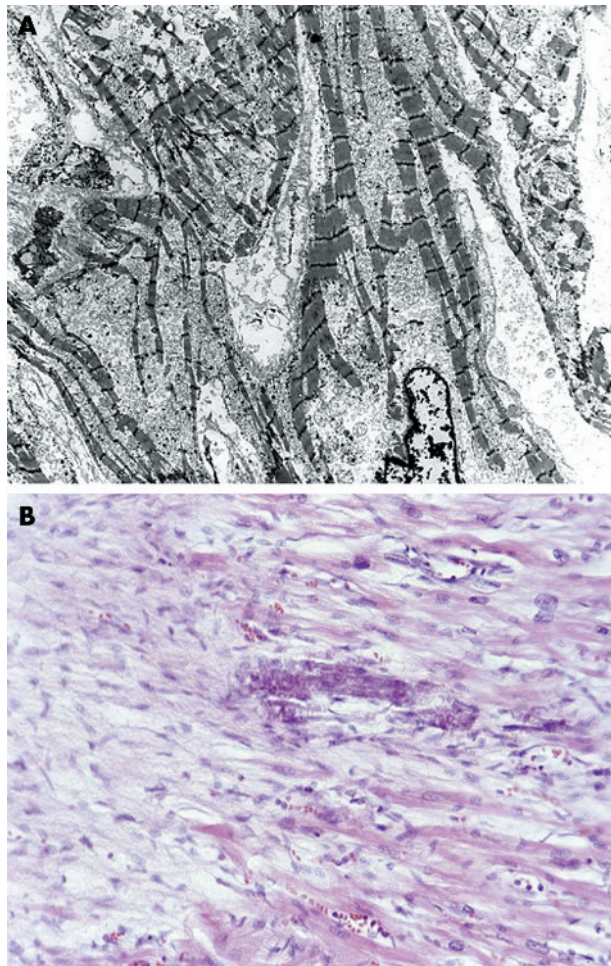Written informed consent was obtained from both families.

MYBPC3 mutation analysis in DNA and RNA

Genomic DNA was isolated from EDTA blood using PUREGENE chemistry (Gentra Systems, Minnesota, Minneapolis, USA). *MYBPC3* coding region and splice junction variation were analysed by DHPLC on the WAVE system (Transgenomics, Santa Clara, California, USA). Aberrant DHPLC patterns were sequenced using the Big Dye Terminatorv3.1 cycle sequencing kit (Applied Biosystems, Foster City, California, USA). Total RNA was isolated using Trizol Reagent from white blood cells obtained using a ficoll gradient from fresh heparinised blood. cDNA was made according to Lekanne Deprez *et al*.[11] A precise description of the methods is available on request. Nomenclature for the description of sequence variations is

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Figure 1 Myocardium of patient 1. (A) Myofibrillar disarray (electron microscopy 6000×). (B) Interstitial fibrosis with calcification (light microscopy 400×).

according to http://www.hgvs.org/mutnomen/using NM_000256.2 as the reference sequence.

RESULTS
Patient 1

A full term girl (gestational age 40 weeks, birth weight 2765 g (10–25 centile)) was born after an uneventful pregnancy and delivery. She was the second child of healthy non-consanguineous parents. On the third day she was admitted because of poor feeding, cyanosis, and difficulty with breathing. Because of her low cardiac output, inotropes and artificial ventilation were started. Physical examination showed an enlarged liver, normal blood pressure, no cardiac murmur, and no dysmorphic features. Chromosomal analysis revealed a normal female karyotype. Functional liver and kidney disorders and a raised lactate of 13 mmol/l (normal <2 mmol/l) were present. A chest *x* ray showed pulmonary oedema and cardiomegaly. On ECG there was an abnormal right superior frontal axis deviation, first degree atrioventricular block, and a clockwise rotation in a horizontal plane, with deep QS complexes in the left precordial leads associated with T wave abnormalities consistent with subendocardial ischaemia.

Echocardiography revealed hypertrophic non-obstructive cardiomyopathy with poor left and right ventricular systolic function. The left ventricular end diastolic diameter measured 17.3 mm (99% of normal), end systolic diameter 12.9 mm (116% of normal), and left ventricular posterior

wall 3.6 mm (111% of normal). The intraventricular septum measured 11.8 mm (288% of normal). The shortening fraction of the left ventricle was decreased to 25%. Multiple abnormal small communications with a minimal flow between a coronary artery and the right ventricle were observed in the apical portion of the interventricular septum. There were no structural congenital heart defects.

The heart failure was progressive despite treatment with diuretics and inotropes, leading to death at the age of five weeks. Extensive diagnostic studies showed no evidence of metabolic, mitochondrial respiratory enzyme, or endocrinological disorders. No evidence of myocarditis or other infection was found.
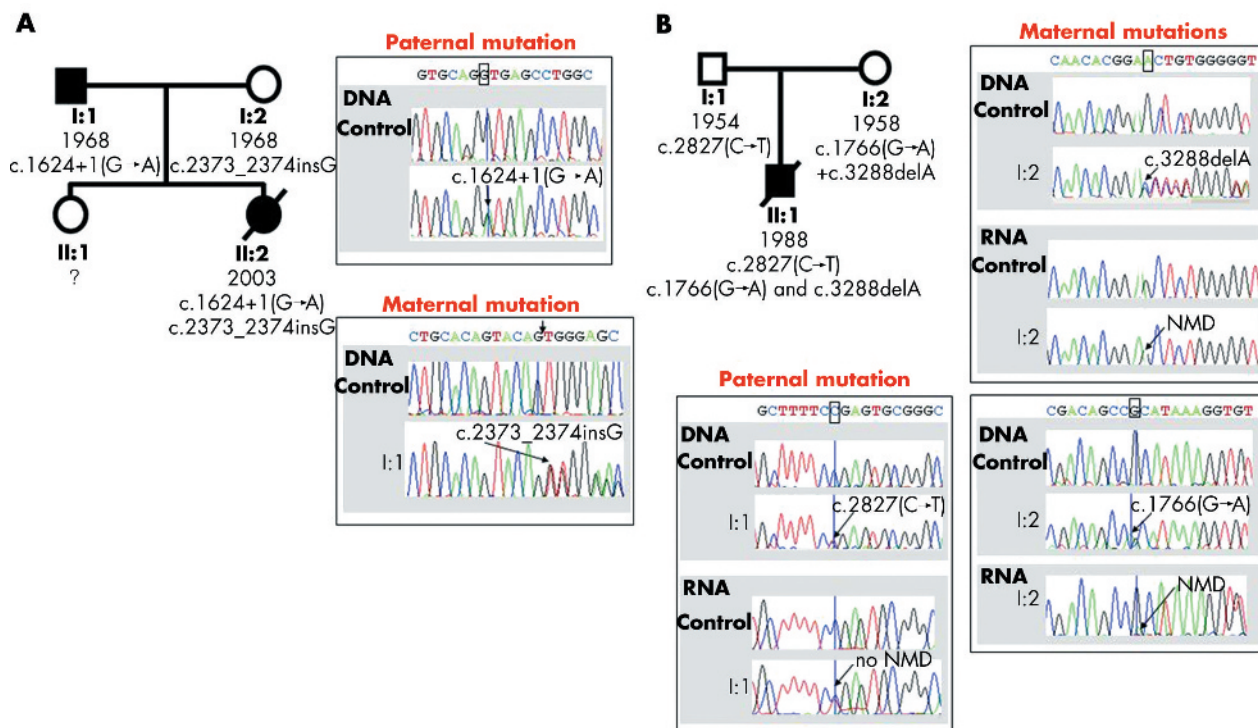
Necropsy confirmed marked hypertrophy of both ventricles and the septum, with ascites ($\pm$20 ml) and pericardial fluid ($\pm$14 ml). Myofibrillar disarray (fig 1A) and interstitial fibrosis (fig 1B) were demonstrated by microscopy.

Echocardiographic findings in the mother and sister of our patient were normal. In the apparently healthy father, echocardiography showed mild dilatation of the left atrium with an abnormal flow pattern in the mitral valve. Cardiac magnetic resonance imaging demonstrated regional hypertrophy of the interventricular septum (anterobasal) of 15 to 18 mm, a left ventricular end diastolic diameter of 52 mm, and mild hypokinesia of some parts of the septum. These findings are consistent with mild hypertrophic cardiomyopathy.

DNA analysis in this patient (see patient II:2 from family 1 in fig 2A) revealed two mutations in the *MYBPC3*-gene. The c.1624+1G→A mutation was inherited from her father (I:1 in fig 2A) and is a putative splice-donor mutation not reported previously and not on the public database (http://genetics.-med.harvard.edu/~seidman/cg3/muts/ MYBPC3_mutations_TOC.html). Unfortunately, neither RNA nor protein was available from the father or the child; we therefore used splicesitefinder (http://www.genet.sickkids.on.ca/~ali/splicesitefinder.html) to predict what might happen at the protein level. This study suggests loss of the mutated splice-donor site. It could be that as a result of this the upstream exon will be skipped, leading to a premature termination (p.Trp486X?). Alternatively one or more of the predicted alternative splice donor sites—which were all located at least 168 nucleotides further downstream in the intron—could have been used, resulting in a premature termination codon after 23 nucleotides and leading to an insertion of eight amino acids (p.Glu542insX9?). The c.2373_2374insG mutation in individual II:2 was also found in the mother (I:2 in fig 2A). This mutation is a Dutch founder mutation, which leads to almost complete loss of the truncated protein (p.Trp792fsX17) in myocardial tissue.[4][12] No DNA study was done on the sister of the patient (II:1).

Patient 2

This was a boy, born at term with a birth weight of 3540 g, the second child of healthy non-consanguineous parents. Two weeks after birth he was admitted to another hospital because of poor feeding, respiratory distress, and cyanosis. The family history was positive for HCM on the mother's side: the maternal grandfather died from HCM at the age of 52 years, and a brother of the maternal grandfather was also affected. On admission to the intensive care unit he had a poor circulation, and on auscultation there was a soft first heart sound and a grade II systolic murmur at the apex; the liver was enlarged. There were no dysmorphic features. A chest *x* ray showed an enlarged heart. The electrocardiogram had an axis of +150, giant P waves, hardly any left sided activity in the precordial leads, and flattened T waves. Echocardiography revealed an enlarged right atrium, a normal left atrium, and hypertrophy of both ventricles with diminished contractility. On cardiac catheterisation, both ventricles were found to be

Figure 2 Mutational analysis of the *MYBPC3* gene in two families with lethal neonatal hypertrophic cardiomyopathy. (A) Pedigree and sequence traces from carriers of the *MYBPC3* mutations from genomic DNA in family 1. (B) Pedigree and sequence traces from carriers of the *MYBPC3* mutations from both genomic DNA and RNA in family 2. DNA from I:2 harbours the c.3288delA frameshift mutation in one allele and a polymorphism c.3288A→G (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1052373) in the other allele. The control is homozygous for c.3288A→G. The presence or absence of NMD was determined by comparing the intensity of the mutated nucleotide relative to the wild type in the sequence traces of the cDNA (see RNA). The position of the mutation in the nucleotide sequence is indicated with a rectangle or arrow. Filled symbols show the individuals that are clinically and genetically affected. Below each individual the year of birth and the mutation(s), if determined, are indicated.

hypertrophic. There was mild shunting at the level of the foramen ovale. The pressures in the right ventricle and pulmonary artery were pathologically high, while the pressures in the left atrium and ventricle were normal (though with high A waves). There was a total left to right shunt of 43% of the pulmonary circulation, partly caused by a low muscular ventricular septal defect. There was no evidence of valve stenosis, and the coronary arteries showed no abnormalities. An extensive work-up for congenital infections revealed no abnormalities. There was no evidence of a metabolic disorder or mitochondrial dysfunction. Electromyography was normal. Despite some initial improvement on drug treatment, he died of heart failure at the age of six weeks.

At necropsy the heart weight was 47 g (normal 23 g), and the ventricular septum was closed. The muscle fibres were broad and in disarray. There was no evidence of viral infection. The parents and siblings of the patient showed no abnormalities on cardiological examination.

This patient (II:1, fig 2B) showed two mutations in the *MYBPC3* gene as well. The c.2827C→T mutation creates a stopcodon at position 943 of the protein (p.Arg943X) and was inherited by his father (I:1, fig 2B). This mutation was more often found in our patient population and described before.[13] Because no heart material was available we studied RNA stability in blood RNA of both the mutation carrier (I:1, fig 2B) and a normal control. The *MYBPC3* gene is not abundantly expressed in blood and therefore the conclusions should be interpreted with care. This truncated transcript results in a stable mRNA because r.2827c→u was found in about an equimolar amount with the wild type nucleotide after sequencing the cDNA (fig 2B). The second mutation in patient II:1, c.3288delA, was maternally inherited, and leads to a frameshift after amino

acid threonine at position 1095 of the protein (p.Glu1096fsX92) and has not been reported before. The stability of this transcript was investigated in RNA isolated from peripheral blood from the mother. This mutation could not be detected, indicating nonsense mediated mRNA decay (NMD).[14]

Besides the c.3288delA mutation, this *MYBPC3* allele harbours another novel mutation, c.1766G→A (p.Arg589His). This mutation is located in a conserved region of the protein (between species and isoforms) and was not found in more than 200 control alleles. At present it is difficult to say if it can function as a pathogenic mutation. The consequence of this unclassified variant is not relevant for this patient, as the mRNA from this allele is unstable. RNA from the mother shows very small amounts of the r.1766g→a containing transcript compatible with NMD, as was already found for the r.3288dela mutation.

DISCUSSION

This study provides mutational analysis and clinical features in two unrelated patients and their families with severe unexplained hypertrophic cardiomyopathy, who both died within the first weeks of life. Both patients turned out to be compound heterozygote carriers of truncating mutations in the *MYBPC3* gene. Other studies also report homozygote or compound heterozygote carriers that harbour two mutations in the same or different sarcomeric genes and indicate that this could be related to the clinical course.[2 5 13 15–17] In none of these studies was such a severe phenotype described in the neonatal period as we observed in our patients. This phenotypic difference probably reflects the fact that our patients are likely to carry null mutations in the *MYBPC3* gene—that is, no functional copy of the gene is expressed—whereas in the other patients at least one (partially) functional copy is still active.

In support of the important role of the *MYBPC3* mutations in causing the severe phenotypes we did not find any pathogenic mutations in *MYH7* in patient 1 (limited DNA availability did not allow mutation analysis of *MYH7* in patient 2). Although protein extracted from cardiac tissue was not available from the mutation carriers in this study, the conclusion that each mutation in the patients function as a null allele is based on the following. The maternal c.2373_2374insG mutation in patient 1 is a well known Dutch founder mutation, which leads to almost complete loss of the truncated protein (p.Trp792fsX17) in myocardial tissue.[4][12] For the paternal splice-donor site mutation c.1624+1G→A, we can only predict what is likely to happen at the protein level, because no RNA or protein was available for further study. All predictions would result in premature termination. The maternal c.3288delA mutation in patient 2 creates a frameshift and leads to premature termination (p.Glu1096fsX92). This mutation could not be detected in RNA from the mother indicating NMD. The paternal c.2827C→T nonsense mutation creates a premature termination as well (p.Arg943X), but did not result in NMD. When the latter mutation would result in a stable protein it could act in a dominant negative way on the structure or function of the sarcomere. This is not very likely, however, because a recent study suggests that *MYBPC3* mutants that create a premature termination codon but do not result in nonsense mediated mRNA decay would lead to accelerated degradation by an impaired ubiquitin-proteasome system.[18] Therefore, our results suggest that both patients could be considered to be true human *MYBPC3* knockouts.

At present it is unclear whether degradation of the truncated cMyBPC3 protein by the ubiquitin-proteasome system (UBS could result in competitive inhibition of other UBS substrates. As the UBS is important in a variety of fundamental cellular processes, impairment of this system by the truncating mutant could play a contributing factor in the pathogenesis of HCM as well. In contrast to these potential human knockouts, homozygous knockout mice survived into adulthood with profound cardiac hypertrophy and impaired contractile function, whereas heterozygous mice were indistinguishable from wild-type littermates.[19] This difference could reflect a critical difference between mouse and human, or could be explained by other factors such as modifying genes or the environment.

The cardiological findings of patient 1 were unique. The patient suffered from a hypertrophic heart with diastolic dysfunction and poor systolic function of both ventricles. Normally HCM manifests with impaired diastolic function, and with normal or even supranormal systolic function. Systolic function usually remains intact until late in the course of the disease. The systolic dysfunction in our case may be characteristic of the combination of the two mutations found.

Conclusion

Lethal neonatal hypertrophic cardiomyopathy can be caused by compound heterozygous truncating mutations in the *MYBPC3* gene, indicating the need for mutational analysis in sarcomeric genes in primary childhood HCM and the possibility of compound heterozygosity in one or several of the candidate genes.

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Conflict of interest: none declared

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