

ELECTRONIC LETTER

Compound and double mutations in patients with hypertrophic cardiomyopathy: implications for genetic testing and counselling

J Ingles, A Doolan, C Chiu, J Seidman, C Seidman, C Semsarian

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Objective: To report the frequency of single and multiple gene mutations in an Australian cohort of patients with hypertrophic cardiomyopathy (HCM).

Methods: Genetic screening of seven HCM genes (β -MHC, MyBP-C, cTnT, cTnI, ACTC, MYL2, and MYL3) was undertaken in 80 unrelated probands. Screening was by denaturing high performance liquid chromatography and direct DNA sequencing. Clinical data were collected on all patients and on genotyped family members.

Results: 26 mutations were identified in 23 families (29%). Nineteen probands (24%) had single mutations (11 β -MHC, 4 MyBP-C, 3 cTnI, 1 cTnT). Multiple gene mutations were identified in four probands (5%): one had a double mutation and the others had compound mutations. Six of 14 affected individuals from multiple mutation families (43%) experienced a sudden cardiac death event, compared with 10 of 55 affected members (18%) from single mutation families ($p=0.05$). There was an increase in septal wall thickness in patients with compound mutations (mean (SD): 30.7 (3.1) v 24.4 (7.4) mm; $p<0.05$).

Conclusions: Multiple gene mutations occurring in HCM families may result in a more severe clinical phenotype because of a "double dose" effect. This highlights the importance of screening the entire panel of HCM genes even after a single mutation has been identified.

Hypertrophic cardiomyopathy (HCM) is a primary cardiac disorder characterised by myocardial hypertrophy, usually of the left ventricle, in the absence of loading conditions such as hypertension.^{1,2} The prevalence of HCM in the general population is thought to be 0.2% (or 1/500),³ making it the commonest known cardiovascular genetic disorder. HCM shows considerable clinical heterogeneity, both between and within families. Variability in clinical presentation ranges from minimal or no symptoms to the most serious complications including heart failure and sudden cardiac death.⁴ This heterogeneity could be explained by environmental influences such as exercise, modifier genes, or the presence of compound or double disease causing mutations in affected individuals.⁵⁻¹²

Over 200 different mutations in at least 11 genes have been identified in HCM. In most cases, HCM is caused by single heterozygote mutations in genes encoding sarcomeric proteins. These include the β -myosin heavy chain (β -MHC), myosin binding protein C (MyBP-C), cardiac troponin T (cTnT), cardiac troponin I (cTnI), α -tropomyosin, essential and regulatory light chain (MYL2 and MYL3), titin and actin (ACTC) genes.^{1,5} Most recently, it has been reported that approximately 5% of HCM patients carry more than one

disease causing gene mutation, leading to a double or compound heterozygote genotype.^{9,10} It appears that these patients may develop a more severe clinical phenotype because of a "double dose" gene mutation effect.⁹⁻¹¹

In this study we report the frequency of single and multiple gene mutations in an Australian cohort of HCM patients. We discuss the implications of the findings for clinical risk stratification and for genetic counselling of families with regard to genetic testing, inheritance risk, and the accuracy of predictive genetic testing for family members.

METHODS

Patients

Patients referred to the HCM clinic, a tertiary referral centre at Royal Prince Alfred Hospital in Sydney, Australia, were included in this study. Clinical evaluation was undertaken as described previously¹³ and included a full clinical history and physical examination, electrocardiography (ECG), and cross section and M mode echocardiography. A sudden cardiac death event was defined as death occurring within one hour of symptoms in an individual with HCM, or a resuscitated cardiac arrest related to HCM. The primary diagnostic criterion for HCM in adults was a maximum left ventricular wall thickness of ≥ 13 mm on echocardiography in the absence of loading conditions such as hypertension. Within the context of a family history, the diagnosis of HCM was made by the above echocardiographic findings and/or at least one of the following major abnormalities on ECG: abnormal Q waves (>0.04 s or depth $>25\%$ of R wave), left ventricular hypertrophy (voltage criteria), or marked repolarisation changes (for example, T wave inversion in at least two leads).

Following informed consent, a 20 ml blood sample was taken from the proband in each family for genetic analysis. All studies were undertaken in strict accordance with the Central Sydney Area Health Service human ethics standards.

Genetic analysis

Analysis of the entire coding sequence of seven genes known to cause HCM (β -MHC, MyBP-C, cTnI, cTnI, ACTC, MYL2, and MYL3) was carried out as a collaborative effort between the Department of Genetics at Harvard Medical School, Boston, and the Agnes Ginges Centre for Molecular Cardiology, Sydney. Genetic screening of these genes was undertaken as previously described.^{14,15} In brief, the entire coding sequence of each of the seven genes was systematically analysed for the proband in each family. Exons were amplified by polymerase chain reaction (PCR) from genomic DNA and sequenced using an ABI Prism 377 DNA analyser. Analysis of DNA sequences was undertaken using Sequencher v3.1. A sequence variant (polymorphism) was

Abbreviations: AICD, automatic implantable cardioverter-defibrillator; HCM, hypertrophic cardiomyopathy

Table 1 Disease causing gene mutations identified in Australian hypertrophic cardiomyopathy probands

Family	Mutation	Exon	Gene	Status
U, AW	Lys146Asn	5	β -MHC	Novel
CT	Val186Leu	7	β -MHC	Novel
AA	Val606Met	16	β -MHC	Known
AF, AS	Arg663His	18	β -MHC	Known
V	Val698Ala	19	β -MHC	Novel
C	Arg719Gln*	19	β -MHC	Known
AH	Arg723Cys	20	β -MHC	Known
AN	Ile736Thr	20	β -MHC	Novel
L	Ala742Glu	20	β -MHC	Novel
BA	Gly1057Asp	25	β -MHC	Novel
C	Arg273His*	8	MyBP-C	Novel
O, AI	Arg326Gln*	13	MyBP-C	Known
BS	Arg502Trp	18	MyBP-C	Known
G	Glu542Gln*	18	MyBP-C	Known
CE	lvs18+4A→T	Intronic	MyBP-C	Novel
D	Asp745Gly*	24	MyBP-C	Novel
CR	lvs24+1G→A	Intronic	MyBP-C	Novel
G	Ala851Val*	26	MyBP-C	Novel
D	Pro873His*	27	MyBP-C	Known
O	Gln1233Ter*	34	MyBP-C	Known
AZ	Arg162Pro	7	cTnl	Known
X	Arg162Gln	7	cTnl	Known
J	Arg204His	8	cTnl	Known
AK	Arg278Cys	16	cTnT	Known

*Indicates those mutations found in the compound and double heterozygotes.

β -MHC, β -myosin heavy chain; cTnl, cardiac troponin I; cTnT, cardiac troponin T; MyBP-C, myosin binding protein C.

considered to be a disease causing mutation on the basis of the following three criteria: co-segregation with affected members in the family; absence of the mutation in 300 unrelated chromosomes from healthy adult controls; and the conservation of the mutated residue among species and isoforms. Once a mutation was confirmed in the proband by

restriction enzyme digestion, other family members were offered genetic testing and evaluated.

Statistical analysis

We used χ^2 analysis and Student's *t* test to determine significant differences for variables. For all comparisons, a

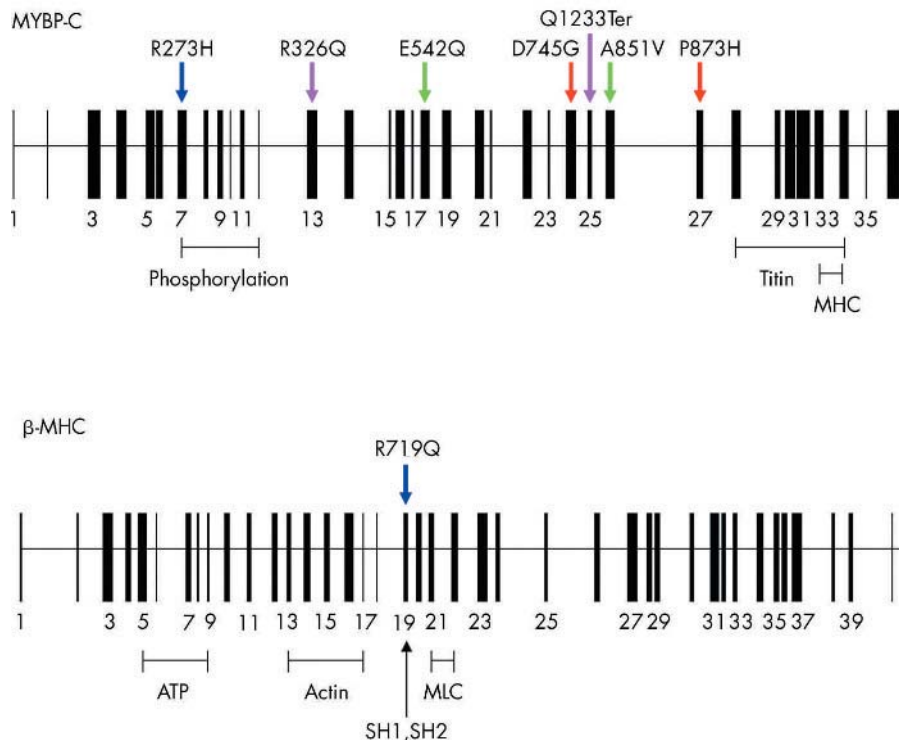


Figure 1 Diagram of compound and double mutations identified in MyBP-C and β -MHC. Family mutations are shown above the gene and are colour coded by family: family C, blue; family D, red; family G, green; family O, mauve. Binding domains are highlighted below the gene. MyBP-C has titin and myosin heavy chain (MHC) binding sites. The phosphorylation site is also shown. Actin, myosin light chain (MLC), and ATP binding sites are represented on the β -MHC gene. Exon 19 of β -MHC contains two "active thiols", SH1 and SH2.

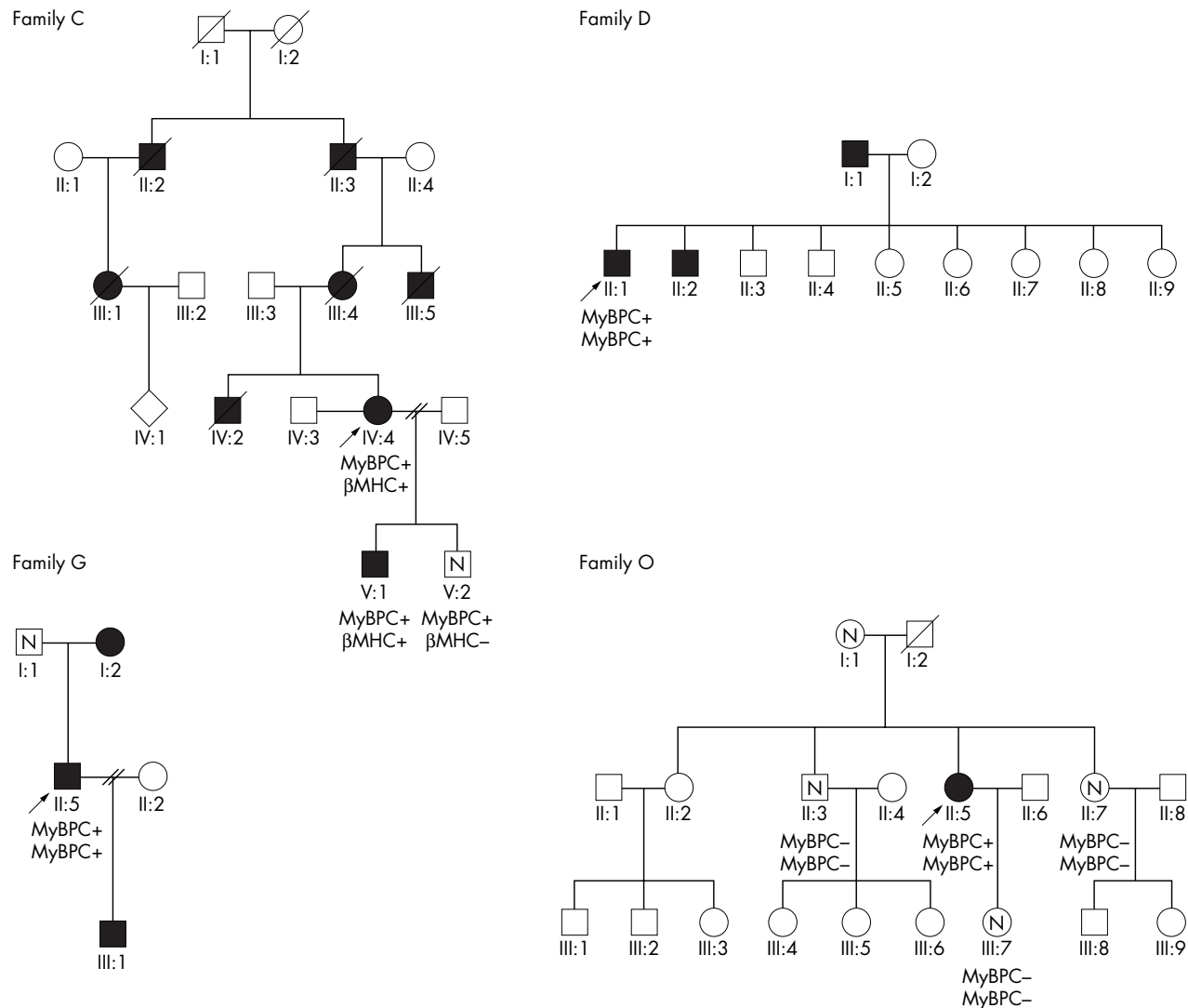


Figure 2 Multiple mutation family pedigrees. Arrow indicates proband; black symbols represent clinically affected individuals. The genotype is shown below the individual, where “+” means that the individual has a mutation in the indicated gene and “-” indicates the individual does not have the mutation. N, clinically unaffected.

probability (*p*) value of <0.05 was considered significant. All values are given as mean (SD).

RESULTS

In all, 80 unrelated probands underwent genetic screening of the seven HCM disease genes. Twenty six gene mutations were identified in 23 probands (29%) (table 1). In these families, where at least one mutation was identified, 52% had a family history of HCM before genetic testing. The same gene defect was found in three families; thus 24 separate mutations were identified. Eleven of the 24 mutations were novel (46%). Of these 23 probands, 19 (24% of all tested) were found to have single mutations (heterozygotes), of which 11 were identified in β -MHC, four in MyBP-C, three in cTnI, and one in cTnT (table 1). Two of the four MyBP-C mutations were identified in intronic regions and were thought to be responsible for alternate splicing. All were missense mutations apart from one nonsense mutation which resulted in truncation of the affected protein (family O; Gln1233Ter).

Four families (5%) were found to carry more than one disease causing gene mutation. The proband from family C was identified as a double heterozygote, with an Arg719Gln

mutation in exon 19 of β -MHC and an Arg273His mutation in exon 8 of MyBP-C. The remaining three probands were found to carry compound mutations in MyBP-C. Family D had an Asp745Gly and a Pro873His mutation, while family G had a Glu542Gln and an Ala851Val mutation. Family O had a nonsense Gln1233Ter mutation and a missense Arg326Gln mutation. The positions of these mutations are shown in fig 1.

The pedigrees of the four multiple mutation families are shown in fig 2. Identification of a mutation in a proband allowed further investigation of other family members. In family C, V:1 was identified as having inherited both the β -MHC and the MyBP-C mutations from his mother (IV:4, proband). This individual is aged 15 years and is clinically affected. Individual V:2 is aged 13 years and is clinically normal but has inherited one of the two genes and is therefore heterozygous for the MyBP-C mutation (genotype positive–phenotype negative). Tissue was not available for genetic analysis in any of the deceased family members, although available clinical and necropsy information allowed accurate diagnosis of HCM and the nature of death in the deceased individuals. All died suddenly except for individual III:4, who had severe biventricular heart failure and died at age 42 years. In family D, only the proband (II:1) had

Table 2 Clinical and genetic characteristics of compound and double heterozygote families

Family	Case	Age (y)	LVWT (mm)	SCD events	AICD	Clinically affected	Genotype	Gene/exon mutation 1	Gene/exon mutation 2
C	III:4	44	20	5	No	Yes	NT	β-MHC/19 R719Q	MyBP-C/8 R273H
	IV:2	16	20		No	Yes	NT		
	IV:4	35	17		Yes	Yes	DH		
	V:1	15	29		Yes	Yes	DH		
	V:2	13	9		No	No	HZ		
D	I:1	72	N/A	1	No	Yes	NT	No mutation MyBP-C/24 D745G	R273H MyBP-C/27 P873H
	II:1	29	30		Yes	Yes	CH		
	II:2	32	N/A		Yes	Yes	NT		
G	II:1	34	34	0	Yes	Yes	CH	MyBP-C/18 E542Q	MyBP-C/26 A851V
	III:1	13	N/A*		Yes	Yes	NT		
O	I:1	82	14	0	No	No	NT	MyBP-C/13 R326Q	MyBP-C/34 Q1233Ter
	II:3	58	11		No	No	No mutation		
	II:5	53	28		No	Yes	CH		
	II:7	52	10		No	No	No mutation		
	III:7	17	8		No	No	No mutation		

*Myectomy ×2.

AICD, automatic implantable cardioverter defibrillator; CH, compound heterozygote; DH, double heterozygote; HZ, heterozygote; LVWT, left ventricular wall thickness; N/A, not available; NT, not tested; SCD, sudden cardiac death; y, years.

undergone genetic testing, although his brother (II:2) has clinical evidence of HCM and had a resuscitated cardiac arrest, during which he sustained hypoxic brain damage. The proband from family G (II:1) has severe left ventricular hypertrophy (34 mm) and has a 13 year old son (III:1) who also has severe hypertrophy requiring myectomy on two occasions and implantation of an automatic implantable cardioverter-defibrillator (AICD). Genetic analysis of family members of family O has not identified any other affected individuals to date.

The clinical features of the compound and double heterozygote patients are summarised in table 2. Proband with compound mutations had a significantly greater left ventricular wall thickness than the single mutation patients (30.7 (3.1) v 24.4 (7.4) mm, $p < 0.05$). No significant difference between other echocardiographic variables was found (including fractional shortening and left ventricular outflow tract obstruction). Fourteen members of the multiple mutation families were found to be clinically affected. Six (43%) of these individuals suffered a sudden cardiac death event (age range 16 to 66 years). In contrast, 55 members of the single mutation families were found to be clinically affected, of whom 10 (18%) suffered a sudden cardiac death event ($p = 0.05$ v sudden death events in multiple mutation families). Six of eight (75%) clinically affected living family members from the multiple mutation families were managed with AICD implantation because of a family history of sudden cardiac death, massive left ventricular hypertrophy (≥ 30 mm), non-sustained ventricular tachycardia, or syncope.

DISCUSSION

In this report we describe the frequency of single and multiple gene mutations in an Australian cohort of 80 unrelated HCM families. A gene mutation was identified in 23 probands (29%), with four probands being compound or double heterozygote genotypes (5%). Proband carrying multiple gene mutations were shown to express a more severe clinical phenotype than single heterozygote HCM patients, as demonstrated by an increase in left ventricular hypertrophy and an increased incidence of sudden cardiac death events. Implications for genetic counselling of patients harbouring multiple mutations include limitations to the accuracy of predictive gene testing in HCM, alterations of inheritance risk in children, and the implications of multiple mutations and clinical severity of disease on decision making processes relating to pregnancy.

Multiple mutations may account for some of the clinical heterogeneity observed in patients with HCM. Most recently, a subgroup of patients has been identified as carrying more than one gene mutation, leading to a compound or double heterozygote genotype. As in the current study, recent reports indicate that this subgroup comprises approximately 5% of HCM patients.^{9,10} Descriptions of large cohorts of HCM patients who have undergone genetic testing have included individuals carrying compound MyBP-C and β-MHC mutations, double MyBP-C/β-MHC, MyBP-C/cTnT, MyBP-C/cTnI, MyBP-C/TPM, β-MHC/cTnT mutations, and homozygous MyBP-C, β-MHC, and cTnT mutations.^{9–11,16} It is thought that these genotypes have a double dose effect, leading to a clinically more severe phenotype.⁹ This may relate to direct effects of these mutations on protein function, affecting actin–myosin interactions within the sarcomere, the overall stability of the sarcomere structure, and alterations in calcium handling within myocyte.

The relatively high incidence of multiple mutations in HCM suggests that the reported prevalence of this disease of around 1/500³ may be an underestimate. This is not surprising as such prevalence studies have been based largely on echocardiographic diagnosis, and we now know that some individuals may have either low or late penetrance—that is, they carry the gene defect but do not show clinical evidence of disease such as symptoms or echocardiographic evidence of left ventricular hypertrophy. Interestingly, the overall rate for mutations in the current study of 29% was significantly lower than in previous studies, and may reflect both a clinically or genetically different HCM population in Australia.

Patients with multiple mutations have been reported to develop more significant left ventricular hypertrophy, are diagnosed at an earlier age, and require more advanced and invasive treatments such as surgical myotomy/myectomy and AICD implantation.^{9,10} In the current study, a consistent finding in a geographically distinct population was observed, whereby patients with multiple mutations had a greater left ventricular wall thickness and a 2.4-fold higher incidence of sudden cardiac death events among family members. Limitations regarding the availability of necropsy tissues did not allow us to confirm the presence of two mutations in all clinically affected individuals who had a sudden death event. Nevertheless, the overall trend towards more sudden death events in this cohort of families compared with single mutation families was of clinical significance. Further elucidation of the phenotype of compound and

double heterozygotes could lead to alterations in their clinical management. Given the important clinical issue of risk stratification for determining which HCM patients are at highest risk of heart failure and sudden cardiac death, the presence of two mutations in an individual may be a major factor in this cardiac event risk algorithm. The available clinical data in those who had sudden death events and carried multiple mutations showed that they had clinical risk factors for sudden death, such as a positive family history of sudden death and severe cardiac hypertrophy (≥ 30 mm). Identification of multiple mutations in an individual may prompt more aggressive treatment including implantation of an AICD to prevent sudden death and management of symptomatic left ventricular outflow tract obstruction.

The role of genetic counselling and testing is an important component of the evaluation, diagnosis, and management of individuals and families with HCM.^{17–19} The occurrence of multiple mutations in a subgroup of this HCM population has raised new questions about what we tell families in the context of genetic testing. For example, based on the current findings, genetic screening should not cease after a single mutation has been identified. As HCM genetic testing moves out from the research phase and in to the clinical arena, it will become more difficult to ensure that families receive the correct information about the limitations of this test. It is important that commercial HCM genetic testing is offered as screening of a panel of common genes, rather than on a single gene basis, which may seem more cost-effective to a patient. This testing panel would ideally be made up of the three most common HCM disease genes as a minimum, as most reports of multiple mutations involve β -MHC, MyBP-C, and cTnT.¹⁰ The implications for a family of not identifying a second gene mutation could be devastating, as currently an individual who receives a negative predictive gene test is released from clinical screening and believes their children are no longer at risk of developing HCM.

An integral part of the genetic counselling process is the explanation of the inheritance of the disease and the risk to children. HCM is an autosomal dominant disorder, whereby most affected individuals have a single heterozygote genotype. However, double and compound mutations significantly alter the chances of inheriting the gene defect in families. If a patient was found to be a double heterozygote carrying a gene mutation on two different genes inherited independently (as in family C), the risk that a child will receive at least one disease causing gene mutation becomes 75%. In addition, there is a 25% chance that the child will inherit both gene mutations and potentially show the more severe phenotype so commonly reported. Similarly, if a patient was identified as being a compound heterozygote (for example, families D, G, and O), the chance of passing on the gene defect depends on whether the two mutations are in *cis* (both mutations on the same allele) or *trans* form (mutations on different alleles). In the former situation, the chance of passing on the gene defect is 50%, while in the latter the risk of passing on one of the two mutations is 100%. These clinical scenarios could have an effect on the uptake of prenatal genetic testing for HCM, which is currently reported to be very low²⁰ owing to the extreme clinical variability seen. Further elucidation of the clinical consequences of multiple mutations may eventually assist some couples in making a more informed decision about prenatal genetic testing and termination of pregnancy. These are important considerations for a family and highlight the value of accurate genetic testing of the proband. It is of equal importance to ensure that cardiologists dealing with HCM families are equipped with the skills to offer appropriate genetic counselling. Ideally, all families with HCM should be referred to a cardiac

clinic where genetic counselling support is available to discuss these issues at length.

Multiple gene mutations within a HCM family are being increasingly reported and raise many issues for genetic counselling. Integral to these issues is the need for accurate HCM genetic testing, which will allow an individual's genotype to be clearly established and understood by clinicians and their patients. Further clarification of the proportion of HCM patients with multiple mutations and how this correlates with an individual's phenotype are important aspects in accounting for the marked clinical heterogeneity characteristic of HCM.

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Authors' affiliations

J Ingles, A Doolan, C Chiu, Agnes Ginges Centre for Molecular Cardiology, Centenary Institute, Sydney, Australia

C Semsarian, Department of Cardiology, Royal Prince Alfred Hospital, Sydney

J Seidman, C Seidman, Department of Genetics, Harvard Medical School, Boston, Massachusetts, USA

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Correspondence to: Associate Professor Christopher Semsarian, Agnes Ginges Centre for Molecular Cardiology, Centenary Institute, Locked Bag 6, Newtown, NSW 2042, Australia; c.semsarian@centenary.usyd.edu.au

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