Genes, calcium and modifying factors in hypertrophic cardiomyopathy

Tatiana Tsoutsman[1], Lien Lam[1], Christopher Semsarian[1,2]

[1]Agnes Ginges Centre for Molecular Cardiology, Centenary Institute and
[2]Department of Cardiology, Royal Prince Alfred Hospital, Sydney, NSW, Australia

Summary

1. Familial hypertrophic cardiomyopathy (FHC) is a primary disorder of the myocardium characterised by remarkable diversity in clinical presentations, ranging from no symptoms to severe heart failure and sudden cardiac death.

2. Over the last 15 years, at least eleven genes have been identified, defects in which cause FHC. Most of these genes encode proteins which comprise the basic contractile unit of the heart, i.e. the sarcomere.

3. Genetic studies are now beginning to have a major impact on diagnosis in FHC, as well as in guiding treatment and preventative strategies. While much is known about which genes cause disease, relatively little is known about the molecular steps leading from the gene defect to the clinical phenotype, and what factors modify the expression of the mutant genes.

4. Concurrent studies in cell culture and animal models of FHC are now beginning to shed light on the signaling pathways involved in FHC, and the role of both environmental and genetic modifying factors. Calcium dysregulation appears to be important in the pathogenesis of FHC.

5. Understanding these basic molecular mechanisms will ultimately improve our knowledge of the basic biology of heart muscle function, and will therefore provide new avenues for diagnosis and treatment not only for FHC, but for a range of human cardiovascular diseases.

Introduction

Familial hypertrophic cardiomyopathy (FHC) is a primary cardiac disorder characterised by hypertrophy, usually of the left ventricle, in the absence of other loading conditions, such as aortic stenosis or hypertension.[1] Population-based clinical studies suggest the prevalence of the condition to be as high as 0.2% (or 1 in 500) in the general population, making FHC the most common cardiovascular genetic disorder known.[2] A prominent feature of FHC is the remarkable clinical diversity observed. Patients with FHC can range in clinical presentation from minimal or no symptoms and have a benign, asymptomatic course, to the development of the most serious complications including heart failure and sudden death.[3] FHC is the commonest structural cause of sudden cardiac death in individuals aged less than 35 years, including competitive athletes.[4,5]

Genetic basis of hypertrophic cardiomyopathy

For many years, clinicians had observed the occurrence of FHC within families, indicating a genetic predisposition. Over the last 15 years, genetic studies have further defined FHC as a "disease of the sarcomere", with several disease-causing gene mutations being identified which encode sarcomeric proteins.[6] Disease mutations in at least 11 different genes are currently known to cause FHC. The disease is transmitted as an autosomal dominant trait, such that offspring of affected individuals have a 50% chance of inheriting the gene mutation. Interestingly, 10 of the 11 genes identified to date encode sarcomere proteins, and include the cardiac β-myosin heavy chain (βMHC), myosin binding protein C (MyBP-C), cardiac troponins T and I, α-tropomyosin, myosin light chains, and more recently, titin and actin genes (Table 1).

The relative frequency of these causative sarcomeric genes in FHC is summarised in Table 1. Over 200 different mutations have now been identified in these genes, with most being of the missense-type, i.e. a single base change resulting in an amino acid substitution (http://cardiogenomics.med.harvard.edu/home). Family studies appear to indicate that defects in different genes may in part be associated with characteristic clinical features. For example, βMHC gene mutations generally result in early onset of disease, usually in the first two decades of life,[7] while MyBP-C mutations result in later onset of disease (age 40-50 years) with less marked symptoms.[8] In contrast, troponin T mutations are associated with minimal cardiac hypertrophy, but significant incidence of sudden death.[9] While these associations have been made, clearly many exceptions have arisen, e.g. individuals with early onset MyBP-C mutations.

Molecular pathogenesis of hypertrophic cardiomyopathy

The demonstration that FHC results from defects in genes which encode sarcomeric proteins has focused attention on the most basic unit responsible for cardiac muscle contraction. The sarcomere is comprised of both thick and thin filaments. The sarcomere units are aligned in parallel, and are attached to each other through the Z discs. The main component of the thick filament is the βMHC protein, while the thin filament is composed of actin, α-tropomyosin and the troponins I, C and T. MyBP-C and titin are major components of the sarcomere and are involved in both stabilisation of the sarcomere structure, and the generation and transmission of force. Following

Table 1: Causative genes in FHC

FHC Gene		Symbol	Chromosome Locus	% of all FHC
β-Myosin heavy chain		MYH7	14q12	30-35%
Myosin-binding protein C		MYBPC3	11p11.2	20-30%
Troponin T		TNNT2	1q32	10-15%
α-tropomyosin		TPM1	15q22.1	< 5%
Troponin I		TNNI3	19q13.4	< 5%
Myosin light chains	-- Essential	MYL3	3p21	< 1%
	-- Regulatory	MYL2	12q24.3	< 1%
Actin		ACTC	15q14	< 0.5%
Titin		TTN	2q24.3	< 0.5%
α-Myosin heavy chain		MYH6	14q12	< 0.5%
Muscle LIM		CRP3	11p15.1	< 0.5%

Modified from Doolan *et al.*[32]

activation by Ca^{2+}, a series of events involving the troponin-tropomyosin complex results in sliding of the thin and thick filaments, resulting in sarcomere shortening and cardiac muscle contraction.

Many questions have been raised following the identification of sarcomeric gene defects in FHC regarding the cell biology of cardiac muscle. What is the mechanism by which a cardiac-specific phenotype results from mutations in sarcomere genes? What signaling molecules and pathways are activated by expression of these gene defects? Is the hypertrophic response compensatory, pathologic or in response to depleted energy stores? What are the key genetic and environmental factors which modify the expression of the mutant gene? Answers to these questions are likely to provide important insights into how and why mutated contractile proteins predispose affected individuals to the diversity of clinical features of FHC including sudden death.

To investigate these issues, human, animal and cell culture studies have been performed. While studies in human families have been particularly informative in identifying disease-causing genes, studies in humans are limited due to a variety of factors including variable genetic backgrounds, environmental stimuli which may differ between individuals (e.g. diet, exercise, life-style), small numbers of individuals with the same mutation, and the relative difficulty in obtaining human cardiac samples as well as inadequate methods of maintaining human heart tissue in cell culture systems. For these reasons, a variety of biochemical, cell and animal models have been engineered to more fully dissect the consequences of human sarcomere mutations on muscle structure and function. The development of animal models in FHC have been particularly useful, where there is essentially an unlimited supply of "patients" with the same mutation, where genetic and environmental backgrounds can be controlled, and where access to tissue samples is essentially unlimited.

Animal models of hypertrophic cardiomyopathy

Animal models of FHC have been of greatest value in addressing the issues of molecular pathogenesis, signaling mechanisms, and modifying factors. Specifically, genetically engineered mice and rabbits that express human FHC mutations have been particularly useful. Transgenic models that over-express mutant forms of myosin heavy chains, cardiac troponin T, MyBP-C, or cardiac troponin I as well as a model that physiologically expresses a particular myosin (Arg403Gln) mutation have been studied.[10-16] Most recently, a bigenic model of FHC has been developed, allowing the mutant troponin T-Q92 gene to be turned "on" or "off" using a ligand-inducible system.[16] All these models exhibit histopathology comparable to that observed in human FHC including myocyte disarray, hypertrophy and myocardial fibrosis (Figure 1). The first and most extensively studied mouse model of FHC (Arg403Gln) illustrates how the human disease is replicated in mice. This mouse model was generated by introducing an Arg403Gln mutation into the α-cardiac myosin heavy chain gene by gene targeting and homologous recombination. The mutation is well characterised in humans with FHC, is associated with high penetrance (>90% express the phenotype by age 20 years) and early sudden death. In brief, Arg403Gln mice develop classical histopathological changes of FHC (myocyte hypertrophy, disarray and fibrosis) by age 15 weeks, and echocardiographically detectable left ventricular hypertrophy by 30 weeks (Table 2).[17] Most fundamentally, these animal models of human FHC provide evidence that a mutation in a sarcomeric gene is indeed the primary cause of FHC.

Furthermore, interesting results have arisen by breeding heterozygote FHC mouse lines to homozygosity. In both myosin heavy chain[18] and MyBP-C[19] mouse models, homozygosity leads to a dilated cardiomyopathy (DCM), i.e. dilatation of all 4 heart chambers with reduced

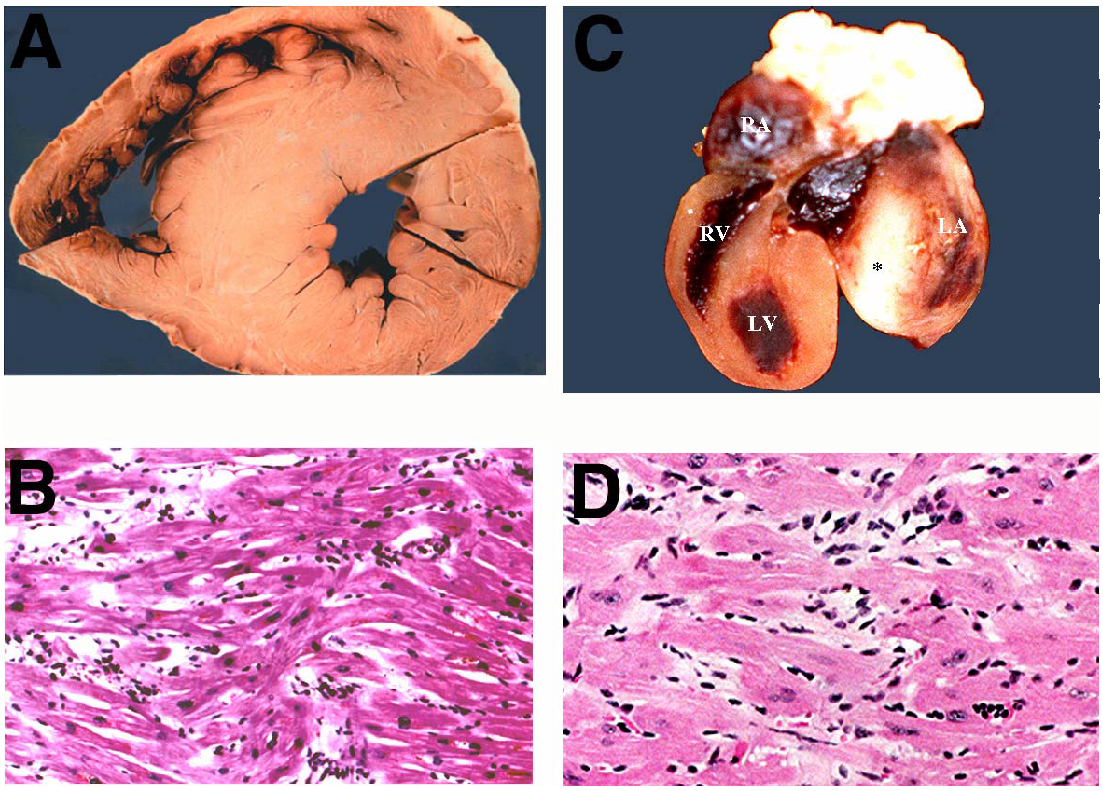

Figure 1: Comparison of mouse and human FHC. *Postmortem* ***human*** *heart sample showing gross hypertrophy and reduction in left ventricular chamber size (Panel A) associated with myocyte hypertrophy, myofibre disarray and interstitial fibrosis (Panel B). Postmortem heart sample from* ***mouse*** *expressing the Arg403Gln mutation in the myosin heavy chain gene, showing myocardial hypertrophy and left atrial enlargement (Panel C; RA=right atrium, LA=left atrium, RV=right ventricle, LV=left ventricle) associated with typical histopathological features of FHC (Panel D).*

Table 2: Comparison of Arg403Gln mutation in mice and humans

FHC Feature	Arg403Gln in Mice	Arg403Gln in Humans
Hypertrophy	Present in 100% by age 30 weeks	in 90% by age 20 years
Histopathology	Myocyte hypertrophy, disarray and fibrosis	Myocyte hypertrophy, disarray and fibrosis
Cardiac Function	Systolic function supernormal Diastolic function impaired early	Systolic function supernormal Diastolic function impaired early
Ventricular Arrhythmias	Common (~60%)	Common (>50%)
Sudden Death	Rare except with vigorous exercise, e.g. swimming	Common (survival at age 45 years is ~50%)
Phenotype Heterogeneity	Yes	Yes
Others	Gender effects observed Exercise capacity decreased	Gender effects observed Exercise limitations unclear

contractile function. In the Arg403Gln homozygote mice, DCM occurs in neonates and all mice die of heart failure by age 7 days.[18] In contrast, homozygous MyBP-C mice develop DCM by age 3 weeks, but subsequently develop compensatory hypertrophy and indeed have a normal lifespan.[19] The ability to breed these mice to both heterozygosity and homozygosity has resulted in clinically relevant models of human FHC and DCM and provides a platform for further studies to both understand pathogenesis, and to potentially identify therapeutic options and targets.

The utility of these animal models have been substantially increased by miniaturisation of many diagnostic procedures that evaluate cardiac function in

humans. For example, the role of vigorous exercise in sudden death and FHC can be evaluated in exercise protocols and provocative electrophysiologic testing in mice. Profound exertion in the Arg403Gln mouse appears to recapitulate sudden death events in some athletes and provocative electrophysiologic testing has demonstrated marked increases in arrhythmogenicity in mutant compared with wild type mice.[10,20] In addition, recent studies in a MyBP-C mouse model of FHC show that while these mice may exhibit very mild disease based on cardiac function studies and histopathology analysis[17], electrophysiological testing suggests that there is a significantly increased vulnerability to ventricular arrhythmias, and therefore sudden death.[21] M-mode and two-dimensional echocardiography in mice has also enabled accurate assessment of left ventricular hypertrophy, changes in cardiac dimensions, and systolic function (fractional shortening).[17-19] More recently application of magnetic resonance imaging (MRI) has enabled detailed assessment of heart structure and even congenital malformations (e.g. atrial and ventricular septal defects) in mice.[22] While there are some differences between humans and mice particularly related to heart rate, leading to differences in Ca^{2+} homeostasis and action potential configuration, murine models of human cardiac disease appear to be valuable reagents for delineating the mechanisms of disease, analyses of complex events such as sudden death and important tools for evaluating pharmacologic therapies and devices.

The role of Ca^{2+} in FHC pathogenesis and sudden cardiac death

Calcium is a key signaling molecule in the cardiac myocyte. The role of Ca^{2+} handling in the development and progression of FHC is currently being investigated. Studies of isolated myocytes and genetically-engineered mice indicate that Ca^{2+} homeostasis is disrupted very early in the pathogenesis of FHC. Specifically, myocytes obtained from Arg403Gln mice which develop FHC, show a significant reduction in sarcoplasmic reticulum Ca^{2+} release in response to caffeine compared to wild-type myocytes (Figure 2A).[23] Furthermore, myofibrillar protein extracts from the hearts of these FHC mice showed reduced levels of expression of the cardiac ryanodine receptor (RyR2) Ca^{2+}-release channel, as well as the sarcoplasmic reticulum Ca^{2+}-storage protein calsequestrin, and the associated anchoring proteins triadin and junctin (Figure 2B). The reduction in RyR2 protein expression was associated with changes in phosphorylation.[23] Interestingly, all these changes were seen very early in life (by 4 weeks of age; Figure 2C), many weeks before the onset of diastolic dysfunction, histopathological changes and cardiac hypertrophy, suggesting an important early cellular event in FHC is dysregulation of the release of Ca^{2+} from the sarcoplasmic reticulum, possibly secondary to Ca^{2+} becoming "trapped" in the mutated sarcomere. The primary defect in FHC is a mutation in a gene encoding a sarcomeric protein. Thus, it is likely that the mutation disrupts normal sarcomeric contraction and relaxation, such that Ca^{2+} release from the sarcomere at the end of systole is impaired, leading to accumulation (or "trapping") of Ca^{2+} within the sarcomere. This could then lead to reduced Ca^{2+} re-uptake into the sarcoplasmic reticulum, which over time may lead to reduced sarcoplasmic reticulum Ca^{2+} stores. Interestingly, administration of the L-type Ca^{2+} channel inhibitor, diltiazem, corrects these Ca^{2+}-related changes (Figure 2B) and prevents hypertrophy in 50% of FHC mice.[23]

The mechanisms by which sarcomere defects increase cardiac interstitial fibrosis have also been probed. Based on pharmacological and molecular studies performed over the last 5 years, it is likely that many factors, including Ca^{2+}, may play a role in the development of cardiac fibrosis, a potential substrate for cardiac arrhythmias and sudden death. L-type Ca^{2+} channel blockade with diltiazem in FHC mice[23], losartan blockade of angiotensin II in FHC mice[24], simvastatin therapy in FHC rabbits[25], and spironolactone in FHC mice[26] have all demonstrated salutary effects by attenuating or preventing profibrotic effects and reducing collagen deposits in different FHC models. Support for these cellular and animal model studies implicating Ca^{2+} as a key molecule in disease pathogenesis, is the recent identification of families with a history of sudden cardiac death in which mutations have been identified in Ca^{2+}-related genes . Two clinically distinct forms of sudden cardiac death in children and young adults have recently been linked to autosomal-dominant mutations in the RyR2 gene.[27,28] These disorders, known as catecholaminergic polymorphic ventricular tachycardia (CPVT) and arrhythmogenic right-ventricular dysplasia (ARVD) share the clinical characteristics of exercise-induced ventricular arrhythmias and sudden cardiac death. Furthermore, autosomal recessive mutations in the sarcoplasmic Ca^{2+}-storage protein calsequestrin have also been described in families with catecholamine-induced ventricular tachycardia.[29,30] Thus, the recent identification of mutations in both the RyR2 and calsequestrin genes in families characterised by sudden cardiac death, coupled with murine and cellular studies showing defects in regulation of the RyR2 channels and diastolic Ca^{2+} "leaks" within cardiac cells suggest Ca^{2+} may play a very important role in development of cardiac arrhythmias leading to sudden death. Indeed Ca^{2+} dysregulation may provide a novel insight into the link between the electrical and mechanical structures in the heart. While it is likely that ARVD and CPVT are disorders clinically distinct from HCM, Ca^{2+} dysregulation may be the underlying primary pathogenic process, while molecular and clinical responses to this dysregulation define the different clinical presentations observed.

Gene Modifiers in FHC

(i) Human studies: the ACE gene

Many gene association studies have been performed in human FHC populations in an attempt to identify secondary genes which may modify the clinical phenotype

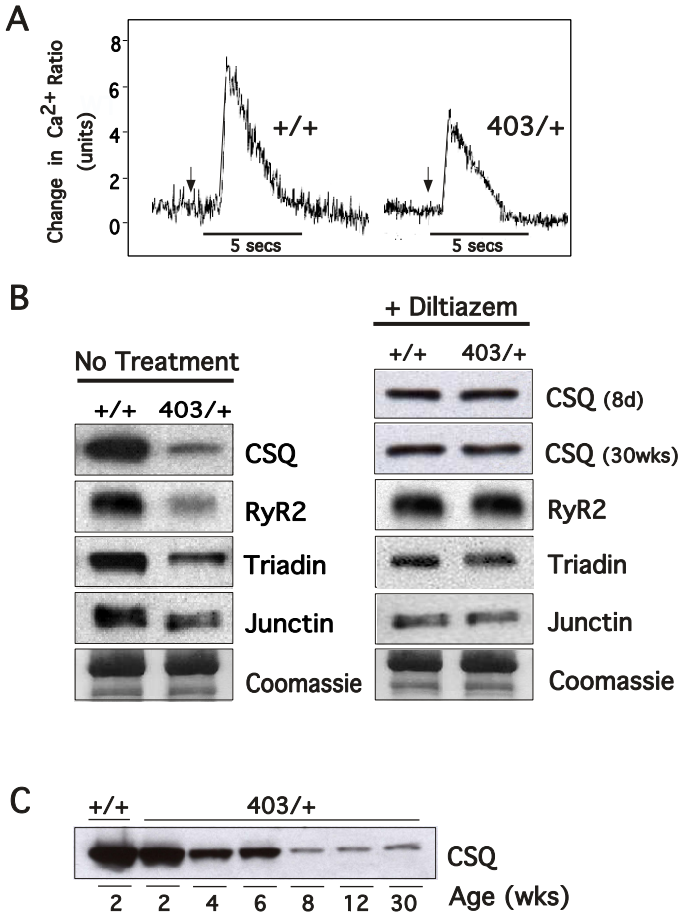

Figure 2: Ca^{2+} changes in mouse FHC. *Sarcoplasmic reticulum Ca^{2+} release and changes in Ca^{2+}-related protein expression.* A, *Ca^{2+} changes, assessed in fura-2 loaded wild-type (+/+) and $\alpha MHC^{403/+}$ (403/+) myocytes, in response to a spritz of 10mM caffeine (vertical arrow indicates time of administration).* B, *Western blot analysis of calsequestrin (CSQ) and components of the quaternary complex in myofibrillar protein extracts from wild-type (+/+) and $\alpha MHC^{403/+}$ (403/+) mice aged 30-50 weeks. Coomassie staining indicates loading of samples in each lane. Normalization of calsequestrin protein levels after 8 days (8d) and 30 weeks (30wk) treatment, and restoration of all 4 components of the calsequestrin-complex in mice treated long-term with diltiazem. C, Time course study of changes in calsequestrin protein expression in equal amounts of myofibrillar extracts from mice aged 2, 4, 6, 8, 12, 30 weeks. (Modified from Semsarian* et al.[23]*)*

through either its own direct effects, or secondary to gene-gene interactions, primarily with the causative gene.[31,32] Perhaps the most widely studied in this setting are genes involved in the renin-angiotensin system. Polymorphisms in a key component of the renin-angiotension system, the angiotensin-1 converting enzyme (ACE) gene, have been studied extensively in cardiovascular diseases such as myocardial infarction, hypertension and dilated cardiomyopathy.[32-36]

The ACE gene, localised to chromosome 17, has a polymorphic region consisting of an insertion (I) or deletion (D) of a 287bp fragment called the I/D polymorphism. The interest in the ACE gene has arisen from its important role in both myocardial growth and blood pressure homeostasis. While no association has been found between the ACE polymorphism and left ventricular mass in some studies[34], other studies have shown significant correlation.[35] In FHC specifically, the D/D genotype has been associated with an increased risk of sudden death. We have most recently shown that the ACE D/D genotype is significantly associated with the rate of progression of left ventricular hypertrophy compared to the I/I and I/D genotypes, independent of other factors such as age, body surface area, and resting blood pressure (Figure 3).[37] The frequency of each polymorphism in the FHC group was similar to that of a control group, suggesting that the D/D polymorphism plays an interactive role with FHC mutant genes, rather than being a marker for hypertrophic growth itself.

The association of the D/D genotype with both sudden death and progression of hypertrophy may relate to the plasma levels of ACE, with 50% of the plasma ACE levels determined by this polymorphism. Higher tissue (cardiac) ACE levels may alter local ACE gene expression and activity. Functionally, a combination of altered ACE homeostasis and presence of the underlying primary sarcomeric gene defect may conceivably lead to abnormal

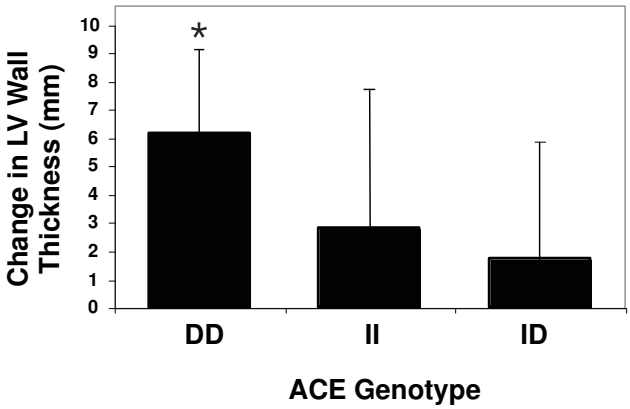

Figure 3: ACE genotype in human FHC. *Progression of left ventricular hypertrophy in patients with the ACE gene D/D, I/I and I/D polymorphism (*p<0.01 vs I/D). (I=insertion, D=deletion).*

patterns of myocardial growth leading to an increased amount of progression of left ventricular hypertrophy in patients with FHC. This may provide the basis for using the ACE D/D polymorphism as one factor in the algorithm for determining the clinical risk profile in patients with hypertrophic cardiomyopathy.

(ii) Mouse studies

The differences in genetic background and environmental influences has limited the impact and utility of studies of genetic modifiers in human FHC populations. In contrast, animal models of FHC have allowed investigators to begin to evaluate the role of modifiers more precisely, primarily due to the unique ability in mice to control both environmental influences, as well as to alter the genetic background by breeding the mutant mice in different mouse strains. Indeed, breeding of the Arg403Gln mice in different genetic backgrounds, both inbred and outbred, has led to the identification of phenotypic differences in terms of hypertrophy, exercise capacity and histopathology, indicating the presence of a gene modifier in FHC (Figure 4).[38] These mice, although carrying the same gene mutation, demonstrate different phenotypic features, e.g. presence of hypertrophy, supporting the notion that a background modifier gene(s) exists which protects mice from developing hypertrophy. Current studies are aimed at identifying the gene locus for this modifier. Definition of such modifying factors by gene mapping strategies, and potentially identifying new signaling pathways triggered by sarcomere protein gene mutations has great promise for defining novel targets for therapeutic interventions in human FHC.

Environmental Influences in FHC

The role of environmental factors in FHC is currently under investigation. There are many diverse influences which may play a role in modifying the phenotypic expression of disease in FHC. Some of the potential environmental influences include the role of exercise, dietary factors, as well as variations in blood pressure and body temperature.

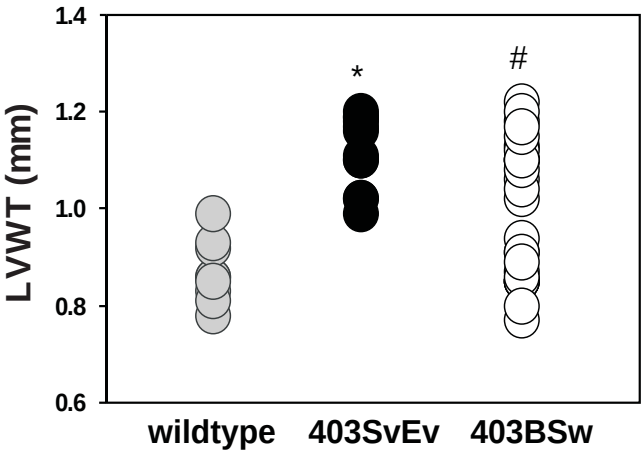

Figure 4: Identification of a gene modifier(s) in mice with FHC. *Left ventricular wall thickness (LVWT) measurements in 3 groups of mice; wild-type mice, and inbred and outbred mutant mice with the Arg403Gln mutation (403SvEv, 403BSw respectively). An LVWT assessed by echocardiography greater than 1.0mm indicates hypertrophy. Approximately 50% of mutant mice in the BSw genetic background are protected from developing hypertrophy (*p<0.01; #p<0.05 vs wild-type mice). (Modified from Semsarian* et al.[38]*)*

The role of exercise as an environmental influence in FHC has been studied in humans for many years. There exists a clear clinical association between high levels of exercise, i.e. sporting activities at a competitive level, and sudden cardiac death in FHC. Indeed, sudden cardiac death in FHC occurs during or immediately after exercise in up to 70% of individuals.[4,5,39] Because of this clinical association, patients with FHC are advised to avoid competitive sports. However understanding the mechanisms of how exercise can trigger sudden cardiac death in FHC, and what the molecular or pathological substrates are, remains unknown. Pathological substrates such as the degree of cardiac fibrosis, myofibre disarray or extent of myocyte hypertrophy have been studied and may be important in determining susceptibility to sudden cardiac death during exercise. Most recently, Ca^{2+} dysregulation and diastolic sarcoplasmic reticulum Ca^{2+} leakiness have been linked to familial syndromes of exercise-induced sudden cardiac death and may play an important role in elucidating the link between exercise and sudden cardiac death in FHC.[27-30,40] Spontaneous Ca^{2+} release from the sarcoplasmic reticulum during diastole might cause a transient inward current, resulting in delayed after-depolarisations (DAD). If this inward current is sufficient to cause a DAD with amplitude greater than the threshold potential, depolarisation will occur, and an arrhythmia can be triggered. Consistent with these findings is the report of DAD-based arrhythmias in

patients with CPVT.[26] Disruption to normal Ca^{2+} handling has also been observed in mice with FHC who have undergone a swimming exercise program (unpublished data, C. Semsarian and L. Nguyen).

Future directions

Major advances have been made in understanding the molecular basis of FHC, particularly over the last decade. The identification of human mutations will allow early and accurate diagnosis, enabling preventative strategies, as well as early therapeutic intervention, to be initiated in an attempt to reduce the serious morbidity and mortality associated with FHC in some patients. Using animal and cell culture models, elucidation of signaling events leading from mutant protein to clinical phenotype, and the identification of factors, either genetic or environmental, which modify the expression of the mutant protein, will provide important insights in our fundamental understanding of the pathogenesis of FHC. It appears dysregulation of Ca^{2+} will be a key focus in evaluating the link between the causative gene mutation, and the clinical outcomes of disease including cardiac hypertrophy, heart failure and sudden death. Further, understanding the molecular aspects of cardiac hypertrophy in FHC may provide new insights into cardiac hypertrophy caused by other more prevalent stimuli, such as hypertension, leading to the identification of novel pharmacological and molecular targets, which could benefit large human populations.

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Author for correspondence:
A/Prof. C. Semsarian,
Agnes Ginges Centre for Molecular Cardiology,
Centenary Institute,
Locked Bag 6,
Newtown NSW 2042
Tel: +61-2-9565 6195
Fax: +61-2-9565 6101
Email: c.semsarian@centenary.usyd.edu.au