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Complexity of Sarcomere Protein Gene Mutations in Restrictive Cardiomyopathy

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Abstract

Restrictive cardiomyopathy (RCM) is characterized by impaired filling of the ventricles in the presence of normal wall thickness and systolic function. Although idiopathic RCM is rare compared to other types of cardiomyopathy, the effects are severe. Until recently, many sarcomere genes previously described to be causative mutations in hypertrophic cardiomyopathy and dilated cardiomyopathy have been reported in RCM. Nowadays, it is accepted that primary RCM is also within the spectrum of sarcomere disease. However, the relationship between the identified mutations in sarcomere genes and clinical manifestation are complex, and the possible pathogenic mechanisms are not fully understood. Besides, many RCM-related sarcomere mutations were reported to cause variable clinical phenotype. Occasionally, “phenotype transition” may also be seen in an individual who was previously diagnosed with RCM.

Keywords: restrictive cardiomyopathy, sarcomere, gene mutation

1. Introduction

Restrictive cardiomyopathy (RCM) is characterized by impaired filling of the ventricles in the presence of normal wall thickness and systolic function. While RCM is rare compared to other primary cardiomyopathies, most affected individuals have severe signs and symptoms of heart failure and majority die shortly after diagnosis unless they receive a cardiac transplant [1]. According to the etiology, RCM has been classified as primary or secondary. Secondary RCM refers to the conditions in association with local inflammation (Loeffler cardiomyopathy, endomyocardial fibrosis, and eosinophilic endomyocardial disease) or infiltrative (amyloidosis and sarcoidosis) or storage disease (hemochromatosis, glycogen storage disease, and Fabry disease, etc.) [2]. Primary RCM includes RCM ascribed to inherited or sporadically acquired mutations or in many cases due to unknown etiology. So far, through mutation

screening of different individuals and families presenting the RCM phenotype, mutations in multiple sarcomere genes have been identified to be linked with RCM, which has greatly expanded our understanding about “idiopathic RCM.” However, the relationship between the identified sarcomeric mutations and clinical manifestation are complex and many puzzles still exist. The mechanism behind the genotype-phenotype correlation is not clearly understood. Most of these RCM-associated sarcomeric mutations, when mutated at specific sites, are also known to induce HCM or DCM. Even people carrying the same mutation of the same sarcomeric gene may exhibit heterogenous manifestations. “Phenotype transition” may even be seen at a late stage of RCM resulting in atypical RCM.

2. Multiple sarcomeric gene mutations in human RCM

The sarcomere contains different protein involved in muscle contraction. Two major components are actin, which constitutes the backbone of the thin filament, and myosin, which makes up the thick filament. The interaction of myosin and actin causing the sliding of the thin filaments along the thick filaments results in muscle contraction and force development. Association and dissociation of myosin and actin are regulated in a Ca^{2+} -dependent manner by the troponin-actin complex. The actin-myosin contractile apparatus, which consists of five thin filament proteins (actin, tropomyosin, and troponin T, I, and C) and three thick filament proteins (myosin heavy chain, essential light chain, and regulatory light chain), plays a key role in regulating the sarcomere function. So far, except for troponin C, mutations in the other thin filament and thick filament proteins have all been identified in RCM. Besides, a series of mutations in other regulatory sarcomere proteins such as myosin-binding protein C, titin, and Z-disc proteins also have been recognized to induce diastolic dysfunction resulting in a restrictive phenotype.

2.1. Cardiac troponin mutation

Cardiac troponin is located at regular intervals along the thin filament and consists of three subunits: cardiac troponin C (cTnC), troponin I (cTnI), and troponin T (cTnT). cTnC acts as a Ca^{2+} sensor, which confers Ca^{2+} sensitivity to muscle contraction [3]. Cardiac TnI is the inhibitory subunit, primarily functioning to prevent actin and myosin from interacting in the absence of Ca^{2+} . The cTnT subunit binds to tropomyosin (Tm) and is responsible for transmitting the Ca^{2+} -binding signal from cTn to Tm [4]. Electrical depolarization of the cell membrane opens the L-type calcium channels and allows Ca^{2+} influx, which incites release of Ca^{2+} from sarcoplasmic reticulum due to opening of the ryanodine receptors. The released Ca^{2+} binds to cardiac troponin C and induces conformational changes in the troponin T-tropomyosin complex, resulting in displacement of cTnI from actin and subsequent association of actin with myosin. Currently, nine mutations in genes encoding cTnI and three dominant mutations in genes encoding cTnT have been reported in human RCM and no mutations have been identified in cTnC yet.

2.1.1. Troponin I mutation

The gene of cardiac TnI (*TNNI3*) is situated on the 19th chromosome (19q13.4) and consists of eight exons and seven introns. The mature molecule of cTnI is 209 a.a long and consists of five

domains: (1) N-terminal domains, (2) IT-arm, (3) inhibitory domain, (4) regulatory domain, and (5) C-terminal mobile domains [5]. Until now, RCM-related cTnI mutations are found located in the inhibitory domain and the C-terminal domain.

2.1.1.1. Missense mutations located at the inhibitory domain

The inhibitory domain of hcTnI spans residues 137–148 [6, 7], some also reported a different border of this region (residues 129–148) [6, 8]. In the absence of Ca^{2+} , residues 138–148 of the inhibitory domain interact with actin [9] and shift the tropomyosin molecule, impeding the antomyosin-complex formation [10]. Two of the RCM mutations are localized within this important region. The first one is 797T→A nucleotide substitution of exon 7, which led to a Leu144Gln (L144Q) amino acid substitution [11]. The second mutation is 799C→T nucleotide substitution of exon 7 that leads to an Arg145Trp (R145W) amino acid substitution [11].

Data from *in vitro* experiment showed that both L144Q and R145W alter myofilament sensitivity to Ca^{2+} . Skinned cardiac fiber experiment, which measures the Ca^{2+} -buffering capacity of the myofilament while measuring the development of tension and maximal force, revealed that these two mutations resulted in increase of Ca^{2+} sensitivity of force development in skinned fibers from transgenic mice. In addition, a significant increase in the basal force was shown compared to WT cardiac fiber [12]. Measurement of myofilament ATPase activity revealed that L144Q and R145W mutant showed an increase in the basal ATPase at low Ca^{2+} concentrations. Besides, fibers from these two mutants exhibited markedly increased Ca^{2+} sensitivity of ATPase activity [13].

2.1.1.2. Missense mutations located at the mobile C-terminal domain

The mobile C-terminal domain of hcTnI is further divided into the H4 α -helix (residues 164–188) and the C-terminal part (residues 199–210) [5]. 865G→A nucleotide substitution of exon 7 that led to an Ala171Thr amino acid substitution and an 886A→G nucleotide substitution of exon 7, which resulted in a Lys178Glu (K178E) amino acid substitution both occurred within the H4 α -helix region and have been identified in RCM patients. Since these two mutations are known to be located within the actin-binding sites (residues 173–181) [14, 15], K178E and Ala171Thr mutations may influence the inhibitory function through actin binding.

Although the structure of the C-terminal part of hcTnI which consists of residues 190–210 is not fully understood, it is critical for a full inhibitory activity and Ca^{2+} sensitivity of force development because it binds to actin and helps to maintain the thin filament in a blocked state [16]. Mutations occurred at the C-terminal domain may destabilize or decrease its interactions with actin in the absence of Ca^{2+} , consequently relieving cTnI inhibition [17]. Up to now, RCM-related mutant Asp190His (D190H), Arg192His (R192H), and Arg204His (R204H) have been localized within the conserved C-terminal region of the protein [11, 18]. *In vitro* experiments revealed that two of these mutants D190H and R192H markedly increased the filament sensitivity to Ca^{2+} , while another mutation R204H has been reported to result in a disruption of the normal interaction between cTnI-cTnC and cTnI-cTnT. It remains to be further determined what conformational changes happened in these mutant that lead to disrupted interaction between cTnI-actin and cTnI-cTnC.

2.1.1.3. Two deletion mutations

Deletion of nucleotides usually causes frame shift and the introduction of a premature stop codon. Two deletion mutations of *TNNI3* which impaired relaxation of myocardium and resulted in a restrictive filling pattern were reported to be located in exon 7 and cause truncation of C-terminal portion of cTnI. The truncated cTnI which lost its C-terminal portion is susceptible to degradation and has reduced inhibitory capacity on the thin filament since C-terminal contains the second binding domain for actin and cTnC [19, 20].

2.1.2. Troponin T mutation

cTnT anchors the troponin complex to Tm and plays a critical role in modulating ATPase activation when Ca^{2+} concentrations achieve threshold levels. Until now, three different mutations in the cTnT gene (*TNNT2*) linked to RCM have been identified. However, the molecular pathogenesis of these cTnT mutations is not clear [20–22].

3. α -Cardiac actin (*ACTC*)

Actin is a major constituent of the thin filament and spans the length of thin filament. Together with myosin, actin generates force and transmits this force from the sarcomere to the surrounding syncytium via the thin filament [23]. RCM mutations in the *ACTC* gene are extremely rare and only one *ACTC* mutation has been reported in RCM (nucleotide substitution in exon 5 g. 4642G→C which lead to an Asp313His amino acid substitution). However, the significance of this variant is uncertain.

4. α -Tropomyosin (*TPM1*)

In cardiac muscles, tropomyosin together with troponin forms the principal mechanism by which contractility is regulated in response to the Ca^{2+} concentration. In the absence of Ca^{2+} , tropomyosin prevents productive myosin head binding. In systole, tropomyosin moves in response to Ca^{2+} binding to allow partial myosin attachment, which resulted in further shift of tropomyosin to expose fully the interaction site on actin [24]. Recently, a novel missense mutation in α -tropomyosin, c.835A>C p.Asn279His has been identified in a patient with primary RCM [25]. However, the significance of this variant is not clear since family evaluation did not show cosegregation.

5. β -Myosin heavy chain (*MYH7*)

Myosin is a dimeric protein consisting of two heavy chains and two associated pairs of light chains. The ~23-kb long human *MYH7* gene, located on chromosome 14, contains 40 exons that direct the synthesis of the 1935 amino acid β -myosin heavy chain [26]. Although over

500 disease-causing point mutations have been found in human *MYH7* gene, most of them are reported in HCM, only four of these variants are identified in patients with primary RCM [27–32]. The understanding about the genotype-phenotype correlation between the reported *MYH7* mutation and RCM are very limited. *MYH7* contains different functional domains, including the globular head domain (S1), the neck or hinge region (S2), and the tail (light meromyosin) [33, 34]. Although many mutations have been identified to cluster in functional hotspots due to the development of high throughput sequencing, all the above mentioned RCM-related *MYH7* mutations located at regions where no definite function has been assigned to so far [28]. Besides, whether haploinsufficiency may contribute to the consequence of the variants is not clear.

6. Ventricular myosin essential light chain (*MYL3*) and ventricular myosin regulatory light chain (*MYL2*)

Myosin assembles into hexamers comprising two heavy chains and two pairs of each light chain isoform. The light chain forms a stabilizing collar around the α -helical neck of the heavy chain, a region of the myosin multimer thought to function as the lever arm. Mutation in the gene encoding the essential light chain of myosin (*MYL3* Met149Val) and the regulatory light chain of myosin (*MYL2* Glu22Lys) was previously reported to correlate with marked diastolic dysfunction and restrictive physiology both in human and transgenic mice [35]. Interestingly, carriers with simultaneous mutation in both *MYL3* and *MYL2* showed different phenotypes. While patients with heterozygous mutation in *MYL2* (p.Gly57Glu) and homozygous mutation in *MYL3* (p.Glu143Lys) had severe, early onset RCM, double heterozygote for these variants have no evidence of cardiomyopathy [25, 36]. It is speculated that Glu143Lys substitution may be responsible for this heterogeneity through a loss of function mechanism. This speculation is based on (1) carriers with one mutant allele were clinically silent [36], while homozygotes for the mutation have severe, early-onset cardiomyopathy [25]. If the cardiomyopathy was caused by a dominant-negative mechanism, an intermediate phenotype in heterozygotes would be expected. (2) The substitution occurs in a surface-exposed loop of the essential light chain [37], which makes it less likely to disrupt protein conformation or stability. The site-directed mutagenesis of the corresponding loop domain was confirmed to have no effect on binding between light and heavy chains [38]. Further study using transgenic animal models may help to answer this question.

7. Myosin-binding protein C (*MYBPC3*)

Cardiac myosin-binding protein C is a modular polypeptide located at the C-zone in the striated muscles and binds myosin heavy chain in thick filament and titin in elastic filaments [39]. More than 150 mutations identified in *MYBPC3* have been reported, which is the most common genetic cause of HCM [40, 41]. Recently, variants of *MYBPC3* have also been reported to be RCM-causing mutation [42, 43].

8. Titin (*TTN*)

The giant protein titin acts as the third filament system of the sarcomere, in addition to the actin and myosin filament. It physically connects myosin fibers to actin polymers and is attached to the Z-line. Titin consists of four structurally and functionally distinct regions. (1) The N-terminal titin binds to various Z-disk proteins and acts as an anchor, which is composed of Z-repeats and multiple immunoglobulin (Ig) domains. (2) Elastic I-band region contains the important PEVK portion (proline, glutamate, valine, and lysine) and acts as the molecular spring. (3) Stabilizing A-band region binds to the thick muscle filaments and contains Ig-like, fibronectin type III (fibronectin3) domain. (4) M-band region contains the unique serine-threonine kinase domain modulating titin expression and turnover, with C-terminus of titin embedding in the M-line [44–46]. When the sarcomere is stretched during diastole, the I-band segments gradually lengthen and develop passive tension and therefore, titin is a major determinant of the stiffness of myocardium [47].

Currently, two mechanisms are known to modulate titin's passive stiffness. (1) Titin-based passive tension is critically defined by the ratio of the two major adult cardiac isoforms (N2BA and N2B). As a general rule, longer titin isoforms with longer PEVK repeats in the I-band have more elasticity, whereas shorter isoforms provide more passive stiffness [48]. In adult cardiac muscle, two major isoforms are present: the long compliant N2BA and the shorter stiff N2B. In healthy adult, the ratio of N2BA to N2B in human left ventricle is 40:60 [49]. Moreover, the right ventricle expresses more N2BA than does the left ventricle [50]. In conditions with concentric remodeling such as hypertension or aortic stenosis with diastolic dysfunction, a decreased N2BA:N2B ratio was shown [51, 52]. (2) Another more short-term mechanism modulating titin's passive stiffness is caused by post-translational modification influencing phosphorylation states. Titin could be phosphorylated at the PEVK domain and the cardiac-specific N2B domain, and phosphorylated titin exhibited decreased titin-based passive tension [53].

Titin, known to be the major disease-causing gene for DCM, is encoded by a single gene *TTN* on chromosome 2q31 [54]. Interestingly, recent clinical and genetic studies have established the role of titin defects in the pathophysiology of diastolic dysfunction and RCM. A *de novo* missense mutation of Titin c.22862A > G replacing adenine by guanine at position 109 of exon 226, resulting in the substitution of an evolutionally conserved tyrosine by cysteine (p.Y7621C) has been reported to result in early-onset family RCM with severe heart failure [55]. Structural analysis revealed that p.Y7621C mutation is likely to disrupt the hydrophobic core within fibronectin3 domain, which locates in the A/I junction region [55]. However, how this *TTN* mutation might affect cardiac function and lead to the consecutive development of RCM is unknown.

9. Myopalladin (*MYPN*)

Myopalladin (*MYPN*) is an Ig-domain family member protein that has been reported to be a key intermediate molecule at the Z-disc involved in sarcomere/Z-disc assembly and regulation of gene expression in cardiac cells [56]. Through genetic screening for *MYPN* mutations in

large cohorts of patients with cardiomyopathy, 15 *MYPN* variants were identified, of which a nonsense mutation (p.Q529X) was identified in an RCM family with variable penetrance [57]. Q529X was found to disturb different functional domains of MYNP. MYNP contains five Ig domains (two N-terminal and three C-terminal). Central and C-terminal domains of MYPN bind at the Z-disc to α -actinin and nebulin (NEBL), respectively. This actinin-MYPN-NEBL complex tethers actin and titin to the Z-disc and may play roles in the signaling and regulation of gene expression in response to muscle stress. The N-terminal domain of MYNP binds cardiac ankyrin repeat protein (CARP), which is involved in the control of muscle gene expression [58]. Q529X mutation truncates the C-terminus of MYNP, including the NEBL- and α -actinin-binding domains. Comparative immunohistochemistry of human heart tissue was performed on specimens from the siblings with RCM and from normal control subjects without *MYPN* mutation as well as in neonatal rat cardiomyocytes (NRCs) expressing green fluorescent protein (GFP) chimeras of WT- and Q529X-MYPN. Disrupted sarcomeric Z-discs with abnormally diffuse MYPN codistributed focally with abnormal sarcomeric α -actin, localization were seen in the specimen from siblings with RCM and NRCs expressing Q529X-MYPN [57]. Therefore, losing the NEBL and α -actinin-binding domain results in severe disturbance of sarcomere/Z-disc assembly, which may have impact on early myofibrillogenesis and resulted in RCM. Besides, since MYNP localizes both at sarcoplasm and nucleus, mutant MYPN-Q529X protein in the nucleus was reported to result in downregulation of CARP expression and upregulation of MLP and desmin, augmenting fibrotic restrictive remodeling [59]. In addition, truncated proteins are usually unstable due to decay of mRNA and/or protein degradation through the lysosome or ubiquitin-proteasome system. Although *MYPN* mRNA was not affected by the Q529X-MYPN mutation in the myocardium, mutant MYNP was relatively unstable compared with WT protein [57]. Therefore, insufficient quantities of the protein (haploinsufficiency) may be partly involved in dysfunction of the protein.

10. Mutations cause variable phenotypes

The molecular mechanisms by which gene mutation cause cardiomyopathy can usually be explained by two alternative ways. One is mutation may cause structural and functional change in the protein, which should be analyzed in four different levels: (1) change in the deoxyribonucleic acid sequence, (2) the actual amino acid change, (3) the changes in Ca^{2+} sensitivity of force development and ATPase activity, and (4) the change in protein-protein interaction. The other mechanism may involve insufficient quantities of the protein due to instability of the mutant protein leading to haploinsufficiency [23].

However, it is now evident that different mutations in one gene could cause multiple phenotypes. It is also intriguing that even for a given single mutation and even within a single family disparate phenotypes can be seen. For example, the cTnT Ile79Asn mutation has been shown to be associated with HCM, DCM, and RCM within a single pedigree [60]. This implies that factors just beyond the pathogenic sarcomere mutation influence the phenotype. Therefore, the model of sarcomeric cardiomyopathy as monogenic disease following simple Mendelian pattern of inheritance is an oversimplification. Theoretically, a discrete number of

reasons can account for phenotypic diversity. (1) First of all, patients may carry more than one disease-associated mutations, which is underestimated in most cases. Recent genotyping suggests that multiple cardiomyopathy patients have more than one mutation in the same gene or mutations in different genes. For example, in a study carried out in 292 HCM patients, 13 were found to carry at least 2 mutations [61, 62]. However, the number of patients carrying at least two mutations is likely to be significantly underestimated since many genetic studies typically investigate less than 15 genes for mutations associated with cardiomyopathy [63]. (2) Secondly, different expression and the incorporation rate of the mutant sarcomeric proteins in the heart may also contribute to the heterogeneity of the disease. A few examples in HCM have been published. Heterozygous individuals carrying the β MHC Lys207Gln mutation developed an HCM phenotype, but a homozygous individual developed a DCM-like phenotype [61]. In another case, homozygous carriers of cTnT Ser179Phe mutation exhibited profound left and right ventricular hypertrophy [64] while heterozygous carriers had little or no hypertrophy [65]. (3) Third, mutant proteins are usually unstable due to decay of mRNA and/or degradation through the lysosome or ubiquitin-proteasome system, especially in the case of nonsense mutation [66]. Because of genetic polymorphisms, individuals with RCM are likely to have substantial difference in their genome sequence including disease-modifier genes which are involved in post-translational or translational regulation, resulting in different amounts of mutant protein. This mechanism may interfere with the development of the phenotype and explain different penetrance of a specific mutation. (4) Lastly, sarcomere gene mutation induces serials of maladaptive features, ranging from the prolongation of cardiomyocyte action potential to microvascular dysfunction, from intracellular calcium abnormality to dysregulation of collagen turn-over, and from energetic derangement to abnormal sympathetic activation [67]. The extent and the rate at which each of these features occur and evolve are quite variable within individual patients. Therefore, the clinical heterogeneity of a specific sarcomere gene mutation may partly be ascribed to different stages of disease progression [68]. Although epigenetics and environmental factors are likely to be relevant in sarcomeric cardiomyopathies, there have been no studies yet describing how epigenetic modification and environmental factors may affect phenotype in sarcomeric RCM.

11. Phenotype transition

Occasionally, with the progression of disease, “phenotype transition” may be seen in a given individual who was initially diagnosed with a specific type of cardiomyopathy. This situation could often be seen in patients who was initially diagnosed with HCM and gradually developed into end-stage of the disease. The morpho-functional manifestation in this advanced stage usually exhibits two extremes: hypokinetic-dilated form or hypokinetic-restrictive form which could be hard to distinguish from primary DCM and RCM [68]. The “HCM to RCM” transition has been discussed above. There is another situation that patients initially diagnosed with primary RCM could undergo persistent cardiac remodeling resulting atypical phenotype at a later stage of the disease. Although the main impairment of RCM is diastolic dysfunction, deterioration of systolic function has been observed in some patients. In a retrospective study, it was reported that 16% of 94 primary RCM patients was observed to have

systolic dysfunction [69]. Another study carried out in pediatric RCM reveals that although all the 18 children had preserved ventricular systolic function at diagnosis, 6 of them later presented a deteriorated ventricular systolic function and eventually required inotropic support [70]. In the RCM model using transgenic mice noticeable impaired systolic was observed over a time of 10 months. By the time, those RCM mice presented with signs of severely aggravated congestive heart failure and some of them died [71]. This disease progression is consistent with some of the clinical observation, suggesting that systolic dysfunction may be responsible for the end-stage lethal heart failure. As for the mechanism, some proposed that ischemia may be a bridge between the progression of diastolic dysfunction and the development of systolic function, based on evidence of ischemia observed in hearts for autopsy from RCM patients and transgenic RCM mice [71–73]. Besides, it is presumed that reduced capillary density due to interstitial fibrosis and increased extravascular compressive force in the restrictive heart may also induce ischemia [74]. Of note, although an RCM patient may appear to have systolic dysfunction and low cardiac output, enlargement of ventricle is seldom seen, probably because the molecular mechanism and sarcomeric mechanics of RCM and DCM are opposite [75].

12. Summary

In the past decades, clinical and genetic studies suggest that primary RCM is part of a spectrum of sarcomeric disease. Since RCM is associated with severe prognosis, ongoing and future basic science will continue to dissect the precise pathways driving how these mutations remodel the heart, and identify rational therapies targeting pathophysiological aspects to interrupt the emergency of pathology. On the other hand, it is also important to realize that genes contain information that is essential for the development of the phenotype but not necessarily complete. The final phenotype is determined not only by the causal mutation but also by the modifier genes, each exerting a modest effect, epigenetic factors, which link the gene to the phenotype, and the environmental factors, as in the case of complex phenotypes observed in single-gene disorders with Mendelian pattern of inheritance. Thus, while advances in molecular genetics of cardiovascular diseases are gradually changing our classical understanding of the disease and the phenotype-based approach to the practice of medicine, currently they are unlikely to be sufficient to trigger a full switch from a phenotype-based to genotyped-based medicines. A comprehensive understanding of molecular mechanism of the pathogenesis integrating the genetic, epigenetic, transcriptomic, and proteomic profiles is necessary.

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