

Malalignment of the sarcomeric filaments in hypertrophic cardiomyopathy with cardiac myosin heavy chain gene mutation

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Abstract

Objective—To investigate changes in the alignment of the sarcomeric filaments in hypertrophic cardiomyopathy and the effects of cardiac β myosin heavy chain (β -MHC) mutation on the sarcomeric ultrastructure.

Design—A retrospective analysis.

Patients—Endomyocardial biopsy samples were examined by transmission electron microscopy in seven patients with hypertrophic cardiomyopathy and β -MHC mutation, six with hypertrophic cardiomyopathy but without the mutation, and five controls (with chest pain syndromes). **Main outcome measure**—Alignment of the sarcomeric filaments and the distance between neighbouring thick myosin filaments.

Results—In controls, cross sections of the sarcomere at the A band showed a highly organised orthohexagonal array with 6 thin actin filaments surrounding one thick myosin filament, whereas in hypertrophic cardiomyopathy the alignment of the sarcomeric filaments was sparse and disrupted. In hypertrophic cardiomyopathy with a mutation, the distance between neighbouring thick myosin filaments was greater than in controls (mean (SD) 45.3 (4.7) v 38.5 (3.5) nm, $p < 0.05$), and the variance of the distance was greater than in controls (8.0 (0.7) v 4.8 (1.0) nm, $p < 0.001$) or in patients with hypertrophic cardiomyopathy without a mutation (6.7 (0.6) nm, $p < 0.05$). In the latter, the variance of the distance was also greater than in the controls ($p < 0.01$). A significant correlation was found between the grade of the myocyte hypertrophy and the variance of the distance ($r = 0.654$; $p < 0.01$).

Conclusions—The alignment of the sarcomeric filaments is disrupted in hypertrophic cardiomyopathy, particularly when there is β -MHC mutation.

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Keywords: hypertrophic cardiomyopathy; β myosin heavy chain; myosin filament; sarcomere

Hypertrophic cardiomyopathy appears to be genetically transmitted in half the patients as an autosomal dominant trait.^{1,2} We and other investigators have identified various mutations of the cardiac β myosin heavy chain (β -MHC) gene, the gene responsible for the disease.^{3–7} In

addition, it has been shown that gene mutations of other components of the cardiac sarcomere, including cardiac troponin T and α tropomyosin, could also be responsible for hypertrophic cardiomyopathy.⁸ Furthermore, it has recently been found that the mutant β -MHC protein has impaired contractility characterised by reduced contraction velocity and impaired interaction with actin filaments.^{9,10} These findings raise the possibility that hypertrophic cardiomyopathy is a disease of the sarcomere.¹¹

The sarcomere is the repeating, morphological and functional unit of the myofibrils of cardiomyocytes, which produces a regular band pattern of dark and light areas on electron microscopy. The dark and light zones of the sarcomere are produced by the periodic, interdigitating relations between the thin actin and thick myosin filaments. The cross section of the sarcomere at the level of the A band, where the thin actin and thick myosin filaments overlap, shows a hexagonal array with six thin filaments surrounding one thick filament. It is proposed that the integrated alignment of the sarcomeric filaments is critical for actin–myosin interaction and the subsequent contraction of the sarcomere.¹² It is plausible that the ultrastructure of the sarcomere is affected in patients with hypertrophic cardiomyopathy, especially when there is a mutation of the sarcomeric proteins. However, little information has hitherto been available on this.¹³

Our aim in this study was therefore to investigate changes in the alignment of the sarcomeric filaments in patients with hypertrophic cardiomyopathy, especially in the presence of a β -MHC mutation. To do this, we assessed the alignment of the sarcomeric filaments in the A band of the sarcomere by transmission electron microscopy in endomyocardial biopsy samples obtained from patients with hypertrophic cardiomyopathy with or without a β -MHC mutation.

Methods

PATIENTS

We enrolled 13 patients with hypertrophic cardiomyopathy and five with chest pain syndromes as controls (table 1). The diagnosis of hypertrophic cardiomyopathy was based on the echocardiographic demonstration of a non-dilated, hypertrophic left ventricle in the absence of other cardiac or systemic disease that could produce left ventricular hypertrophy.¹⁴ In all subjects, cardiac catheterisation was performed, including a left

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Table 1 Patient profile and histological findings

Case	Age (years)	Sex	β -MHC mutation site	Echocardiography			Histological study		
				IVST	LVDd	LVEF	Cell size	Disarray	Fibrosis
<i>Mutation positive HCM</i>									
1	57	F	⁵⁶ Val → Ile	17	41	0.77	1	1	2
2†	46	M	⁷³⁶ Ile → Met	10	49	0.79	1	0	2
3	63	M	⁷⁷⁸ Asp → Gly	22	43	0.69	2	2	2
4	18	M	⁷⁷⁸ Asp → Gly	13	49	0.81	2	1	2
5	16	M	⁸⁷⁰ Arg → His	20	38	0.83	1	1	2
6	36	F	⁸⁷⁰ Arg → His	19	31	0.80	1	1	1
7	27	M	⁹³⁵ Glu → Lys	26	50	0.53	1	2	2
Mean (SD)	38 (19)			18 (5)*	43 (7)	0.75 (0.11)	1.3 (0.5)*	1.1 (0.7)*	1.9 (0.4)*
<i>Mutation negative HCM</i>									
8	19	F	–	14	26	0.98	2	1	2
9	15	M	–	15	45	0.64	1	2	3
10	49	F	–	20	47	0.80	1	2	2
11	24	F	–	13	50	0.58	1	2	1
12	31	M	–	30	36	0.77	1	1	2
13	36	F	–	12	40	0.83	0	2	1
Mean (SD)	29 (12)			17 (7)*	41 (9)	0.77 (0.14)	1.0 (0.5)*	1.7 (0.5)*	1.8 (0.8)*
<i>Controls</i>									
14	54	F	–	7	46	0.82	0	0	0
15‡	49	F	–	8	42	0.81	0	1	0
16	31	F	–	8	46	0.78	0	0	0
17	62	F	–	8	52	0.68	0	0	1
18	28	M	–	11	48	0.78	0	0	0
Mean (SD)	45 (15)			8 (2)	47 (4)	0.77 (0.06)	0.0 (0.0)	0.2 (0.5)	0.2 (0.5)

Myocyte hypertrophy was graded as 0 for average cell diameter < 15 μ m; 1+ for 16–20 μ m; 2+ for 21–25 μ m; and 3+ for 26 μ m or more. The area of fibrosis was graded as 0 for 0–5%; 1+ for 6–15%; 2+ for 16–20%; and 3+ for \geq 21% fibrosis. The myocardial disarray was graded as follows: a parallel arrangement of the myocardial fibres was graded as 0; disarray was graded as 1 to 3 according to the extent of cellular branching and disarrangement.

* $p < 0.05$ v controls.

†These patients showed significant hypertrophy of the lateral, inferior, and apical wall although measurements of their septal thickness were not necessarily diagnostic for HCM.

‡This patient had frequent ventricular ectopic beats accompanied by no apparent findings suggesting organic heart disease, except for mild myocardial disarray.

β -MHC, β myosin heavy chain; HCM, hypertrophic cardiomyopathy; IVST, interventricular septal thickness (mm); LVDd, left ventricular diastolic dimension (mm); LVEF, left ventricular ejection fraction.

ventriculogram and selective coronary angiograms. Patients with valvar heart disease, coronary artery disease with significant atherosclerotic lesions, or apical hypertrophy were not included in the study (in patients with hypertrophic cardiomyopathy and significant left ventricular outflow tract obstruction, we considered that the pressure overload caused by the outflow obstruction would modify the histological and ultrastructural features specific to hypertrophic cardiomyopathy). Left ventricular outflow tract obstruction was assessed at cardiac catheterisation, and patients with a significant pressure gradient across the left ventricular outflow tract (mean pressure gradient > 30 mm Hg), either at rest or after provocative tests such as post-extrasystolic potentiation or an isoprenaline challenge test, were excluded. Controls had neither organic heart disease nor cardiac hypertrophy assessed by echocardiographic and histological studies. No subjects had a history of hypertension. The research protocol was approved by the institutional review committee for clinical research, and written informed consent was given by each subject.

MUTATION SCREENING OF THE CARDIAC β -MHC GENE

Polymerase chain reaction-DNA conformation polymorphism (PCR-DCP) analysis followed by direct DNA sequencing was performed using peripheral leucocyte genomic DNA for the mutation screening of cardiac β -MHC and troponin T. Preparation of genomic DNA and the procedures of the PCR-DCP analysis were

performed as described previously.⁴ PCR products containing unusual DNA fragments were subjected to sequencing analysis to identify the sequence variances.⁷

ENDOMYOCARDIAL BIOPSY AND ELECTRON MICROSCOPIC ANALYSIS

Several endomyocardial biopsy samples were obtained from the right side of the interventricular septum using a standard transfemoral approach. Biopsy samples were fixed in 2% osmium tetroxide and then embedded in Epon 812. Thin sections of the samples (3 μ m) were subjected to transmission electron microscopy (Phillips 201, Phillips, Eindhoven, Netherlands). Photographs of the cross section of the sarcomeres were taken at 20 000 power magnification at the level of the A band where the thin actin and thick myosin filaments overlap, and were finally enlarged to 75 000 power magnification for printing. The photographs were scanned with an image scanner to analyse the alignment of the thick myosin filaments using "NIH image" software on a Macintosh computer. The area of interest selected was where the cross sectional image at the A band showed a hexagonal pattern, and eight areas were investigated. The distance between neighbouring thick myosin filaments was measured for at least 100 thick filaments, and the average and the standard deviation of the measures were computed in each subject. The standard deviation of each subject was regarded as a parameter of the variance of the thick filament distance.

HISTOLOGICAL ANALYSIS

Biopsy samples were fixed in 10% formalin, dehydrated with ethanol, embedded in paraffin, and sectioned with a thickness of 4 μm . Cardiomyocyte hypertrophy, myocardial disarray, and interstitial fibrosis were semiquantitatively evaluated using previously described criteria.^{15,16} Briefly, a transverse diameter of at least 30 myocytes was measured in haematoxylin-eosin stained sections, and myocyte hypertrophy was graded as 0 for an average cellular diameter of < 15 μm , 1+ for 16–20 μm , 2+ for 21–25 μm , and 3+ for 26 μm or more. The per cent area of fibrosis was calculated in Mallory–Azan stained sections, and interstitial fibrosis was graded as 0 for 0–5%, 1+ for 6–15%, 2+ for 16–20%, and 3+ for \geq 21% fibrosis. The myocardial disarray was graded for phosphotungstic acid stained sections as follows: a parallel arrangement of the myocardial fibres was graded as 0, and disarray was graded as 1 to 3, according to the extent of cellular branching and disarrangement. Concordance of each criterion was more than 0.95 between the two observers.

STATISTICAL ANALYSIS

Data are expressed as mean (SD). Unpaired Student's *t* tests, analysis of variance followed by Scheffe's *F* test, and the Kruskal–Wallis test followed by the Mann–Whitney *U* test with Bonferroni modification were used for statistical comparison of the thick myosin filament distance and the clinical and histological variables. Analysis of covariance was performed for statistical analysis of the variance of thick filament distance, to eliminate the effects of the latter as a covariate. Correlation between the grade of cardiomyocyte hypertrophy and the thick filament distance or the variance of the thick filament distance was analysed using Spearman's rank correlation test. A *p* value < 0.05 was considered statistically significant.

Results

β -MHC GENE MUTATION AND CLINICAL FEATURES
PCR-DCP analysis of the genomic DNA showed that seven patients with hypertrophic cardiomyopathy had a point mutation of the β -MHC gene and six did not (table 1). The site of mutation was variable. A troponin T mutation was not detected. No gene mutation was found in the five controls. Age and echocardiographic findings relating to left ventricular dimension and ejection fraction were similar among the three groups. The patients with hypertrophic cardiomyopathy showed significant thickening of the interventricular septum and characteristic histological findings such as cellular hypertrophy, myocardial disarray, and interstitial fibrosis; these variables did not differ between the mutation positive and mutation negative cases.

ALIGNMENT OF SARCOMERIC FILAMENTS IN HYPERTROPHIC CARDIOMYOPATHY

Figure 1 shows representative transmission electron microphotographs of the cross section of the sarcomere at the level of the A band. In the control, six thin actin filaments surround-

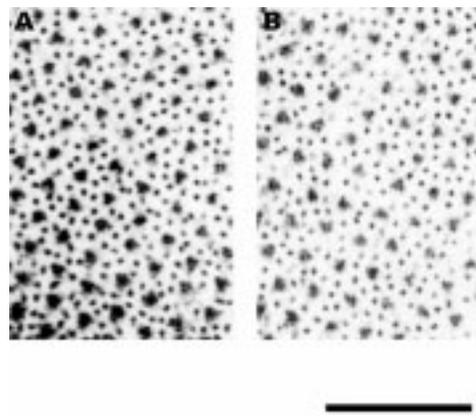


Figure 1 Representative electron microphotographs of cross section of the sarcomere at the A band ($\times 75\,000$) in a control patient (A) and a patient with hypertrophic cardiomyopathy (B). The bar represents 100 nm.

ing one thick myosin filament form an organised orthohexagonal array, which gives a crystal-like structure (fig 1A). In contrast, the patient with hypertrophic cardiomyopathy and a β -MHC mutation shows sparse and disrupted alignment of the sarcomeric filaments (fig 1B).

To assess the malalignment of the sarcomeric ultrastructure quantitatively, the distance between neighbouring thick myosin filaments was evaluated (fig 2A). The thick myosin filament distance was significantly greater in patients with hypertrophic cardiomyopathy than in the controls: mean (SD) 43.9 (4.5) *v* 38.5 (3.5) nm (*p* < 0.05). The variance of the thick filament distance was also greater in the cardiomyopathy patients than in the controls: 7.4 (0.9) *v* 4.8 (1.0) nm (*p* < 0.001). The variance of the thick filament distance was considered to reflect the malalignment of the sarcomeric filaments. The effects of the β -MHC mutation on the ultrastructural changes were then evaluated. The thick myosin filament distance was greater in the index patients with a β -MHC mutation than in the controls (*p* < 0.05), whereas there was no significant difference between the index patients with and without the mutation, or between the mutation negative index patients and the controls (fig 2B). The variance of the distance was significantly greater in index cases with a β -MHC mutation than in either the controls (*p* < 0.001) or the index cases without the mutation (*p* < 0.05; fig 2C). The mutation negative index cases also showed a larger variance of the thick filament distance than the controls (*p* < 0.01). As shown in fig 3A, there was a trend towards a correlation between the grade of cardiomyocyte hypertrophy and thick myosin filament distance, but this was not statistically significant (*r* = 0.457; *p* = 0.091). In contrast, a significant correlation was found between the grade of myocyte hypertrophy and the variance of thick filament distance (fig 3B, *r* = 0.654; *p* < 0.01). Neither the distance between the thick myosin filaments nor the variance of the distance showed a significant correlation with age, myocardial disarray, or interstitial fibrosis.

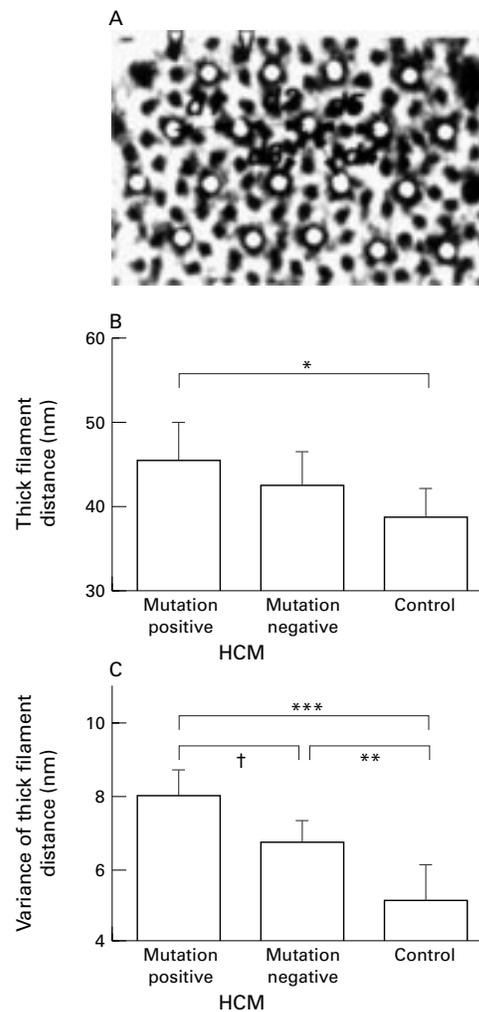


Figure 2 (A) The distance ($d_1, d_2, d_3, d_4, \dots, d_n$) between neighbouring thick myosin filaments (arrows) was measured for at least 100 thick filaments in the cross section of the sarcomere at the A band (arrow head, actin thin filament). The average and the standard deviation of the measures ($d_1, d_2, d_3, d_4, \dots, d_n$) were regarded as the thick myosin filament distance and the variance of the thick filament distance in each subject, respectively. (B) Averages of the thick myosin filament distance in patients with hypertrophic cardiomyopathy (HCM) with and without the β myosin heavy chain (β -MHC) mutation and in controls ($n = 7, 6,$ and $5,$ respectively). (C) Averages of the variance of the thick filament distance in HCM patients with and without the β -MHC mutation and in controls. Error bar denotes 1 SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ v controls; † $p < 0.05$ v HCM patients without a β -MHC mutation.

Discussion

Our study revealed a novel feature of the sarcomeric ultrastructure in hypertrophic cardiomyopathy, namely malalignment of the sarcomeric filaments, which was characterised by increased variance of the distance between neighbouring thick myosin filaments. The disorganisation of the sarcomeric ultrastructure was more marked when a β -MHC mutation was present. Disorganisation of the contractile apparatus is a histological feature of hypertrophic cardiomyopathy at various levels, including the whorled pattern formation of the cardiac muscle bundles, the myocardial disarray resulting from the abnormal cell to cell arrangement, and the myofibrillar disarray manifested by the loss of integrity of the myofi-

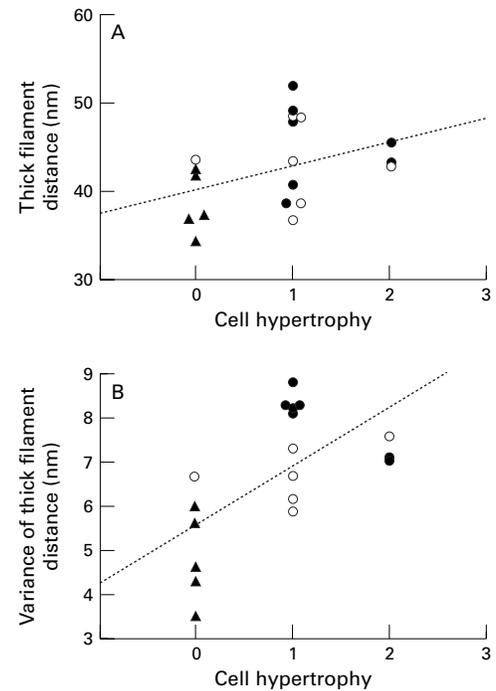


Figure 3 Correlation between the grade of cardiomyocyte hypertrophy and the thick myosin filament distance (A), and the variance of the thick filament distance (B). Controls, triangles; patients with hypertrophic cardiomyopathy and a β myosin heavy chain (β -MHC) mutation, filled circles; patients with hypertrophic cardiomyopathy without a β -MHC mutation, empty circles.

brillar architecture.¹⁷ To our knowledge, this report is the first in vivo demonstration of the disorganisation of the contractile unit at the level of the sarcomeric filaments in hypertrophic cardiomyopathy.

We measured the variance of the distance between the thick filaments and found it to be markedly increased in patients with hypertrophic cardiomyopathy, irrespective of the presence of the mutation. We considered that this reflected the destruction of the uniform distribution of the thick filaments. Although the distance between the thick myosin filament and surrounding thin actin filaments was not directly evaluated in the present study, the malalignment of the thick filament apparently increased the variance of the distance between the thick and thin filaments, as each thin filament is supposed to interact with two neighbouring thick filaments (fig 2A). Thus it seems plausible that the loss of the integrity of the alignment of the sarcomeric filaments results in impaired efficiencies of the actin-myosin interaction, the sliding of the filaments, and the coordinate force generation in the diseased sarcomere. It was noteworthy that the variance of the thick filament distance was well correlated with the grade of myocyte hypertrophy, which supports the hypothesis that impaired sarcomeric proteins may result in increased fibre stress leading to compensatory hypertrophy in hypertrophic cardiomyopathy.¹¹ It is not surprising that the disorganisation of the sarcomeric filaments was more marked in the presence of a β -MHC mutation, as it is expected that the binding affinity of the mutant β -MHC molecules to actin or other sarcomeric

proteins is altered and subsequent assembly of the sarcomeric filaments is impaired.^{9 10 18} In contrast, it was suggested that changes in the sarcomeric ultrastructure are not dependent upon aging and that sarcomeric ultrastructural disorders have no apparent direct relation to the myocyte disarray and interstitial fibrosis which are histological features of hypertrophic cardiomyopathy at the tissue level.

The greater distance between myosin thick filaments in patients with hypertrophic cardiomyopathy compared with controls may be a result of cardiomyocyte hypertrophy. However, this difference was found to be significant for index cases with the β -MHC mutation and not in those without the mutation, although the grade of myocyte hypertrophy was similar in the two groups. Also, the thick filament distance was not significantly associated with the grade of the myocyte hypertrophy. These findings imply the involvement of another factor apart from cellular hypertrophy in the thick filament difference. Malalignment of the thick filament could be one possible factor. When the sarcomeric filaments form a regular pattern, it is considered that the intermolecular assembly force among the filaments is uniform, allowing the filaments to take up a compact alignment. The uniformity of the assembly force would be destroyed by increased variance of the distance between the filaments, so in this case the compact alignment of the sarcomeric filaments would be lost, resulting in the more marked increases in the distance between the filaments seen in patients with hypertrophic cardiomyopathy with the β -MHC mutation. Increasing the number of subjects and examining other types of hypertrophy would clarify this issue.

In the present study, the small number of patients prevents us from drawing any conclusions about the relation of the ultrastructural changes to the site of the particular β -MHC mutation, although the location of the mutation could be crucial for conformational changes of the mutant β -MHC molecules and for alterations in the binding affinity of the mutant β -MHC to actin and other sarcomeric proteins. We cannot rule out the possibility that patients with hypertrophic cardiomyopathy without either a β -MHC or a troponin T mutation may have a gene mutation of some other contractile protein, or a mutation which has not yet been identified. Furthermore, it is possible that a mutation of non-sarcomeric proteins causing cardiomyopathies¹⁷ may indirectly affect the ultrastructure of the cardiac

sarcomere. Thus mutation of other genes may have different effects on the sarcomeric ultrastructure.

In conclusion, this study is the first to report on the disorganisation of the sarcomeric ultrastructure in hypertrophic cardiomyopathy, which was manifested by a loss of integrity of the alignment of the myosin thick filaments, more marked with a β -MHC mutation. The molecular mechanism and the functional significance of the ultrastructural changes should be addressed in future studies.

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