Variable expression of wild type and mutant β-myosin mRNA for different myosin mutations in Familial Hypertrophic Cardiomyopathy

Von der Naturwissenschaftlichen Fakultät der
Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Grades
Doktorin der Naturwissenschaften
Dr. rer. nat.
genehmigte Dissertation
von

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Tag der Promotion: 20.03.2009

My Parents

Acknowledgements

It is a sheer delight for me to see that the research that I carried out for my doctoral work at the Medizinische Hochschule Hannover is coming to fruition in the form of the present thesis. In carrying out this work, I learnt a lot of new things and techniques, broadened my horizon of knowledge, and received ceaseless and selfless guidance, help, support, and cooperation from several corners and many people, and, therefore, it will be most imprudent on my part not to acknowledge all such help and assistance – scientific and otherwise, that I constantly got during my sojourn at the Medizinische Hochschule Hannover (MHH), its Molekular-und Zellphysiologie department, in particular. As such, I take this opportunity to express my gratitude and thankfulness to all those who, directly or indirectly, have ungrudgingly and smilingly extended their helping hands at different stages of my work till its completion.

At the outset, I would like to express my gratitude and indebtedness to Frau Prof. Dr. Theresia Kraft – for not only agreeing to be my guide but also for supervising the work throughout and offering her constructive criticism and many helpful discussions and advice. In equal measure I express my gratitude and thankfulness to Prof. Dr. Bernhard Brenner, Head, Department of Molekular-und Zellphysiologie, MHH, for constant encouragement, support, helpful discussions, valuable advice, and allowing me to freely work in different Labs of MHH. Under the tutelage of both these Gurus, I have indeed learned a lot. I have no hesitation in admitting that without their support and ungrudging help at every stage of the work, it would not have been possible for me to submit my thesis in the form it is now being done. I have no sufficient words to express my gratitude to both of them in the limited space at my disposal.

In the same strain, my gratitude flows very strong to Prof. Walter Müller, for his kindly acceding to my request to be my '*Doctorvater'* and being available to answer any queries.

I would like to thank Prof. Dr. Ulrich Lehmann of the Institute for Pathology, MHH, for allowing me to do pyrosequencing experiments in his laboratory and for the helpful discussions. I am also grateful to his very competent technical assistant, Britta Hasemeier, for introducing me to the technique.

I am also grateful to Imke Schulte for providing some of her data for comparison with my data. Thanks are also due to Edgar Becker for providing some data of protein quantification. Two other scientists namely Dr. Walter Steffen and Dr. Tim Scholz of the Molekular-und Zellphysiologie department, MHH deserve special mention and to whom I owe grateful thanks for their encouragement and valuable advices whenever I needed them.

A big 'Thank you" to Dr. Ante Radocaj, who has always been very kind, friendly and helpful to me, especially for helping me out with some elementary statistical analyses of my experimental data.

I would like to place on record my appreciation and special thanks to Frau Evelyn Harder for helping me out with all the official work. My dear friend, Steffi Schmell, I would like to thank for all her help in numerous ways. Thanks are also due to Frau Birgit Piep, and Frau

Petra Uta for providing all the technical assistance whenever required, as also to all the colleagues and friends of the Molekular-und Zellphysiologie department for their selfless help throughout in so many ways.

I would particularly like to mention and thank my all-time friends, Mamta Amrute-Nayak and Meera Shah, Sarvari Velaga, Sukhada Chaturvedi and Reena Singh for all their help for being there and encouraging me when things would not go as smoothly as I would have liked them to.

I'd then like to thank all my family members, who have ever been a constant source of inspiration and encouragement to me and who always stood by me, lending their support, comforting me, and making the bad days easier to handle, from miles away.

In the fitness of the things, I like to thank Frau Petra Marotz, my German teacher at the Medizinische Hochschule, Hannover, for her painstaking efforts in teaching me the German language and its nuances. It was through her effective and interesting way of teaching that I could grasp the intricacies of the language and hone my conversational skills to the state it is today, which made my life easier in Germany in general.

Lastly - but never in the least, I would like to heartily acknowledge the financial support provided to me by the Deutsche Forschungsgemeinschaft (DFG) for this work, without which it would not have been possible for me to stay at MHH and complete my doctoral work.

As is evident from the above roll call - and as I mentioned in the beginning, I received tremendous amount of help along the way during the course of my doctoral work from many individuals, and any omissions - though quite unintentional, in mentioning some names are mine and mine alone for which I beg their forgiveness.

Place : MHH, Hannover **SNIGDHA TRIPATHI**
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Zusammenfassung

Hypertrophische Kardiomyopathie (HCM) ist eine Erkrankung des Herzmuskels, die durch linksventrikuläre Hypertrophie und unregelmäßige Anordnung der Herzmuskelzellen charakterisiert ist. Eine sehr häufige Ursache der autosomal dominant vererbten HCM sind Punktmutationen in der schweren Kette des β-Myosins (β-MHC), welches außer im Myokard auch in langsamer Skelettmuskulatur exprimiert wird. Überraschenderweise fanden wir bei Quantifizierung des Anteils mutierter β-MHC in Biopsien des *M. soleus* von HCM-Patienten signifikante Abweichungen von dem aufgrund des Erbgangs erwarteten 50:50-Verhältnis von Wildtyp- zu mutierter β-MHC. Eine erste Quantifizierung des Anteils mutierter β-MHC-mRNA ergab für eine Mutation auch eine ähnliche Abweichung auf mRNA-Ebene. In der vorliegenden Arbeit wurde untersucht, ob es auch bei anderen β-MHC-Mutationen eine Abweichung vom Verhältnis 50:50 gibt und ob im *M. soleus* und im Myokard von HCM-Patienten eine vergleichbare Abweichung vorliegt. Um den der Abweichung zugrunde liegenden Mechanismus zu erforschen, wurde überprüft, ob bei Geschwistern, bei Patienten verschiedener Generationen der gleichen Familie und bei nichtverwandten Patienten mit der gleichen Mutation die Abweichung vom 50:50 Verhältnis ähnlich ist.

Um mögliche Artefakte durch Bildung von Heteroduplexen aus einem Wildtyp und einem mutiertem DNA-Strang zu minimieren und sichere Aussagen über das Verhältnis von Wildtyp- zu mutierter β-MHC-mRNA machen zu können, wurde eine besondere PCR-Methode mit einem Rekonditionierungsschritt entwickelt. Die Methode basiert auf *reverse-transcription PCR* mit nachfolgender PCR und rekonditionierender PCR. Quantifizierung erfolgt durch enzymatischen Verdau und Restriktions-Fragment-Analyse.

Die Ergebnisse zeigten, dass der Anteil an mutierter β-MHC-mRNA bei Patienten mit der I736T-Mutation 38.4±7.6% und bei Patienten mit der R723G-Mutation 67.2±3.3% beträgt. Die Abweichung vom 50:50-Verhältnis war sehr ähnlich unter Geschwistern (I736T: 37.6%, 39.1% und 38.6%; R723G: 66.4% und 62%). Darüber hinaus fanden wir sowohl bei HCM-Patienten einer jüngeren Generation und aus einer anderen Familie mit der Mutation R723G, wie auch im Myokard von zwei Patienten mit dieser Mutation etwa den gleichen Anteil mutierter β-MHC-mRNA (71.8% und 67.2% im Myokard, 69.6% und 66.1% *im M. soleus)*.

Diese Daten, die auch durch ähnliche Beobachtungen an zwei nichtverwandten HCM-Patienten mit der Mutation V606M unterstützt werden, zeigen, dass der Anteil mutierter β-MHC-mRNA im langsamen Skelettmuskel und im Myokard etwa gleich ist, und typisch für die jeweilige Mutation zu sein scheint. Interessanterweise beobachteten wir bei den Familien eine Korrelation zwischen Schweregrad der Erkrankung und Anteil mutierter β-MHC-mRNA bzw. β-MHC im Muskel. Dies bedeutet, dass das Verhältnis von Wildtyp- zu mutiertem Protein im Myokard der Patienten zur Heterogenität des HCM-Phänotyps beitragen könnte.

Funktionelle Untersuchungen in unserer Gruppe und erste mRNA-Quantifizierung in einzelnen Skelettmuskelfasern von HCM-Patienten in der vorliegenden Arbeit deuten auf eine variable Expression der β-MHC-mRNA von Faser zu Faser und damit auf einen variablen Anteil an mutierter β-MHC in den Sarkomeren dieser Fasern hin. Um dies in Zukunft weiter untersuchen zu können haben wir eine neue Methode zur relativen mRNA-Quantifizierung in viel kleineren Gewebeproben, bis hin zu einzelnen Myokardzellen, etabliert, die auf einem Pyrosequenzier-Verfahren basiert. Ein variabler Anteil mutierter β-MHC von Zelle zu Zelle würde zu einem funktionellen Ungleichgewicht zwischen den Herzmuskelzellen führen und könnte so die Entstehung von „*disarray*“ und anderen Charakteristika des HCM-Phänotyps auslösen.

Abstract

Hypertrophic Cardiomyopathy (HCM) is a myocardial disease characterized by left ventricular hypertrophy and myocyte disarray. The disease is transmitted in an autosomal dominant manner. Mutations in genes encoding different sarcomeric proteins have been found as cause of HCM. Missense mutations in the β-myosin heavy chain (β-MHC), which is the MHC of ventricular myocardium and slow skeletal muscle like *M. soleus*, account for nearly 40% of all genetically characterized HCM cases. Our group found, unexpectedly, that in *soleus* muscle biopsies of HCM patients, heterozygous for β-MHC mutations, the ratio of wildtype vs. mutated β-MHC deviated significantly from the expected 50:50 ratio. In preliminary work, quantification of the fraction of mutated β-MHC-mRNA showed a similar deviation for one mutation also at the mRNA level. Here we investigated whether a deviation from the 50:50 relation is also found for other β-MHC mutations and whether it is similar in biopsies of slow skeletal muscle and myocardium of HCM patients. Since the underlying mechanism for development of the deviation is unclear, we also studied whether the deviation is similar in HCM patients of the same family of different generations and of unrelated families with that mutation.

To ensure that quantification with the previously used restriction digest method is not falsified by artifacts like formation of heteroduplexes of one wildtype and one mutant DNA strand in PCR at high cycle numbers, a 'reconditioning PCR' method was established. The method is based on reverse-transcription polymerase chain reaction (RT-PCR) followed by PCR and a reconditioning PCR step to ensure that no heteroduplexes underwent the subsequent enzymatic cleavage of the PCR products and restriction fragment analysis.

We found that for patients with the β-MHC mutations I736T and R723G the fraction of mutated β-MHC-mRNA in the samples studied on average was 38.4±7.6% and 67.2±3.3%, respectively. The deviation from 50% was found to be quite similar for siblings (I736T: 37.6%, 39.1% and 38.6%; R723G: 66.4% and 62%). Furthermore, essentially the same fraction of mutated β-MHC-mRNA was also found in younger family members, in an unrelated family also with the R723G mutation, as well as in myocardium of two patients with this mutation (71.8% and 67.2% for myocardium, 69.6% and 66.1% for soleus, respectively).

These data, which are confirmed by similar findings for two unrelated patients with mutation V606M, indicate that the level of expression of the mutated β-MHC mRNA for each mutation not only is essentially the same in slow skeletal muscle and in myocardium but also appears to be characteristic of each mutation. Interestingly, for the families studied the malignancy of the disease is correlated with the fraction of mutated β-MHC-mRNA and β-MHC in the muscle tissue, which may indicate that the expression level of a mutation contributes to the heterogeneity of the HCM phenotype.

Functional studies in our group and preliminary mRNA quantifications in this work on single slow muscle fibers indicate a variable expression of mutated β-MHC-mRNA from fiber to fiber and hence a variable amount of mutated β-MHC in these fibers. To test this in the future we established a new method for relative mRNA quantification in much smaller samples, possibly even single cells, using a pyro-sequencing approach. A variable proportion of mutated β-MHC from cell to cell is expected to cause functional imbalances among the cardiomyocytes, and may thereby trigger the development of cardiomyocyte disarray and other features characteristic for the HCM phenotype.

Keywords / Stichworte

Hypertrophic Cardiomyopathy, β-myosin heavy chain, relative mRNA quantification

Hypertonische Kardiomyopathie, β-Myosin schwere Kette, relative mRNA-Quantifizierung

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1 Abbreviations

α-MHC	alpha myosin heavy chain
APS	Adenosine 5' phosphosulfate
β-MHC	beta myosin heavy chain
bp	base pair
BSA	bovine serum Albumin
CCD	Charge coupled device
cDNA	complementary deoxyribonucleic acid
dATPaS	Deoxyadenosine alpha thio-triphosphate
dNTP	deoxyribonucleotide tri-phosphate
DTT	Dithiothreitol
ECG	Electrocardiogram
EDTA	Ethylene diamine-N, N, N', N' tetra acetic acid
ELC	Essential Light Chain
EtBr	Ethidium Bromide
G	Glycine
GSP	Gene Specific Primer
HCM	Hypertrophic Cardiomyopathy
I	Isoleucine
ICD	Implantable Cardioverter Defibrillator
IOD	Integrated Optical Density
KDa	Kilo Dalton
M	Methionine
mg	milligram
min	minute
ml	milliliter
μl	microliter
mm	millimeter
Mut	Mutant/mutated
MYBPC3	Cardiac Myosin Binding Protein C
MYH7	Myosin Heavy chain
NYHA	New York Heart Association

PCR	Polymerase Chain Reaction
P_i	inorganic phosphate
PP_i	pyrophosphate
Q	Glutamine
R	Arginine
RLC	Regulatory Light Chain
RT	Reverse Transcription
RTase	Reverse transcriptase
SNP	single nucleotide polymorphism
T	Threonine
TBE	Tris, Boric acid, EDTA
TNNI3	Cardiac Troponin I
TNNT2	Cardiac Troponin T
V	Valine
W	Tryptophan
WT	Wild Type

2 Introduction

2.1 The human heart and Hypertrophic Cardiomyopathy

The human heart is one of the most efficient pumps known. This organ, composed entirely of involuntary muscles called myocardium, in a normal condition, pumps blood between two independent systems, the pulmonary and the systemic circulations, respectively. During cardiac relaxation or ‘diastole’, the ventricular chambers get filled with blood from the atria; the right ventricle with deoxygenated blood and the left ventricle with oxygenated blood. During the contractile phase or ‘systole’, the left ventricle pumps blood into the various parts of the body and the deoxygenated blood in the in the right ventricle is pumped via the pulmonary artery into the lungs where it can be oxygenated again (Guyton and Hall, 1998).
Impairments in the systolic or diastolic functions may lead to a chronic disorder called heart failure. As a root cause of heart failure are always one or a series of abnormalities such as valve disease, congenital malformations, rhythm disturbances or cardiomyopathies (Ahmad et al., 2005). These ‘initiating insults’ lead to an overall morphological remodeling and hemodynamic changes.

Hypertrophic Cardiomyopathy (HCM), also known by other names such as idiopathic subaortic hypertrophic stenosis or hypertrophic obstructive cardiomyopathy, as the name suggests, is a disease of the cardiac muscle. In 1957 HCM was described for the first time by Sir Russell Brock, who found a functional subvalvular obstruction in patients undergoing corrective surgery for aortic stenosis. In 1958 came a description from D. Teare, who described asymmetric septal hypertrophy with bizarre muscle bundle orientations in the hearts of 8 patients who died suddenly. It was only in the 1960s when Braunwald and colleagues reported a disease condition in which asymmetric septal hypertrophy, myofibrillar disarray and dynamic outflow tract obstruction were encountered.

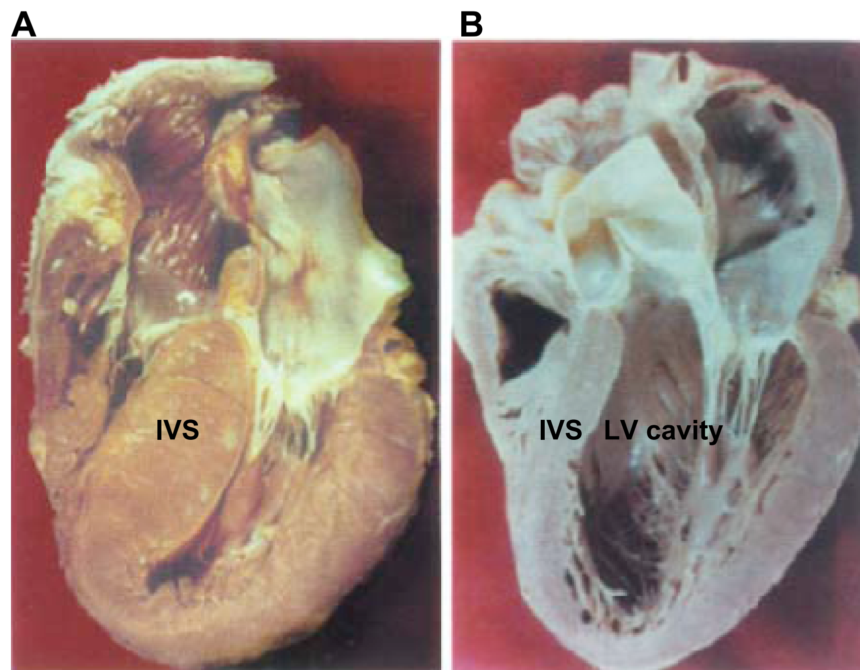


Fig. 1. The morphological remodeling of the heart in HCM. A normal heart (B) and a hypertrophic heart (A). The reduction in the left ventricular (LV) chamber volume and hypertrophy of the free wall of the left ventricle and inter-ventricular septum (IVS) are the characteristic feature of HCM. Figure modified from Chung et al., 2003.

HCM is a disease of the myocardium and is characterized by an increase in cardiac mass typically represented by asymmetric hypertrophy of the left ventricle which involves the inter-ventricular septum, in the absence of any external causes such as hypertension or aortic valve stenosis. Fig. 1 shows a typical hypertrophied heart in comparison to a normal heart. Usually the left ventricular volume is reduced and systolic pressure gradients are common. It was not until 1990 when for the first time a point mutation of a sarcomeric protein was identified as the primary cause of HCM (Geisterfer-Lowrance et al.,1990). The increase in cardiac mass is an outcome of the increase in the size or volume of the cardiomyocytes, rather than an increase in their number (hyperplasia). The hypertrophy is accompanied by interstitial fibrosis, which is characterized by the presence of more fibroblasts and associated extracellular matrix. The presence of intertitial fibrosis increases cardiac stiffness and causes further changes in the heart's anatomy. The other most remarkable and distinguishing feature of HCM is myocyte and myofibrillar disarray (Richardson et al., 1996). (Fig. 2). The disease

is characterized by various symptoms such as, angina pectoris, shortness of breath or dyspnea, arrythymias, unexplained syncopes and one of the features of the disease which cannot be defined as a symptom is unexplained sudden death.

2.2 Pathological hallmark of HCM

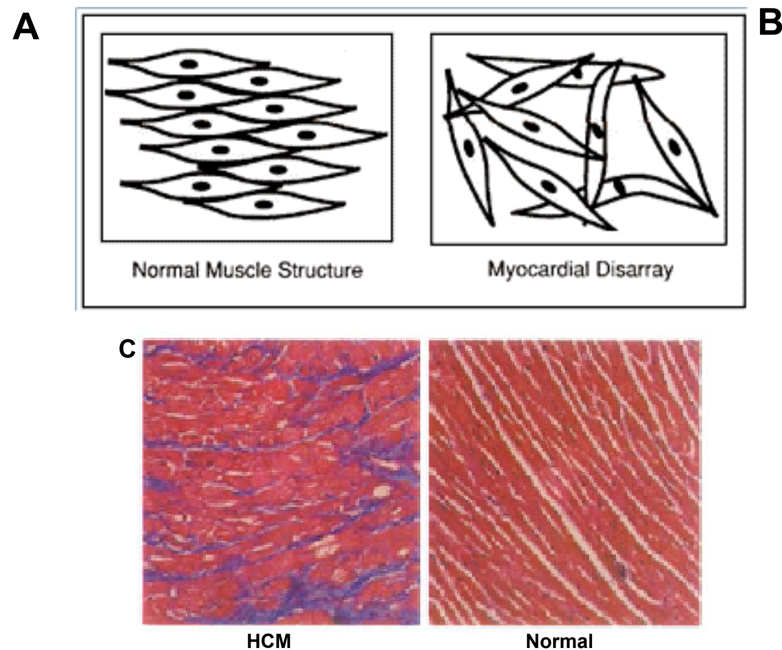


Fig. 2. Structure of the normal (A) and hypertrophied (B) myocardium. Myocte disarray is characterized by myocytes arranged around areas of interstitial fibrosis (C) Figure of sections of Hypertrophic and normal hearts showing the disarray and normal arrangements of myocytes, respectively. Figure modified from (Chung et al., 2003)

Histological examination of the myocardium is fundamental to the diagnosis of HCM. The chief pathological features of HCM are myocyte hypertrophy and disarray along with increase in interstitial collagen deposition (Elliot and McKenna, 2004). In myocyte disarray the orderly alignment and organization of the myocytes is disrupted and adjacent myocytes get obliquely or perpendicularly oriented to one another around areas rich in collagen. The nuclei of the myocytes become enlarged, show different shapes and sizes (pleomorphic) and become darkly stained (hyperchromatic). However, even though the different pathological arrangement of the myocytes in a hypertrophied heart has no prognostic implications (S.E.

Hughes, 2004), yet the development of myocyte disarray possibly causes ventricular arrhythmias.

2.3 Genetics of Hypertrophic cardiomyopathy

According to linkage analyses in different families, genes present on separate chromosomes are known to cause a disease with common phenotypic effects e.g., myocyte hypertrophy and disarray (Fokstuen et al., 2008). To date, at least 16 different genes have been identified (Table 1), mutations in which cause hypertrophic cardiomyopathy. Most of these disease causing genes encode proteins of the structural and functional unit of the muscle cell – the sarcomere.

The allusion of HCM as a disease of the sarcomere is justified, as mutations in several sarcomeric proteins are known to be the chief cause of this disease. These proteins include myosin heavy chain (MHC), essential and regulatory light chains (ELC, RLC), cardiac troponin I (TNNI3), cardiac troponin T (TNNT2), cardiac myosin binding protein C (MYBPC3), α-tropomyosin (TPM1) and cardiac actin (ACTC). Mutations in several other non-sarcomeric proteins like Caveolin-3 (Hayashi et al., 2004) and the phospholamban promoter (Minamisawa et al., 2003) have also been implicated as causes of the disease, but these mutations are very rare in HCM so far.

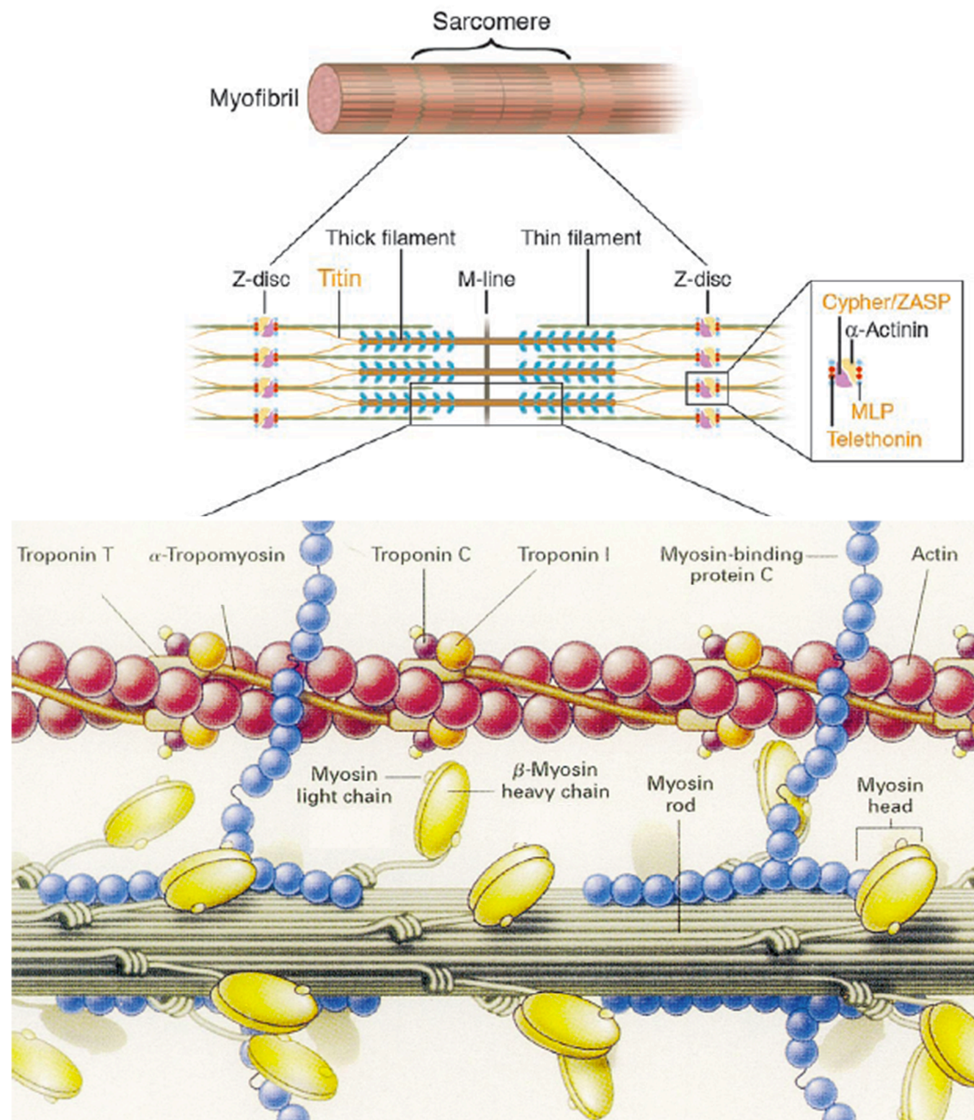


Fig. 3. Schematic representation of a sarcomere. The myofibril shows the alternating light bands (I bands) composed of thin filaments and dark bands (A bands) composed of thin and thick filaments. The thin filament proteins include actin, tropomyosin and troponins C, T and I. The thick filament proteins include myosin heavy chain, myosin regulatory and essential light chains and myosin binding protein C. Titin serves as an anchor between the Z disk and the thick filaments. (Figure modified from Morita et al., 2005 and Redwood et al., 1999).

Gene	Locus	Protein	Number of mutations
MYH7	14q12	β-Myosin heavy chain	193
MYBPC3	11p11	Cardiac myosin binding protein C	138
TNNT2	1q32	Cardiac troponin T	33
TNNI3	19q13	Cardiac troponin I	32
CSRP3	11p15	Cardiac muscle Lim protein	12
TPM1	15q22	α-tropomyosin	11
MYL2	12q23	Regulatory myosin light chain	10
ACTC	15q14	Cardiac actin	7
MYL3	3p21	Essential myosin light chain	5
PRKAG2	7q36	AMP-activated protein kinase	4
PLN	6q22	Phospholamban	2
TNNC1	3p21	Cardiac troponin C	1
TTN	2q31	Titin	2
MYH6	14q11	α-myosin heavy chain	2
TCAP	17q12	Telethonin	2
CAV3	3p25	Caveolin-3	1
Total			455

Table 1. HCM as a disease of the sarcomere. The above table illustrates the chromosomal loci and mutation frequency of the chief sarcomeric and non-sarcomeric proteins which are implicated in hypertrophic cardiomyopathy. The table is modified from Fokstuen et al., 2008.

Out of the above-mentioned sarcomeric proteins, mutations in the β-myosin heavy chain are the most frequently occurring and account for about almost 40% of all HCM cases (Fokstuen et al., 2008). The disease is transmitted chiefly as an autosomal dominant disorder, in which case it is familial. Most HCM patients are heterozygous for the causative mutation. The frequency in the general population is 1:500 (Maron et al., 1995). When the causal mutation occurs de novo in an individual neither of whose parents are clinically or genetically affected, the disease is said to be sporadic. It has, however been difficult to estimate the fraction of HCM that occurs sporadically. In two different studies it has been suggested that HCM was sporadic in 44% and 33%, respectively (Maron et al., 1984; Greaves et al, 1987).

2.3.1 Beta myosin heavy chain gene

Myosin is the chief component of the muscle thick filaments. It has been shown that the MYH7 gene is 22,883 bp long (Jaenicke et al., 1990) and the locus for this gene is 14q13 (Matsuoka et al., 1989). The gene comprises 40 exons, out of which the first two are non-coding (Liew et al, 1990). This gene encodes a 1935 amino acids long polypeptide. In humans the β-myosin is expressed in the ventricles and also in slow skeletal muscle fibers.

2.3.2 Myosin structure and ventricular myosin mutations

Myosin is an actin activated ATPase that converts the chemical energy of ATP into mechanical energy. Following the hydrolysis of ATP, the myosin heads can bind to actin with high affinity. Conformational changes of the myosin head upon P_i and ADP release will pull the actin towards the center of the sarcomere. Repeated cycling of the acto-myosin cross-bridge will cause sliding of the actin filaments and muscle shortening.

A myosin molecule comprises of six subdomains with a total molecular weight of 520 KDa. It consists of two globular heads formed by two heavy chains (each about 220 KDa) and four light chains, two ELCs and two RLCs, each of approximately 20 KDa. The myosin heavy chain has two major functional parts. The amino terminal globular domain to which the light chains bind, is the motor domain consisting of the ATP binding site and the actin binding site. The catalytic activity of the myosin molecule also resides in the head domain. The rod of the molecule comprising an alpha-helical coiled-coil elongated structure forms the backbone of the thick filaments. Myosin heads which can form physical links between the thick and the thin filaments are called cross bridges.

The cycling crossbridge model links events of ATP hydrolysis with force generation. Between the cross bridge conformation at the beginning and end of the power stroke, the lever arm of the myosin molecule is thought to rotate approximately 60 to 70 degrees, causing the displacement of actin (Geeves et al., 1999).

Most of the HCM causing β-myosin mutations are missense mutations causing a change of a single amino acid in the mutated molecule, and the patients are mostly heterozygotes. β-myosin head domain mutations studied in our laboratory are examples of such mutations. Fig. 4 shows the location of the mutations in the myosin head domain reported in this study

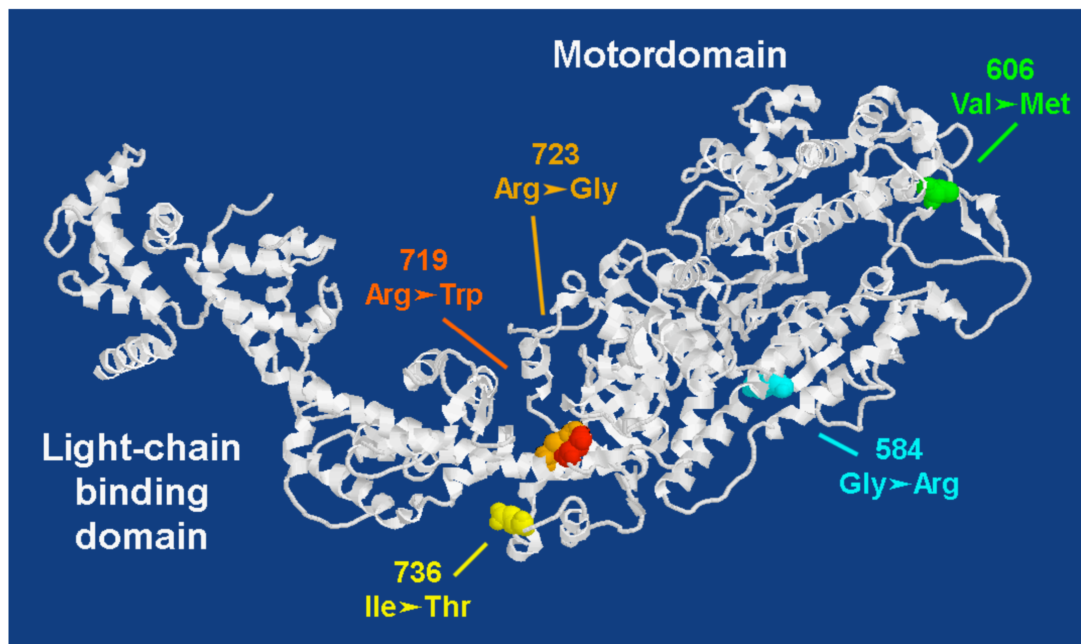


Fig. 4. Structure of a β-MHC head domain with the locations of the mutations under study.

However, very few cases have also been reported where the affected patients have either the identical mutations on both β-MHC alleles (homozygotes) (Nishi et al., 1994) or different mutations on each allele (compound heterozygote) (Mohiddin et al., 2003).

2.4 Putative HCM causing Mechanisms

Different mechanisms of phenotype development in HCM have been postulated in the past. To date there is, however, no conclusive evidence for a common denominator or mechanism common to all HCM causing mutations which would trigger the development of HCM. In the following paragraphs examples will be given for different mechanisms how a mutation might initially affect myocardial function.

2.4.1 Dominant-negative mechanism

According to the dominant-negative mechanism, the mutant and the wild type alleles produce the mutant and wild type proteins, respectively. Both kinds of proteins are integrated into the sarcomere. The mutant protein has a new and/or enhanced and aberrant function. The

incorporation of the mutant (Mut) protein, which acts as a ‘poison polypeptide’, into the sarcomere affects the function of the wild type (WT) protein.

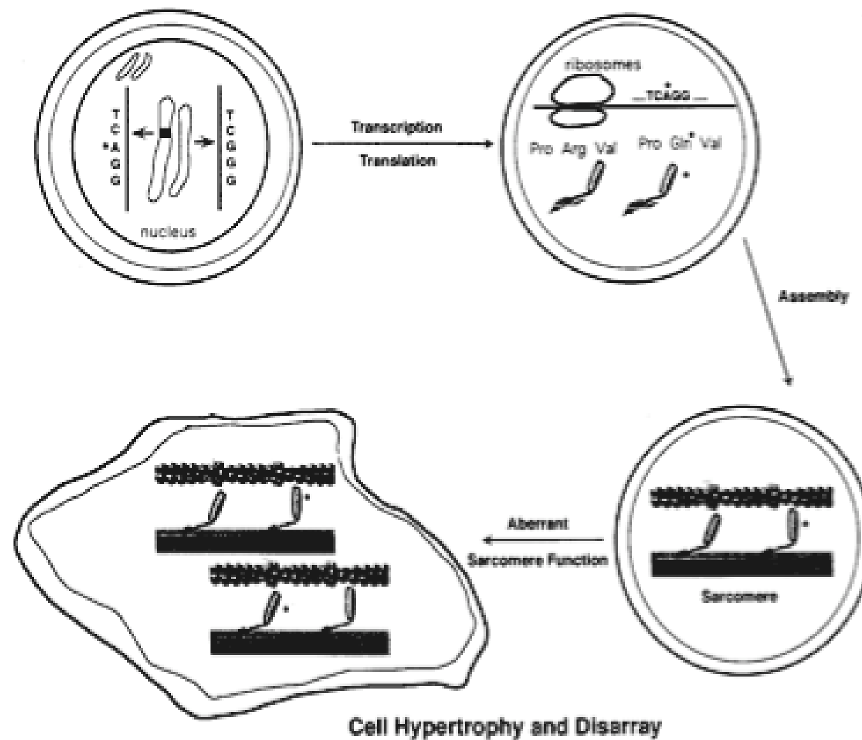


Fig. 5. Dominant-negative mechanism of HCM. From the Mut (asterisk) and WT genes, Mut and WT mRNAs are transcribed which are translated into the corresponding proteins. The proteins are incorporated into the contractile apparatus where the aberrant Mut protein exerts its functional effects and causes myocyte hypertrophy and disarray. Figure taken from Seidman et al., 1998.

Protein quantification done in our laboratory on different β-MHC missense mutations implicated in HCM, performed by chemical permeabilization of muscle fiber membranes to wash off any soluble and free myosin that has not been incorporated into the sarcomere, has demonstrated that mutated protein is present in the sarcomeres (Nier et al., 1999; Becker et al., 2007). Mechanical and X-ray diffraction experiments done by Köhler et al., 2002 have shown that integration of the mutated β-myosin into the sarcomeres does not cause alterations

in the structure and assembly of thick filaments. Therefore, the myosin heavy chain mutations cause their HCM effects due to a change in the functional properties of the myosin molecule.
R403Q mutation in the actin-binding loop of the myosin heavy chain (Geisterfer-Lowrance et al., 1990) is one of the most lethal HCM-causing mutations and has been important in the studies trying to uncover the mechanisms underlying hypertrophic cardiomyopathy. It was shown by Tyska et al., 2000 that myosin isolated from mice hearts engineered to have the R403Q, exhibited a higher actin activated ATPase activity, greater generated force and accelerated actin filament sliding. Recently, Belus et al., 2008 have also shown that in single ventricular myofibrils carrying the R403Q mutation in the myosin heavy chain, tension generation and relaxation were much faster, compared to controls, when Ca^{++} was suddenly increased and decreased. Such gain in function, although it accounts for improved motor performance of R403Q molecules, yet the associated increased ATPase activity results in the increased energy consumption by the hypertrophic heart and might lead to cell death. Since the myocardium is a terminally differentiated tissue, the cells essentially do not replicate or regenerate and proliferation of cardiac fibroblast occurs. The occurrence of replacement fibrosis is a well-documented feature of HCM.
However, in another study to investigate the functional effects of the HCM associated β-myosin mutation R403Q, it was found that the mutant myosin had normal ATPase activity in the absence of actin. In the presence of actin, the mutant functioned like a poison polypeptide causing a decrease in the ATPase activity and the velocity of actin translocation in *in vitro* motility assays (Sweeney et al., 1994).
This indicates that results for the R403Q mutation are not consistent in all studies and in all tissues. When HCM mutations are expressed in transgenic animals, they do not necessarily induce the same functional changes in the sarcomeres of these animals as in human myocardium. This has been shown in a study by Lowey and colleagues in 2008, where either the cardiac α- or the β-MHC isoforms were expressed in mice. It was demonstrated in *in vitro* motility assays that the actin filament velocity was enhanced by 30-40% in the R403Q α-MHC animals when compared with the wild type animals, whereas the mutation R403Q in the β-MHC backbone did not enhance the velocity of actin. It was also observed that the actin activated MgATPase activity of the R403Q-α-MHC was approximately 30% higher than the wild type, whereas, that for R403Q in the β-MHC backbone was reduced by approximately 10%. Thus, the contradictory results in the literature for mutation R403Q may depend on the

source of the mutated myosin. These observations underline the importance of studying the effects of HCM mutations in the respective human protein isoform and if possible, in human tissue.

2.4.2 Loss-of-function mutations

Normally the wild type allele of a given gene codes for a protein that is necessary for a specific biological function. When a mutation occurs in this allele, the function associated with its protein product may be lost. The mutation, in this case, is referred to as a loss-of-function mutation. The extent to which the function of the protein is lost varies. When there is a complete loss of function, then the mutation is referred to as a null mutation. Loss-of-function mutations confer a recessive phenotype to the organism. Usually the protein encoded by the wild type allele is sufficient to compensate for the loss of function in the protein due to the mutated allele. In this case the wild type allele is said to be dominant and the mutated allele is referred to as recessive.

Missense mutations in the β-MHC have been reported to cause a loss-of-function. The mutation G741R, located in a mutational hot-spot area of the β-MHC (Bonne et al., 1998), decreased the maximum velocity of shortening (39% of normal) and caused reduced isometric force generation (42% of normal) (Lankford et al., 1995). This mutation seems to cause a decreased power output and therefore, the hypothesis was developed that to ensure normal mechanical performance of the heart, compensatory hypertrophy might follow.

2.4.3 Haploinsufficiency

Haploinsufficiency is described as a condition in diploid organisms when one allele of a given gene is inactivated through a dominant mutation producing amounts of protein from the wild type allele that is 'insufficient' for normal function. This mechanism involves a quantitative rather than a qualitative change in the polypeptide and therefore leads to impaired myocyte mechanical function (Marian et al., 2001; Dalloz et al., 2001). Some deletion mutations can lead to a 'null allele' in which the spurious gene product is absent and only 50% of the total protein is normal and responsible for executing its given function. Since the proper functioning of muscle cell depends on the structural and functional integrity of the

sarcomere, it is important that each component of the contractile apparatus be present in appropriate stoichiometry.

Most HCM causing cardiac Myosin Binding Protein C (cMyBPC) and cardiac Troponin T (cTnT) mutations are defects in splice signals which encode either truncated or aberrant polypeptides (Bonne et al., 1995; Rottbauer et al., 1997; Thierfelder et al., 1994) In a mouse model of cTnT null allele, cTNNT (-/-), the thick and thin filaments and alpha actinin positive Z disk like structures were not assembled into the sarcomere leading to embryonic lethality. On the other hand, the cTnT(+/-) mice had a normal life span (Nishii et al., 2008). Thus for survival, at least one normal allele of a sarcomeric gene is necessary.

Yet, most likely haploinsufficiency is not a common mechanism in HCM. Quite a few studies have shown that the mutated alpha fast tropomyosin (Bottinelli et al., 1998) and mutated β-MHC (Köhler et al., 2002; Kirschner et al., 2005; Becker et al., 1997; Cuda et al., 1993; Palmiter et al., 2000; Malinchik et al., 1997; Nier et al., 1999; Becker et al, 2007,) peptides, instead of being degraded, are incorporated into the sarcomere. Thus, the stoichiometry of the sarcomeric components is maintained through the expression of both the mutated and wild type sarcomeric proteins.

2.5 Animal models of HCM

Since human muscle tissue is a limiting factor in the study of the disease and is usually obtained *post mortem* or during heart transplantation operations, animal models of HCM with human mutations engineered into genes of sarcomeric components provide an important tool to study the functional impacts of such mutations on disease etiology. So far, mutations of the myosin heavy chain, cardiac MYBPC and TNNT that cause HCM in humans have been engineered into the genomes of animals.

2.5.1 Mouse models

In mice, at the embryonic and fetal stages β-MHC is the predominant isoform, whereas, soon after birth the α-MHC expression is up-regulated and becomes the chief myosin isoform in the ventricles (Weiss et al., 1996). The first reported β-MHC mutation, R403Q, was introduced into the mouse genome at the α-MHC locus by targeted homologous

recombination (Geisterfer-Lowrance et al., 1996). Therefore, in an α-MHC$^{+/403}$ mouse a glutamine codon is present at the nucleotide position corresponding to the 403 amino acid position in the α-MHC polypeptide, instead of the normal arginine codon in one allele. The other allele is wild type and hence expresses the polypeptide with the arginine residue. The R403Q mutation in the β-MHC of humans presents a very severe form of HCM in which approximately 50% of the affected individuals die even before they are 45 years old. Seemingly consistent with this finding, Geisterfer-Lowrance and colleagues have shown that one out of five α-MHC$^{+/403}$ mice died suddenly when subjected to vigorous physical activity like swimming. The dead mouse had an enlarged heart and marked left ventricular hypertrophy (Arad et al., 2002). On the contrary, these mice show no overt symptoms and have a normal life span when they are physically inactive.

Murine models provide important information as regards the mechanism by which mutated sarcomeric proteins cause HCM. These models confirm the notion that the mutated proteins are synthesized and are stably incorporated into the contractile apparatus of the mice.

Although the transgenic murine models elucidate the basic principles of HCM, yet they suffer from certain drawbacks. There exists a significant difference in the sarcomeric protein isoform composition of humans and mice. In humans, the β-MHC is the chief adult ventricular myosin isoform, whereas, α-MHC is the principal ventricular myosin isoform in adult mice. Therefore the exact progression of HCM in mice due to mutations in the β-MHC, other than R403Q, and the corresponding phenotype is a matter of speculation due to the differences in amount of myosin isoforms in the human and murine hearts.

2.5.2 Rabbit models

In adult rabbits β-MHC comprises about 80% of the total endogenous myosin similar to humans where β-MHC is the predominant isoform comprising >90% of total ventricular myosin (Swynghedauw B, 1986). One major advantage of rabbit models over mouse models is that the human and rabbit β-MHC proteins are approximately 98% homologous (Jaenicke et al., 1990; Kavinsky et al., 1984). A transgenic rabbit was created by injecting into fertilized rabbit zygotes, the transgene formed by cloning the mutant and wild type human β-MHC cDNAs, with or without the R403Q mutation, downstream of murine β-MHC promoter (Marian et al., 1999). In these animals also the expression of the mutated transgene mRNA

and protein was demonstrated. The animals with the mutated transgene showed marked myocyte disarray and an increased interstitial collagen in the myocardium. The septum-thickness was significantly increased in animals expressing the mutated transgene compared to wild type transgenic animals. It was also found in this study that in mutant transgenic rabbits, premature death was more common.
Another study on transgenic rabbits with the R403Q mutation in the β-MHC gene has been reported (Nagueh et al., 2004) which tries to elucidate the human HCM by tracing the chain of evolutionary events that lead to the develoment of the cardiac phenotype. It was observed that the Ca^{++} sensitivity of the myosin ATPase activity was decreased in the absence of other phenotypes. Myocyte disarray in the rabbits carrying the mutated transgene occurred quite early and was independent of hypertrophy and interstitial fibrosis, which occurred during puberty as a result of activation of stress-related signaling pathways.
It should however, be noted that also the rabbit model has its drawbacks. It is not clear whether the observed effects are solely caused by the mutation. The effects could also be partially due to the fact that in these animals, human wild type and mutant β-myosin is expressed together with endogenous rabbit β-myosin resulting in a mixture of different myosin isoforms present.

2.5.3 Limitations of animal models

Although the animal models recapitulate various features of HCM, there are differences between these models and human disease. The use of murine models is complicated by the fact that the different ventricular MHC isoforms are expressed in the mouse (α-MHC) and human (β-MHC) myocardium. Also, the experimental animal models produced by transgenic approaches may result in higher levels of mutant protein encoded by the transgene than would be produced endogenously by a single mutated allele for a dominant human disorder. Hence, the effects of the transgene may be exaggerated. Moreover, inherent electrophysiological differences in the cardiac function, e.g. heart rate, between mice and humans has been recognized; the heart rate is ten-fold lower in humans than in mice (London B, 2001). Furthermore, the effects of exercise on these animals may be underestimated in a laboratory environment. Therefore, the utility of murine models as an accurate predictor cardiac arrhythmias and sudden death remains a point of debate.

This further emphasizes that studies on human tissues are important where the genes encoding the mutated protein are present in their natural milieu and thus the effects of extraneous factors on HCM expression and progression can be negated.

2.6 Genotype-phenotype correlations

HCM in humans is a genetically heterogenous disease caused by mutations in various sarcomeric proteins. Mutations in different proteins seem to lead to a different clinical outcome. For example, it is now known that mutations in cardiac TNNT are associated with mild hypertrophy, but high incidence of sudden death, which sometimes occurs even without a pronounced left ventricular hypertrophy (Moolman et al., 1997). Mutations in the cardiac MYBPC are associated with mild phenotype, delayed age at the onset of symptoms and a favorable prognosis before the age of 40 (Niimura et al., 1998; Charron et al., 1998). However for mutations in the β-MHC, the expression of the phenotype varies considerably with different mutations as well as with the identical mutation. Hence, the prognosis for various patients differs. Several mechanisms could account for the large variability in the phenotypic expression of the disease. For example, the type of mutation and the functional alterations or impairment it causes in the sarcomere, which would also depend on the location of the mutation within the protein that carries it. The effect of postulated modifier genes also accounts for the phenotypic heterogeneity of HCM. Moreover environmental factors and the lifestyle of an individual are also factors that contribute toward the phenotypic variation of disease manifestation (Marian AJ, 2002).

Some HCM causing missense mutations in the β-MHC protein, which alter the charge of an amino acid, have been found to be associated with a shorter life expectancy. Examples of such mutations are R403Q and R453C (Marian et al, 1998). These mutations are associated with increased risk of sudden death. Another mutation associated with a high risk of premature death is R719W. This mutation also produces a charge change, in the mutated protein, of -1 (Anan et al., 1994).

The mutations F513C, V606M (located in the motor domain) and L908V (located in the head/rod junction) have been described as benign mutations (Anan et al., 1994). Each of these mutations is a conservative mutation because there is no change of charge associated with the mutated protein. The lack of change of charge may partly account for the favorable

prognosis associated with these mutations. Yet for mutation V606M there are contradictory reports that associate the mutation also with poor prognosis and the risk of sudden death (Havndrup. et al., 2001, Fananapazir et al., 1994). This example shows that the severity of the clinical outcome of an HCM causing mutation apparently depends not only on the net change of charge in the β-MHC due to the amino acid exchange. Attempts to establish a genotype-phenotype correlation for myosin heavy chain mutations based on the net charge change have failed. In addition, despite a very large number of known myosin mutations to date no clear correlation of the phenotype with e.g. location of the mutation within the head domain or any other typical feature of the mutations has been found.

2.7 Aims and Objectives of the current work

From the work in our group we have evidence that besides the type and location of a mutation in the myosin molecule there might be another factor that could contribute to the variability in phenotype and particularly in severity of the disease. We previously observed a lack of functional effects of some β-myosin mutations in studies on single slow human muscle fibers from HCM patients (unpublished data). Based on this we determined the proportion of mutated β-myosin, which was incorporated into the sarcomeres of these muscle biopsies. The result was quite surprising because we found that in a patient with mutation V606M, only 12% of total myosin carried the mutation (Nier et al., 1999). In a patient with mutation G584R only 23±1% was mutated myosin in the contractile apparatus (Nier et al., 1999). These findings were quite unexpected because HCM is inherited in an autosomal dominant fashion and thus approximately 50% mutated and 50% wild type myosin would be expected in the muscle tissue of the patients.

Since a deviation from a 50:50 ratio in the expression of wild type and mutated protein occurred at the protein level, we asked whether the deviation was already present at the mRNA level. First experiments on two mutations (V606M and R719W) in skeletal muscle biopsies of HCM patients, which were carried out by I. Schulte in a previous dissertation (Schulte, 2001) showed that the fraction of mutated β-MHC-mRNA for mutation V606M was similarly low as found at the protein level. However, for mutation R719W, it was close to 50% at the protein and β-MHC-mRNA level. Therefore several open questions remained, which were addressed in the current work.

- It was not clear from the previous work whether the deviation from a 50:50 ratio observed for mutation V606M could also have been caused by formation of heteroduplexes between wildtype and mutant DNA strands. Such heteroduplexes are formed in a multi-template reaction at high cycle numbers during PCR, under limiting primer concentrations, when the denaturation/reannealing steps cause such heterologous sequences to hybridize (c.f. Fig 8). Formation of heteroduplexes would cause errors in the restriction digest used for relative quantification of the mutated and wild type β-MHC-mRNA. Therefore, one important part of the present work was to extend and modify the previously used method for the quantification of the fraction of mutated β-MHC-mRNA in muscle tissue of HCM patients. A control for heteroduplex formation which would affect the quantification had to be established together with generation of defined mixtures of mutant and wild type cDNA cloned into plasmids to test the method.
- Since previously only for one mutation (V606M) a clear deviation from the 50:50 ratio was found, in the current work we had to find out whether the deviation in the expression of wild type and mutated β-MHC-mRNA from 50:50 in *soleus* muscle also exists for other HCM causing mutations.
- Another important question regarding the underlying mechanism of the deviation from a 50:50 ratio in the wild type and mutant β-MHC-mRNA was, whether the deviation is similar for a given mutation in several patients. We had muscle tissue available from five patients with mutation R723G and from three patients with mutation I736T so that we could address this question. In addition, since not all patients with the same mutation were siblings, we could also investigate whether the fraction of mutated β-MHC-mRNA in the muscle tissue exhibits an intra- and inter-familial similarity.
- Quite important with respect to the relevance of our results from soleus muscle biopsies of HCM patients for the myocardial disease in these patients was the question, whether the fraction of mutated β-MHC-mRNA is similar in the myocardium for a given patient and/or mutation. Although myocardial biopsies are very difficult to obtain, we were able to address this question with the myocardial tissue from two HCM patients with mutation R723G.

- Finally to further investigate possible reasons for the observed deviation from a 50:50 ratio also in myocardium we set out to determine relative β-MHC-mRNA levels also in single cardiomyocytes. To achieve this goal in the future, a more sensitive method for the relative quantification had to be introduced to be able to perform relative quantification even on much smaller tissue samples like needle biopsies or even single myocytes obtained by the laser capture microdissection method.

2.8 Methods used to address the question

The above questions were mainly addressed with the newly established reconditioning PCR method for relative quantification of β-MHC-mRNA in muscle tissue. This method is based on reverse-transcription polymerase chain reaction (RT-PCR) followed by PCR and a reconditioning PCR step to ensure that no heteroduplexes are formed which would affect the subsequent enzymatic cleavage of the PCR products and falsify quantification of restriction fragments by densitometry.
Yet, since the restriction digestion method requires large amounts of tissue, it is at its limit for single fibers and it cannot be used for single cell quantification, which is one of our future aims. Therefore we established a pyrosequencing method for relative β-MHC-mRNA quantification.

Pyrosequencing

In order to study the β-MHC-mRNA expression possibly at the single cardiomyocyte level we have started establishing a pyrosequencing method for relative mRNA quantification. In the work presented here, the method was used on larger biopsies. In principle, the method is based on sequencing PCR amplified DNA molecules (Diggle et al., 2004).

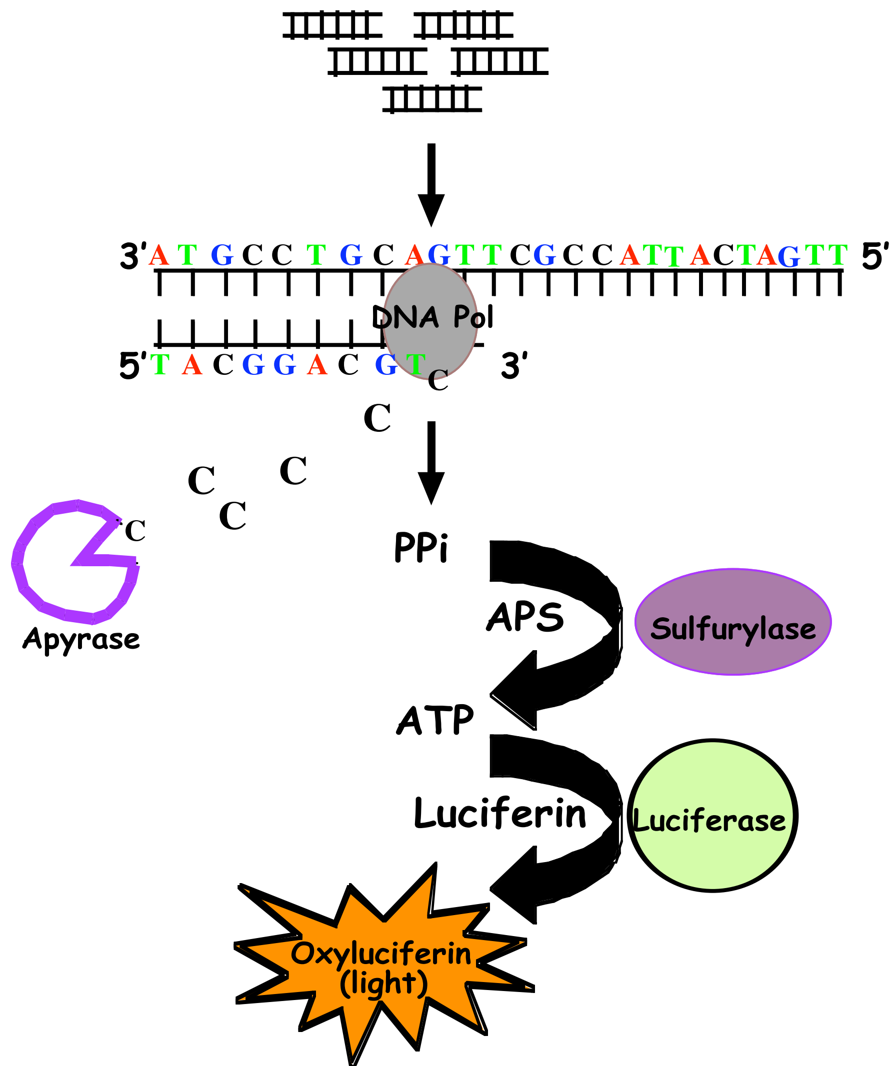


Fig. 6. Schematic representation of the principle of pyrosequencing. The light produced in the luciferase catalyzed reaction is detected by a CCD camera and is seen as a peak in the pyrogram.

In the first step of the reaction (Fig. 6), the PCR amplified single stranded PCR product is hybridized to a sequencing primer along with the enzymes DNA polymerase, ATP sulfurylase, luciferase and apyrase and substrates adenosine 5' phosphosulfate (APS) and luciferin.

The dNTPs are added in a stepwise manner and if a complementary base is found in the template strand, the nucleotide is incorporated into the growing strand accompanied with the release of the pyrophosphate (PP_i) in an amount equimolar to the incorporated nucleotide.
The released PP_i is quantitatively converted into ATP by the enzyme sufurylase in the presence of substrate APS. The ATP that is generated drives the conversion of luciferin into oxyluciferin in the presence of the enzyme luciferase and produces visible light that is proportional to the amount of ATP that was generated. The emitted light is detected by a charge coupled device (CCD) camera and is seen as a peak in the pyrogram. Since the light emitted at a specific step of the sequencing procedure is proportional to the the amount of PP_i released and hence, to the amount of the respective dNTP incorporated into the growing DNA strand at this step, this method can be used for quantification of the fractions of mutated and wild type alleles in a sample.
Apyrase enzyme continuously degrades the unincorporated dNTPs. The addition of dNTPs is performed one at a time. Deoxyadenosine alpha thio-triphosphate (dATPaS) is used as a substitute for the natural deoxyadenosine triphosphate (dATP). dATPaS is efficiently recognized by the DNA polymerase, but not by luciferase.

3 Materials and Methods

3.1 Patients and muscle biopsies.

The present study was performed on muscle biopsies from individuals who were previously clinically and genetically characterized as HCM patients with point mutations in the β-myosin head domain. All of these patients were heterozygous for the mutations they carried. *Soleus* biopsies with the R723G mutation in the β-MHC head domain were obtained from two brothers H27 (57 years) and H28 (50 years) and another younger patient H71 (34 years) of a Catalan family (Enjuto et al., 2000). For H27, after heart transplantation, myocardium from anterior wall of the left ventricle was also available. Left ventricular myocardium from a female patient (H29, 53 years) of another family, with mutation R723G, was also obtained. From a third family, *soleus* muscle was available from another young patient, H72 (30 years). All the above-mentioned five patients carried R723G mutation in the β-MHC head domain (Enjuto et al., 2000).

Another patient (H13), 22 years old at the time of biopsy, was a female from a British family and was characterized to be carrying β-MHC mutation Arg719Trp (Anan et al., 1994). She had moderate left ventricular hypertrophy (22mm) with reduced exercise capacity and received an ICD after cardiac arrest and resuscitation (NYHA Class I). Another patient (H5), 30 years old and nearly asymptomatic, was from a British family and carried the mutation V606M. The other three patients (H17, H18 and H19) were of a Krygyz family (Caucasian origin): two males and one female, and they all carried mutation Ile736Thr (Perrot et al., 2005). They were 52, 55 and 50 years old, respectively, at the time of biopsy. Furthermore, they all were in functional NYHA class II with insignificant to moderate septal hypertrophy (14 mm, 18mm, 17mm, respectively). In addition, biopsies of the *M. soleus* of healthy control individuals, H24, H25 and H26, i.e., volunteers without any known impairment of cardiac function were obtained.

The *M. soleus* biopsies of HCM patients and control individuals were excised under local anesthesia. All biopsies were approximately 10 X 10 X 15 mm. Immediately after surgery the biopsies were cooled and small muscle pieces were instantaneously shock-frozen in liquid nitrogen. Informed consent was obtained according to approved Ethics Committee protocols of all involved centers. The investigations conformed with the principles outlined in the

Declaration of Helsinki (World Medical Association, 1997). Due care was taken that this procedure occurred very fast and with immediate cooling of the muscle tissue. The entire procedure of sample preparation was the same for all biopsies taken and was carried out by the same people at the respective hospitals in Barcelona, London or Hannover (for the Kyrgyz patients). Only for the mRNA analysis of muscle tissue with mutation V606M, tissue was used that had originally been prepared at the time of biopsy for cryo-sectioning and histological examination. Prior to shock-freezing in liquid nitrogen this muscle biopsy was mounted on cork using tissue freezing medium. The biopsy was stored at –80°C at all times except for cryo-sectioning, so that intact mRNA could be obtained. All the other biopsies were stored in liquid nitrogen until mRNA-isolation.

3.2 RNA isolation, synthesis of sscDNA and PCR

Total RNA was isolated from around 3mg of frozen samples from *M. soleus* biopsies of the different patients with mutations R719W and I736T, including the biopsy with mutation V606M, using Trizol (GIBCO BRL Life Technologies, Karlsruhe, Germany). Isolated RNA was reverse-transcribed into cDNA (SuperScript First-Strand Synthesis System for RT-PCR, GIBCO BRL Life Technologies, Karlsruhe, Germany). For mutation R723G the RNA isolation, from both myocardium and *soleus* muscle, and cDNA synthesis steps were performed using a cells-to-cDNA kit (Ambion) according to the manufacturer's recommendations. For reverse-transcription (Fig. 7A and 7B) a common gene specific primer RTR719W (Table 2) was used for all mutations. The primer was placed over an intron22-exon23 junction (all nucleotides are numbered according to the GenBank accession number X52889) to reverse transcribe only mRNA and to increase the specificity of the product.
Primers were designed to specifically amplify regions of cDNA encompassing the mutation site. A summary of all the primers is found in Table 2. All PCR was carried out in a final volume of 50µl. The PCR conditions using a thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) were the same for mutations R719W, I736T and V606M. It comprised 1 minute of initial denaturation at 94°C, 22-28 cycles of 30 seconds of denaturation at 94°C and 3 minutes of annealing and primer extension at 68°C, final elongation for 3 minutes at 68°C. To obtain highly specific PCR products and prevent background due to DNA synthesis from nonspecifically primed sites, the polymerase mix

contained a proofreading enzyme and polymerase antibodies. For mutation R723G, the thermal-cycling included a step of enzyme activation at 95°C for 15 minutes as recommended by the manufacturer. The other steps included denaturation at 95°C for 1 minute, primer annealing at 68°C for 30 seconds and an extension at 72°C for 2.5 minutes. The denaturation/annealing/extension cycle was repeated for 35 cycles. A final extension at 72°C was done for 5 minutes.

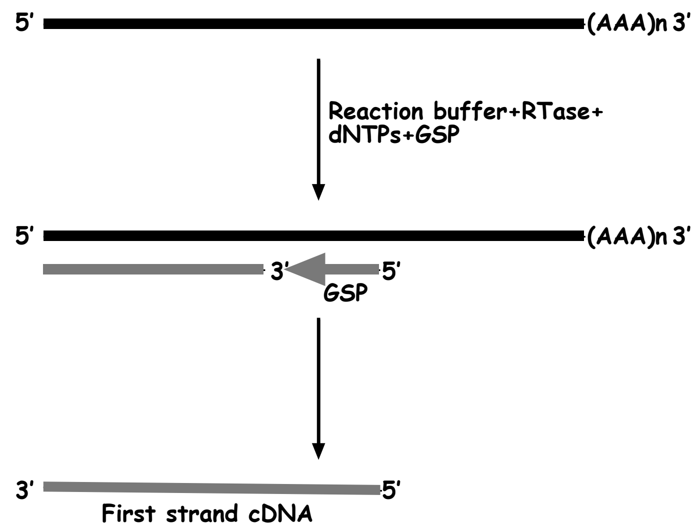


Fig. 7A. Synthesis of cDNA from mRNA using a gene specific primer (GSP). Using a GSP, instead of random hexamers or oligo-dTs ensures the generation of specific cDNAs from the mRNA; RTase, reverse transcriptase.

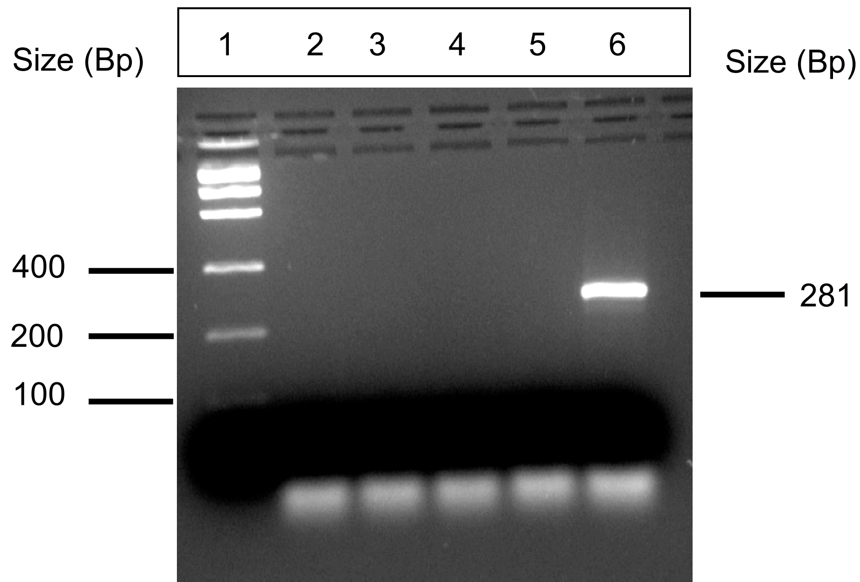


Fig. 7B. An example of the reverse transcription polymerase chain reaction (RT-PCR) performed on ventricular biopsy of patient H27. Lane 1, Low DNA Mass Ladder; Lane 2, Blank for PCR in which nuclease free water was used as template instead of the cDNA; Lane 3, Blank at the reverse transcription level wherein nuclease free water was used as template instead of mRNA; Lane 4, Blank for RT step wherein lysis buffer was used as template instead of water or mRNA; Lane 5, -RT reaction where the template mRNA was used for RT without the addition of the reverse transcriptase enzyme to the reaction; Lane 6, +RT reaction where the template mRNA was reverse transcribed into cDNA which was subsequently used for PCR. Each lane was overloaded in order to facilitate the detection of even the slightest trace of contamination.

	Primer name	Primer sequence 5'-3'
β-MHC gene specific reverse transcription primer	RTR719W	TGC CAG GTT GTC TTG TTC CG
Myosin mutations		
V606M	FEx16 606 Forward	TCC CCA AGG CCA CCG ACA TGA CCT TCA AGG CCA AGC TGT TT
	RExon 16 II Reverse	CTT TGC CCT TCT CAA TAG GCG CAT CAG
R719W	719F Forward	ACG AAG TCT CCA GGC GTG ATG GAC AAC C
	719R Reverse	CTT TTT GTA CTC CAT TCT GGC GAG CAC A
I736T	736F' Forward	ACA AAG TCT CCA GGC GTG ATG G**T**C AAC C
	719R Reverse	CTT TTT GTA CTC CAT TCT GGC GAG CAC A
R723G	R723G Mut F Forward	CCA ACC GCA TCC TCT ACG GGG ACT TCC GGC AGA GG**G** AT
	719R Reverse	CTT TTT GTA CTC CAT TCT GGC GAG CAC A

*Table 2. Summary of primer sequences. The bold **(T)** in 736F' forward and the bold **(G)** in R723G Mut F primers are the base changes introduced into the β-MHC sequence for the respective mutations.*

3.3 Restriction Digests

The PCR products were digested with the following restriction enzymes, according to the manufacturers' recommendations, for discriminating between the wild type and the mutated fragments: NdeII for R723G, MspI for R719W, Hpy8I for I736T and NlaIII for V606M. In order to distinguish the uncleaved PCR product from the wild type PCR product e.g., for mutation I736T (cf Fig. 11A), the PCR products were completely digested for all mutations. Primers and enzymes were chosen such that all PCR products contained at least one cleavage site for the respective enzyme. Conditions of digestion were optimized to ensure complete digestion in all cases.

3.4 Reconditioning PCR to avoid heteroduplex formation

A relatively high cycle number (35 cycles) was used to obtain the PCR product. To avoid the formation of heteroduplexes between wild type and mutant strands in the final PCR product, which was used for restriction digest and relative quantification, a reconditioning PCR method was used (Thompson et al., 2002; Becker-Andre et al., 1989). To achieve this, the PCR product of 35 cycles was diluted 1:5 in nuclease free water (Ambion) and 2.5µl were used in a 50µL reaction so that the final dilution was 1:100. To ensure that the cDNA used for quantification was obtained from a linear range of PCR, several such reaction mixtures were prepared. Each of these reactions were run for different cycle numbers (McCarrey et al., 1992) so that a PCR curve could be generated to determine the exponential range of the reaction (Fig. 8B, an example for mutation R723G, patient H28 is shown). For the example shown in Fig. 8B, this would be from the 1st until the 7th cycle. For quantification, the samples were taken from the exponential range of the reaction only. About 10 aliquots of PCR product from a particular cycle in the exponential range were pooled. The pooled PCR product was precipitated overnight with 3M sodium acetate (final concentration 200 mM) and 3 volumes of absolute alcohol at -80°C.

Cloning the 125bp fragment for estimation of heteroduplex formation

From the mutant PCR product (of 35 cycles) of patients with mutation R723G, the 125bp fragment (Fig. 8A) was excised from the agarose gel. The DNA in the gel piece with this fragment was purified using Gel purification kits (Qiagen). The isolated fragments were

cloned into pAlli10 vector using the Alligator Cloning Kit (Trenzyme Biotechnology). Cloning of this fragment was carried out to estimate the possible formation of heteroduplexes in a PCR where the product is obtained at high cycle numbers. The clones were sequenced at GATC (Konstanz, Germany) in the forward as well as reverse orientations.

3.5 Relative quantification

The DNA-fragments generated by the restriction digests were separated on 2,5% or 3,5% ethidium bromide (EtBr) agarose gels, using an agarose specific for high resolution of DNA bands (Metaphor or NuSieve agarose, FMC Bioproducts, Rockland, USA). For relative quantification of the different bands it was necessary to ensure that the intensities of the relevant bands were all in the linear intensity range of the gel and that the intensities corresponded to the amount of DNA in the respective bands. Therefore, different volumes of an equimolar DNA-standard (Low DNA Mass Ladder, Invitrogen AG, Basel, Switzerland) were run on each gel parallel to the restriction digests (Fig. 8C). This ladder consists of six DNA fragments. The lengths of the three faster-migrating fragments (400bp, 200bp and 100bp) relevant for our method are in the ratio 4:2:1. Since the ladder is equimolar, the fragments are present in equal numbers, i.e. the amount of DNA contained in the equimolar standard for each of these fragments is also in a 4:2:1 ratio (known from the company). Hence, if 1µl of DNA-standard is run on the gel, the amount of EtBr incorporated into each of these three fragments corresponds to their respective lengths i.e., also in the ratio 4:2:1. If IODs of the bands from lanes with different volumes of the DNA standard are plotted vs. the amount of DNA in each band, a linear relationship is expected. In an equimolar standard the bands in one lane should all have the same IOD/bp ratio or the same relative amount so that the ratio IOD/bp for the three bands of 400bp, 200bp and 100bp should appear in a 1:1:1 relation.

Intensity profiles of the individual bands of the equimolar DNA-standards and of the restriction digests of patient cDNA on the gels were obtained densitometrically using the Gel-Pro Analyzer software (Media Cybernetics, Maryland, USA and Total Lab Software (Biostep). The intensities of the peaks from the individual bands were obtained by curve fitting using the software Origin™ (Microcal™ Software, Northampton, MA, USA).

The bands of at least three lanes of standard on each gel were analyzed to check for linearity

of the plot and to ensure that intensity saturation had not been reached.

A measure for the amount of DNA in each band of the restriction digests was obtained by calculating the ratio of the total integrated intensity of this band over length of the respective fragment (IOD/bp). The fraction of mutated mRNA in each restriction digest was calculated from the IOD/bp ratio of a fragment exclusively from the mutant (e.g., the 125bp fragment in Fig. 8A) vs. the IOD/bp ratio of a fragment exclusively from the wild type (e.g. the 90bp fragment in Fig. 8A) or vs. the average of the IOD/bp values of two wild type fragments (mutation R719W). This ratio was taken as a measure for the fraction of mutated β-MHC mRNA isolated from the myocardium and/or *soleus* muscle samples of each patient. In some cases the ratio of the IOD/bp values of two fragments cleaved from the mutant or from the wild type, respectively, was calculated as internal control (e.g., the ratio of the 149bp and the 205bp fragments of mutation I736T).

Control experiments to test the relative quantifications with known plasmid mixtures

281bp wild type and mutated MYH7 sequences, encompassing the site of mutation R723G and thus, differing only by a single nucleotide (c/g), were cloned into plasmid pGA4 between the restriction sites KpnI and SacI (Geneart, Regensburg, Germany). We generated several defined mixtures of these constructs containing either wild type or the mutant sequences. The mutant:wild type ratios in these mixtures were 20:80, 40:60, 50:50, 70:30, 90:10. Amplification occurred with the primers for R723G (Table 2). For the plasmid mixtures we generated a PCR curve, like for patient cDNA (Fig 8B), to ascertain the PCR cycle number at which the reaction was still exponential. Since our objective was to determine whether the input ratios of mutant:wild type would be the same as the output ratios, reconditioning PCR was not done on plasmid mixtures. The restriction digestion, gel analysis and quantification of the fraction of mutated vs. wild type plasmid in the mixtures were done as described earlier for the DNA standard and patient samples.

3.6 Single fiber analysis

Single fibers were isolated from a freeze-dried *soleus* muscle biopsy of patient H27 with mutation R723G for relative β-MHC-mRNA quantification. Presence of water in the tissue is detrimental to biomolecules because it facilitates enzymatic hydrolysis or chemical oxidation. Freeze-drying was advantageous in the extraction of single fibers from the muscle piece

because the removal of water rendered the muscle piece quite brittle. Isolation was done in small plastic chambers surrounded by silica gel to keep the muscle sample dry. The procedure of cDNA preparation, PCR (for single fibers reconditioning PCR was not used) and relative quantification was the same as described for larger biopsy pieces.

3.7 Pyrosequencing

Pyrosequencing experiments were carried out at the Institute for Pathology in collaboration with Prof. Dr. Ulrich Lehmann. First we optimized and tested the pyrosequencing method to ensure that it can be used for the relative quantification of β-MHC-mRNA. We prepared plasmids with genomic DNA and cDNA, respectively, which carried the R723G mutation. Subsequently, control experiments were carried out also on cDNA prepared from tissue of healthy controls and HCM patients.

Plasmids carrying genomic DNA inserts

Wild type and mutant genomic DNA inserts of size 275bp from the MYH7 sequence (intron 19, position 12111- exon 20, position 12385) containing the site of the R723G mutation were cloned into pGA4 vector between KpnI and SacI restriction enzyme sites (Geneart, Regensburg, Germany).

Plasmids carrying cDNA inserts

MYH7 cDNA inserts, 281bp in size, encompassing the site of mutation R723G were cloned into plasmid pGA4 between the restriction sites KpnI and SacI (Geneart, Regensburg, Germany).

The wild type and mutant genomic DNA plasmids and wild type and mutant cDNA plasmids were mixed in specific ratios. The ratios of the wild type vs. mutant plasmids in these mixtures was 100:0, 90:10, 75:25, 50:50, 25:75, 10:90 and 0:100, respectively. PCR was performed on these mixtures using the primers listed in Table 3.

Pyro-PCR

The genomic DNA or cDNA sequences in the plasmid mixtures were amplified. The amplification conditions included an initial denaturation step of 95°C for 1 minute, primer

annealing at 60°C and extension at 72°C for 2.5 minutes. The denaturation-annealing-extension cycle was repeated 45 times. The primers used for the pyro-PCR are listed in Table 3. The reverse primer has a biotinylated tail. Therefore, the PCR product that is obtained is biotinylated. This is important for a subsequent step where the biotinylated PCR product is bound to streptavidin sepharose beads. These beads are then vacuum sucked onto a hand-grip and released into a denaturing solution, where the hydrogen bonds between the two strand of the DNA double helix are broken.
As a next step, pyro-PCR was also performed on cDNA from patients and controls. Yet the conditions for this non-circular cDNA of tissue origin has not yet been fully optimized.

Template	Primer sense	Primer name	Primer sequence
cDNA	Forward	cBMH-F1	5’-CCC CAA CCG CAT CCT CTA-3’
	Reverse	cBMH-R1-tail	5’-(GGG ACA CCG CTG ATC GTT TA) G GAT GGC CGC TGG GT-3’
Genomic DNA	Forward	BMH-F2	5’-CAG ACT CAC TGC AGA GCA TGG-3’
	Reverse	cBMH-R1-tail	5’-(GGG ACA CCG CTG ATC GTT TA) G GAT GGC CGC TGG GT-3’

Table 3. List of primers used for pyro-PCR.

Template	Sequencing primer	Primer sequence
cDNA	cBMH-pyro	5’-TTC CGG CAG AGG TA-3’
Genomic DNA	BMH-pyro	5’- CTT CCC TCC TCA GGT A-3’

Table 4. list of sequencing primers used for pyrosequencing.

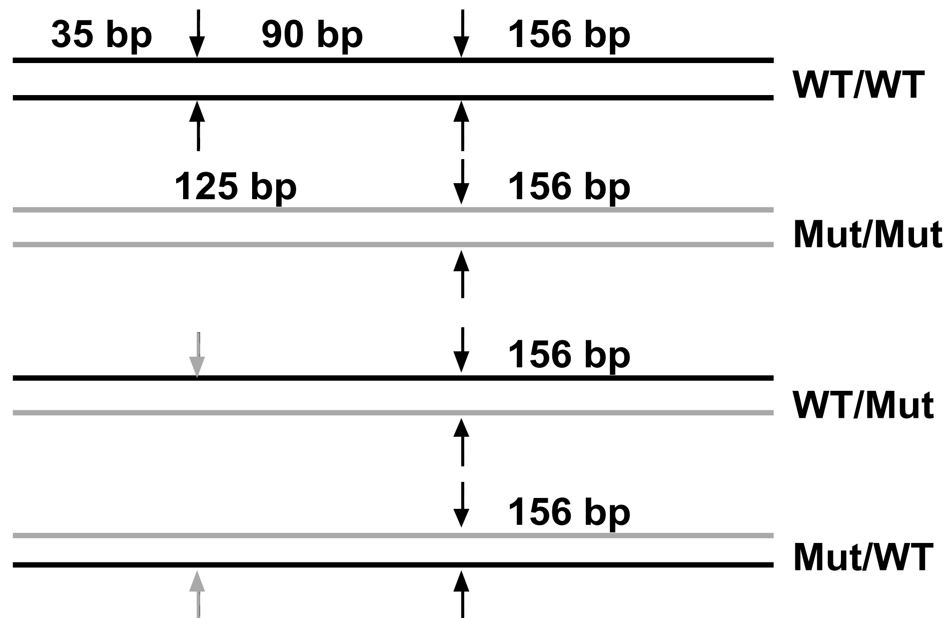
Statistics

Results are presented as the mean±SD of at least 7 or more restriction digests from at least 2 or more pieces of the muscle biopsies of each patient. Stastical comparison between two experimental groups were performed using Student’s 2-tailed *t*-test (p values ≤0.05 were considered significant).

4 Results

4.1 Relative quantification of mutant fragment in defined mixtures of plasmids.

To test our experimental method and detection system, we investigated whether in artificially generated heterozygous samples the input fraction of a specific allele would be equal to the output fraction after PCR. We used plasmids, which carried as inserts a MYH7 sequence containing the site of the R723G mutation, which upon amplification with the R723G primers yielded a 281bp PCR product. These plasmids carried either the wild type or the mutant inserts differing only by a single nucleotide (c/g). Known mixtures of these plasmids were generated in which the mutant and wild type sequences were present in the ratios 0:100, 20:80, 40:60, 50:50, 70:30, 90:10 100:0. Fig. 9A shows an example of the restriction digest products of the different mixtures. The increasing ratio of the mutant resulted in brighter bands of 125bp and weaker bands of 90bp. For these experiments the loading of the gel was done such that same intensity range as used for patient samples was achieved, as could be judged from the DNA standard. From three independent experiments performed to calculate the fraction of mutant in the known mixtures, the results were summed up and are presented in Fig. 9B**.** These data evince an estimate of the deviations that could arise in our experiments due to errors in pipetting or band analysis by densitometry and curve fitting. The fraction of mutant determined for the above-mixtures of mutant and wild type plasmids were 0%, 23.3±1.5%, 39.3±3.7%, 49±5.2%, 68±5.1%, 86.5±3.5% and 100% respectively. Therefore, during PCR, the mixed allelic templates were amplified in direct proportion to the stoichiometric fraction of each template.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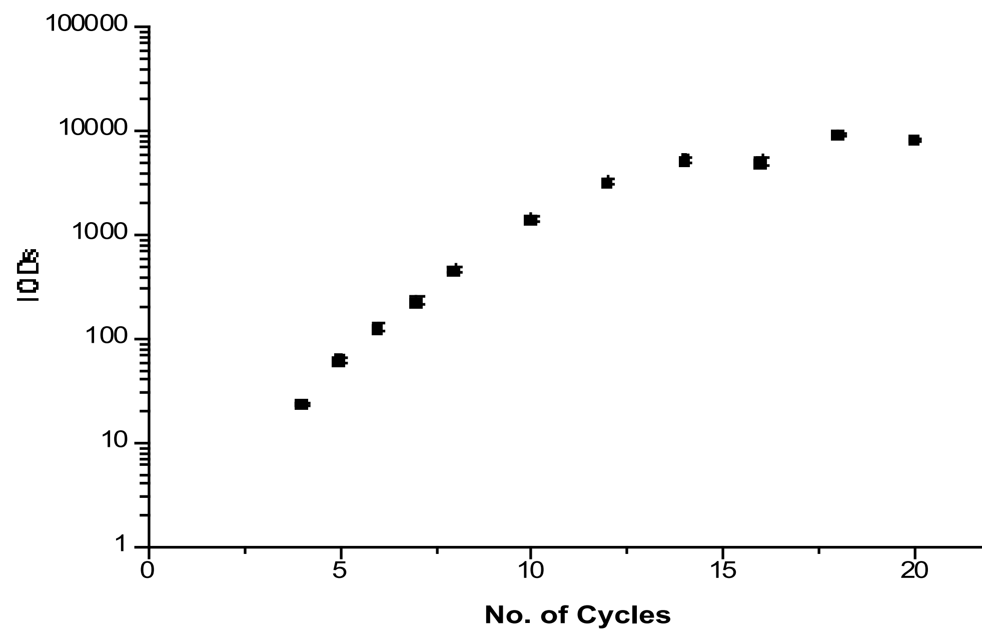
90 bp
156 bp
WT/WT
125 bp
156 bp
Mut/Mut
156 bp
WT/Mut
156 bp
Mut/WT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1000
100
10
1
IODs
0
5
10
15
20
No. of Cycles

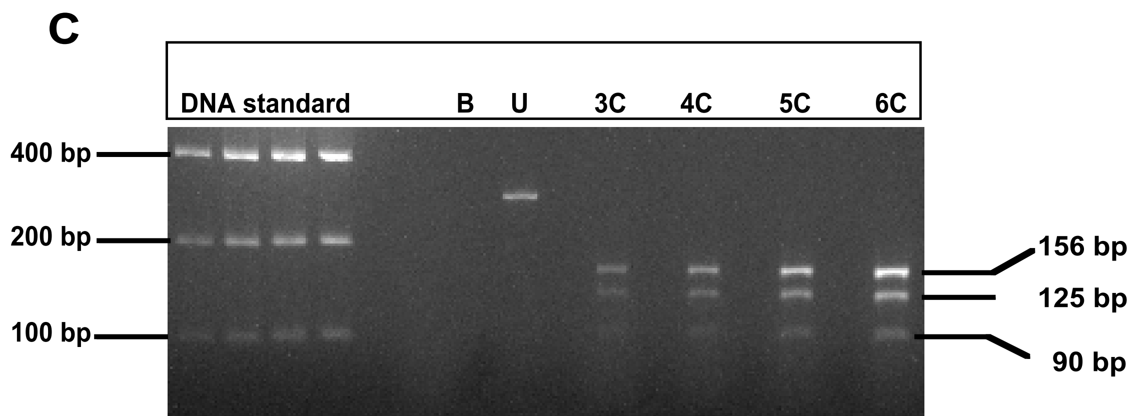


Fig. 8. Experimental approach to minimize heteroduplex formation. (A) Schematic representation of restricton fragments produced by Nde II digestion of the wild type and mutant homoduplexes for mutation R723G. The 90bp fragment is produced exclusively by the wild type and the 125bp fragment is produced exclusively from the mutant. Formation of heteroduplexes between a wild type and mutant strand makes a restriction site to become refractory (grey arrows), thus producing a 125bp fragment also from the heteroduplex. (B) Reconditioning PCR to ascertain that the samples taken for quantification are in the exponential range of the reaction. For this particular example the samples were in the exponential range approximately until the 7th cycle. The small error bars of the PCR product at a given cycle number are obtained from the averaging the intensities of the PCR product at different different exposure times. (C) A 3.5% agarose gel with restriction digests of R723G PCR products from the 3rd, 4th, 5th and 6th PCR cycle from the experiment shown in (B). The fraction of mutated β-MHC-mRNA is 70%, 67%, 63% and 63% for cycles 3, 4, 5 and 6, respectively. In case of heteroduplex formation, an increse of the relative amount of mutated β-MHC-mRNA would be expected due to the uncleaved 125bp fragment. Left four lanes, increasing volumes (6µl, 12µl, 15µl and 20µl, respectively) of Low DNA Mass Ladder; B: Blank; U: undigested 281bp PCR product.

4.2 Controls for heteroduplex formation

Because our assays contain the wild type and the mutant templates differing only by a single nucleotide, heteroduplexes might form between the two kinds of strands under denaturation/reannealing conditions in the plateau phase of PCR. As an example, Fig. 8A depicts schematically for mutation R723G how heteroduplexes which may form during the plateau phase eliminate a restriction site in such a chimeric species. The elimination of the restriction site in this case for the enzyme NdeII impairs the ability of this enzyme to recognize its cognate sequence, which is now present only on one strand. Hence, a fragment of size 125bp which is derived from the heteroduplexes would co-migrate with the fragment of the same size derived from the mutant homoduplex species on agarose gels, resulting in an overestimation of the mutant species.

In order to circumvent the problem of generation of heteroduplexes, we used an approach of a 'reconditioning PCR' whereby, the PCR products from a 35 cycles reaction were diluted and re-amplified from 1 to about 20 cycles for generation of a PCR curve (Fig. 8B). It is seen that the curve enables the determination of the exponential phase of the reaction i.e., in this case, from the 1st until at least the 7th cycle where the efficiency of the reaction is close to 2. The samples for quantification were taken from the exponential parts of the PCR curve (Fig. 8C) e.g., 4th cycle. Since at the 4th cycle the intensity of the PCR product was not strong enough for detection on ethidium bromide agarose gels, the signal was intensified by pooling several aliquots collected after the 4th cycle, before performing the restriction digestion.

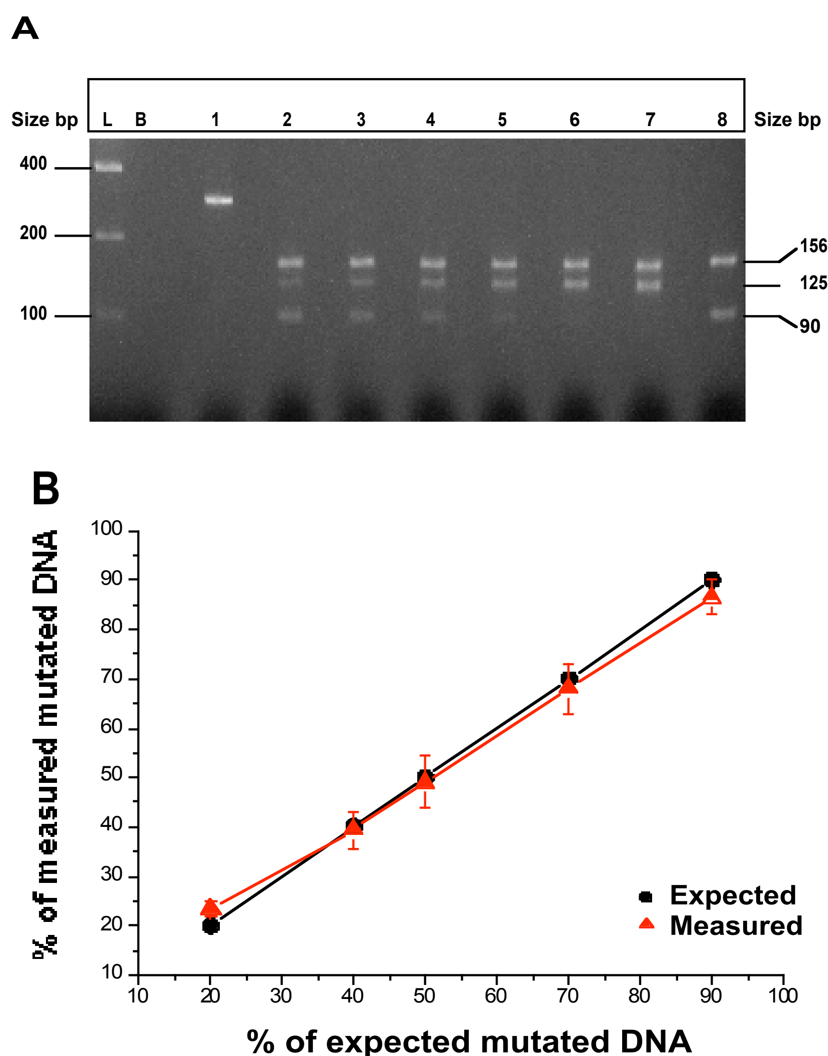


Fig. 9. Relative quantification of mutant and wild type fragments in defined mixtures of plasmids with R723G mutant and wild type sequences. (A) 3.5% agarose gel with NdeII restriction fragments of a 281bp PCR product (lane 1) amplified by the R723G primers. Lanes 2-8; restriction fragments of PCR products in plasmid mixtures with ratios of 20:80, 40:60, 50:50, 70:30, 90:10, 100:0 and 0:100, respectively, of the mutant:wild type plasmids. Lane L; Equimolar DNA standard, lane B; blank. (B) Expected (circles) vs. measured (triangles) percentage of mutant PCR product in plasmid mixtures from three independent experiments. For the above-mentioned plasmid mixtures, the fraction of mutant we found was 23.3±1.5%, 39.3±3.7%, 49±5.2%, 68±5.1%, 86.5±3.5%, 100% and 0% respectively.

Estimation of heteroduplex formation

In addition, to further estimate the extent of the possible formation of heteroduplexes for mutation R723G in a reaction of 35 cycles, the mutant-specific 125bp fragment after the NdeII cleavage (Fig. 8A) was isolated and cloned into a vector. Sequence analysis in both forward and reverse orientations of 105 cloned fragments showed only two fragments to contain sequences that result from formation of heteroduplexes.

Finding just 2 positives out of 105 samples, we put forward the question how reproducible this result is. If we repeat the experiments how sure can we be that we get a similar small number of positives. If we propose a confidence limit of 95% how large could the number of positives be? We calculated this using the binomial distribution. Given, the actually measured number of positives and sample size, the true fraction of positives lies in an interval from 0 to 0.06 with 95% confidence.

This actually means that with only 5% probability the fraction of positives measured could be more than 0.06. In our case this means that even by pushing the limits of PCR it is quite improbable that we get more than 6% heteroduplexes.

4.3 Quantification of the relative amount of mutated β-MHC mRNA for the mutation R723G

The ratio of mutated vs. wild type β-MHC-mRNA in the myocardium and *M. soleus* biopsies of one patient (H27, 57 years old) of a Catalan family (Enjuto et al., 2000) carrying the R723G mutation in the converter domain of β-MHC was quantified. Mutation R723G is caused by a c/g transversion in exon 20 of the MYH7 gene. Since this mutation neither creates not abolishes a restriction enzyme site, we used a mutagenic forward primer to create a cleavage site. Fig. 10A depicts a quantification from a *soleu*s biopsy. On the agarose gel the restriction fragments of the cDNA from this patient are shown in lanes 2, 3, 4 and 5, respectively. The uppermost 156bp band is derived from, both the mutant and the wild type PCR products, the 125bp exclusively from the mutant and the 90bp exclusively from the wild type (c.f. Fig. 8A). A 35bp fragment, also produced by the digestion of the wild type PCR product is not visible, owing to its small size. Hence, the intensity of the 35bp fragment was excluded from our calculation of the proportion of mutant mRNA present in the heterozygous patient cDNA. The 156bp fragment served as an internal control in the calculations as it is

derived from both the wild type and the mutant PCR products. We calculated the ratio of IOD/bp values of the sum of 125bp fragment and 90bp fragment over the IOD/bp value of the 156bp fragment to ensure that this ratio was close to 1.

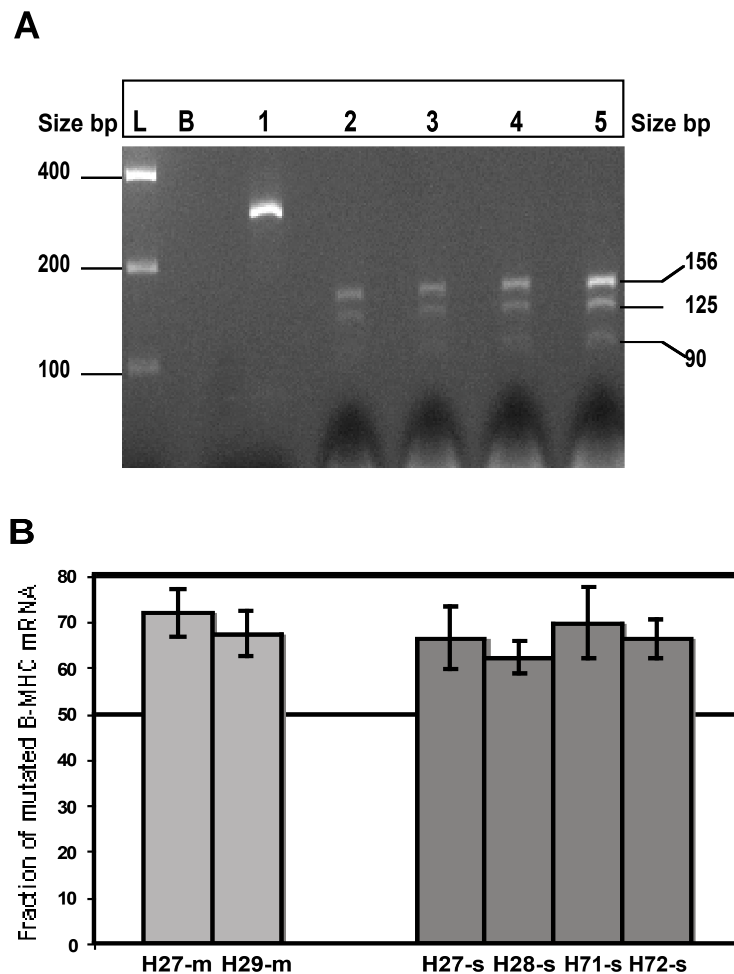


Fig. 10. Relative quantification of wild type and mutant β-MHC-mRNA for mutation R723G. (A) 3.5% agarose gel with restriction fragments (lanes 2-5) of a 281bp PCR product (lane 1) from the soleus muscle of patient H27. Restriction fragments were obtained after reconditioning PCR for 3, 4, 5 and 6 cycles respectively. Fragments are as depicted in Fig. 8A. (156bp from mutant + wild type, 125bp from mutant and 90bp from wild type PCR products, respectively). L, equimolar DNA standard; B, blank. (B) Fraction of mutated mRNA in the myocardium and M. soleus of several patients of three families with mutation R723G. The fraction of mutated β-MHC-mRNA in the myocardium (m) of patient H27 was 71.8±5.3% (n=12 restriction digests) and that in his soleus (s) muscle was 66.4±6.9%

(n=10). Patient H29 had 67.2±4.9% (n=28) of mutated β-MHC-mRNA in the myocardium (m). For other patients, H28 (n=7), H71 (n=16) and H72 (n=15) the fractions of mutated β-MHC-mRNA in the soleus muscle (s) were 62±3.5%, 69.5±7.8% and 66.1±4.3%, respectively.

We found that the fraction of mutated β-MHC-mRNA in the *soleus* muscle of patient H27 was 66.4±6.9% (n=10 restriction digests) and in the anterior wall of the myocardium of this patient was 71.8±5.3% (n=12 restriction digests) (Fig. 10B). Thus, the fraction of mutated mRNA for the mutation R723G deviates clearly from the 50% expected for an autosomal dominant disorder. Yet this deviation is very similar for myocardium and *soleus* of the same patient. In H28 (50 years), the brother of H27, the fraction of mutated mRNA in the *soleus* muscle biopsy was 62±3.5% (n=7 restriction digests). We also analyzed *soleus* muscle samples from a younger patient (H71, 32 years) of the same family and found that the fraction of mutated β-MHC mRNA for this patient was 69.6±7.7% (n=16 restriction digests). A female patient, H29, with mutation R723G, from a second family, for whom only the myocardium samples were available, had 67.12±4.9% (n=28 restriction digests) mutated β-MHC-mRNA in the left ventricle. In yet another younger patient, H72 (30 years old) from a third Catalan family, the *soleus* muscle had 66.1±4.3% (n=15 restriction digests) mutated β-MHC-mRNA.

The difference in expression of mutated β-MHC-mRNA in the two tissues of H27 is not significant (p=0.064). In addition, the expression level of mutated β-MHC-mRNA between the other patients is not significantly different (p≥0.05), yet for H28 it is slightly lower (p=0.05) compared to H27 *soleus*. For all these patients the wild type:mutant ratio of β-MHC-mRNA deviates from the expected 50:50 ratio. The data further indicate that the expression of the mutated β-MHC-mRNA seems to be very similar among patients with this mutation, even when they are from different families. From the presented results of patient H27, and also from H29 in comparison to the *soleus* biopsies from the other patients with R723G, it can be inferred that the fraction of mutated β-MHC-mRNA in the slow skeletal muscle appears to be reflective of its expression in the myocardium.

4.4 Relative quantification of mutated mRNA for the mutations I736T, V606M and R719W in *soleus* biopsies

To establish whether the deviation from 50:50 of the wild type and mutated mRNA was specific only to the above shown R723G mutation or whether it could be generalized for other HCM missense mutations in the coding region of the β-MHC gene, several other mutations were characterized with respect to the fraction of mutated β-MHC-mRNA expression in *soleus* muscle biopsies. The IOD/bp values for mutation V606M and R719W were obtained previously by I. Schulte (Schulte, 2001). These data are shown here to be able to compare all the patient data we have until now.

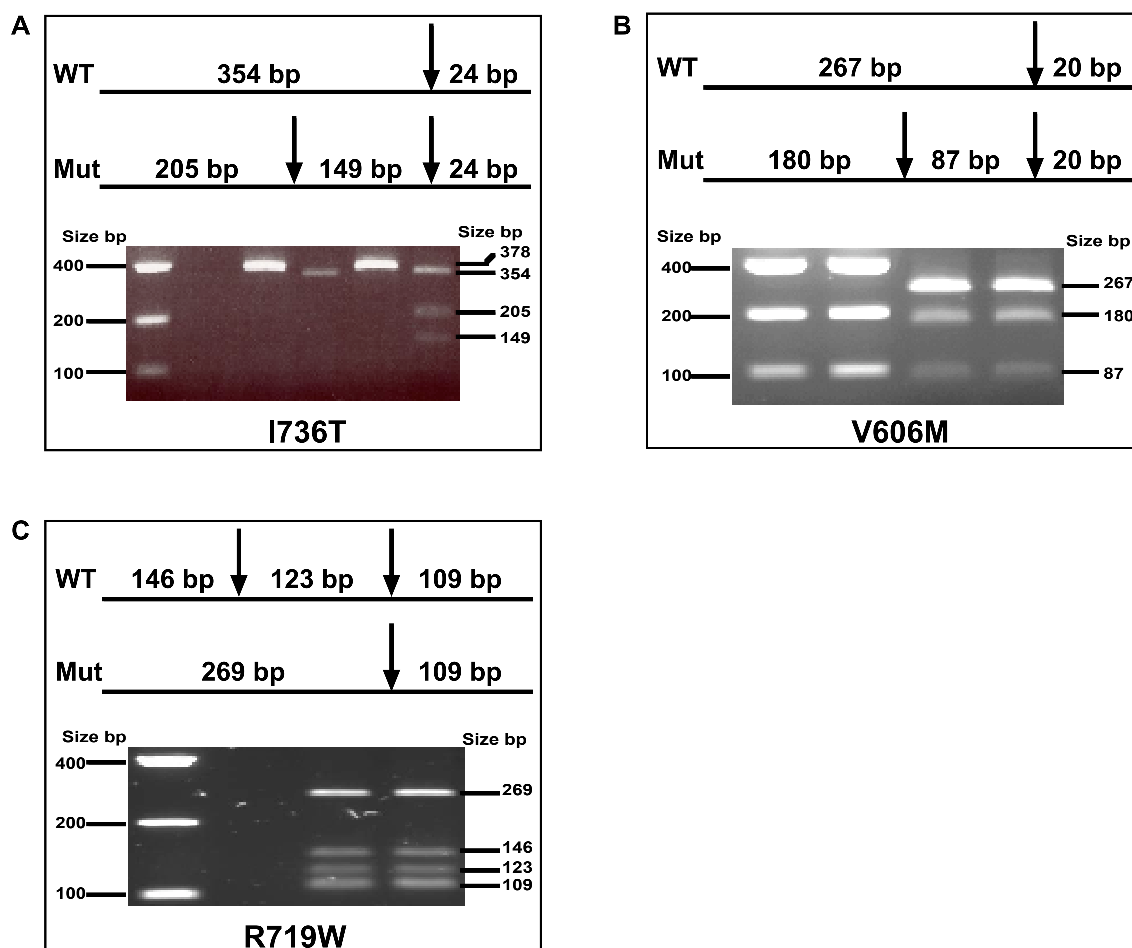


Fig. 11. Relative quantification of the fraction of mutated β-MHC-mRNA for different myosin point mutations. (A) Hpy8I restriction fragments for the quantification of mutated β-MHC-mRNA for mutation I736T. The 378bp heterozygous PCR product yields 205bp, 149bp and

24bp fragments from the mutant and 354bp and 24bp fragments from the wild type PCR products, respectively. Lane 1, Low DNA Mass ladder; Lane 2, Blank; Lanes 3 and 5, undigested PCR product; Lane 4, digest of PCR product of control cDNA; Lane 6, restriction fragments of PCR product of heterozygous patient PCR product. The 24bp fragment is not seen on the agarose gel owing to its small size. (B) Restriction digestion of a 287bp heterozygous PCR product by restriction enzyme NlaIII for mutation V606M. The digestion yields a 180bp and 87bp and 20bp fragment from the cleavage of the mutant PCR product and a 267bp and 20bp fragment from the wild type PCR product. The faster migrating 20bp product is not seen on the gel. Lanes 1 and 2, Equimolar DNA standard; Lanes 3 and 4, restriction fragments of heterozygous patient PCR product. (C) Restriction fragments generated by MspI cleavage of a 378bp heterozygous PCR product for mutation R719W. The wild type PCR product upon cleavage furnishes 146bp, 123bp, 109bp fragments, while the mutant PCR product yields a 269bp and 109bp fragment. Lane 1, Equimolar DNA standard; Lane 2, Blank; Lanes 3 and 4; restriction fragments of heterozygous patient PCR product. The gel pictures in (B) and (C) were obtained by I. Schulte (2001) during her doctoral thesis in our laboratory (Molekular-und Zellphysiologie, MHH).

I736T

Total mRNA was isolated from *soleus* biopsies of three HCM-patients with the mutation I736T, all of them members of the same family, and β-MHC-mRNA was reverse transcribed into cDNA. The I736T mutation is caused by a T2293C transition in exon 20 of MYH7 gene, which creates a cleavage site for *Hpy*8I in the mutant sequence. The wild type sequence has no cleavage site for *Hpy*8I. However, to distinguish wild type and undigested PCR product, we had to ensure that the wild type product was cleaved too. Therefore a cleavage site was introduced into the PCR product by using a mutagen forward primer (Table 2). The 378bp PCR product when cleaved with *Hpy*8I, generates fragments of sizes 354bp and 24bp from the wild type sequence and of sizes 205bp, 149bp and 24bp respectively, from the mutant sequence. A restriction digest of β-MHC-cDNA obtained from the *soleus* muscle tissue of a heterozygous patient and a control individual along with the equimolar DNA ladder used for analysis is shown in Fig. 11A. For all three patients the fraction of mutated β-MHC-mRNA was calculated based on the IOD/bp ratio of the 205bp fragment of the mutant and the IOD/bp ratio of the 354bp fragment of the wild type, i.e., only the larger restriction digest

fragments which are clearly in the linear IOD/bp range of the equimolar ladder were used. The fraction of mutated β-MHC-mRNA was found to be 37,6 ± 4,2 % (n=22 restriction digests), 39,1 ± 4,1 % (n=20 restriction digests) and 38,6 ± 5,3 % (n=24 restriction digests) for patients H18, H19 and H20 respectively. Thus, for all the three patients a significant deviation in wild type:mutant ratio from 50:50 was observed. As an internal control for the restriction digest analyses of mutation I736T, we also calculated the ratio of the IOD/bp values for the mutant fragments of 149bp and 205bp. The ratio was 0.96±14, 1.01±0.09 and 0.99±0.07 for patients H18, H19 and H20, respectively. This indicates in most cases, a rather small deviation from the expected 50:50 ratio of these control fragments. Altogether, the analysis of mutation I736T consolidates the observation also made for R723G, that within a family, i.e., for a given mutation, the expression of the mutated mRNA does not vary significantly.

V606M

For amplification of the cDNA from the patient with the V606M mutation forward primer FEx16 606, located within exon 16, and reverse primer RExon 16 II (Table 2), located at the splice junction between exons 16 and 17 were used (Schulte, 2001). These primers specified a 287bp PCR product. The V606M mutation is caused by a G1902A transition in exon 16, which creates an additional *Nla*III cleavage site in the mutant sequence. *Nla*III cleavage of a heterozygous 287bp PCR product, containing both wild type and mutant sequences, yields fragments of sizes 267bp, 180bp, 87bp and 20bp, respectively (Fig. 11B). The 20bp fragment is not seen due to its small size. The cleavage of the mutant PCR product yields two mutant-specific fragments of 180bp and 87bp. Due to uncertainties in determination of the IOD values for the smaller and weaker 87bp fragment, we used the larger 180bp fragment to calculate the fraction of mutated β-MHC-mRNA in these biopsies. The relative amount of mutated β-MHC-mRNA in the *M. soleus* from the patient with mutation V606M on an average is 28±6% (n=40 restriction digests, IOD/bp values from I. Schulte). The uncertainties in analysis of the 87bp fragment of the mutant PCR product is also evident when we calculate as internal control the ratio of the 87bp fragment vs. the 180bp fragment which was 0.85±0.17. The quite large deviation from the expected 1:1 ratio most likely is due to difficulties in measuring the intensity of the rather weak 87bp band. It is not expected to be due to incomplete digestion of the PCR product, since no undigested product and no cleavage

products of sizes other than the expected ones were found on the gels. Nevertheless, this mutation clearly shows a quite large deviation in expression of mutated:wild type β-MHC-mRNA from the expected 50:50 ratio.

R719W

The R719W mutation results from a C2241T transition, which abolishes an *Msp*I restriction site in the mutant sequence. From five *soleus* muscle pieces of the patient with mutation R719W, mRNA was isolated and separately reverse transcribed into cDNA (Schulte, 2001). Table 2 lists the primer pairs used to amplify the cDNA for this mutation. These primers specify a 378bp PCR product. Fig. 11C shows an example of a gel used for the analysis of mutation R719W. The *Msp*I digest of the 378bp wild type PCR product resulted in three fragments of 146bp, 123bp and 109bp, as expected for a control individual. The heterozygous patient shows the three bands characteristic for the wild type allele, with an additional band of 269bp, which is characteristic for the mutant allele (Fig. 11C). The fraction of mutated PCR product and thus β-MHC-mRNA in the patient sample was calculated by taking the average of the almost identical IOD/bp values of the two wild type fragments (the 146bp fragment was 50.4±1.8% of the total amount of the 146bp and the 123bp fragment together) and the IOD/bp value of the 269bp fragment into account. For the patient with the mutation R719W, the fraction of the mutated β-MHC-mRNA was 57.1 ± 5.4% mutant β-MHC mRNA (n=41 restriction digests, IOD/bp values from I. Schulte, 2001), and thus deviates somewhat from 50:50. As an internal control and to obtain an error estimate of the relative quantification for mutation R719W, we also calculated the ratio of the two wild type fragments of 123bp and 146bp on the gels. This yielded a ratio of 0.99±0.07, which is rather close to the expected 1:1 ratio.

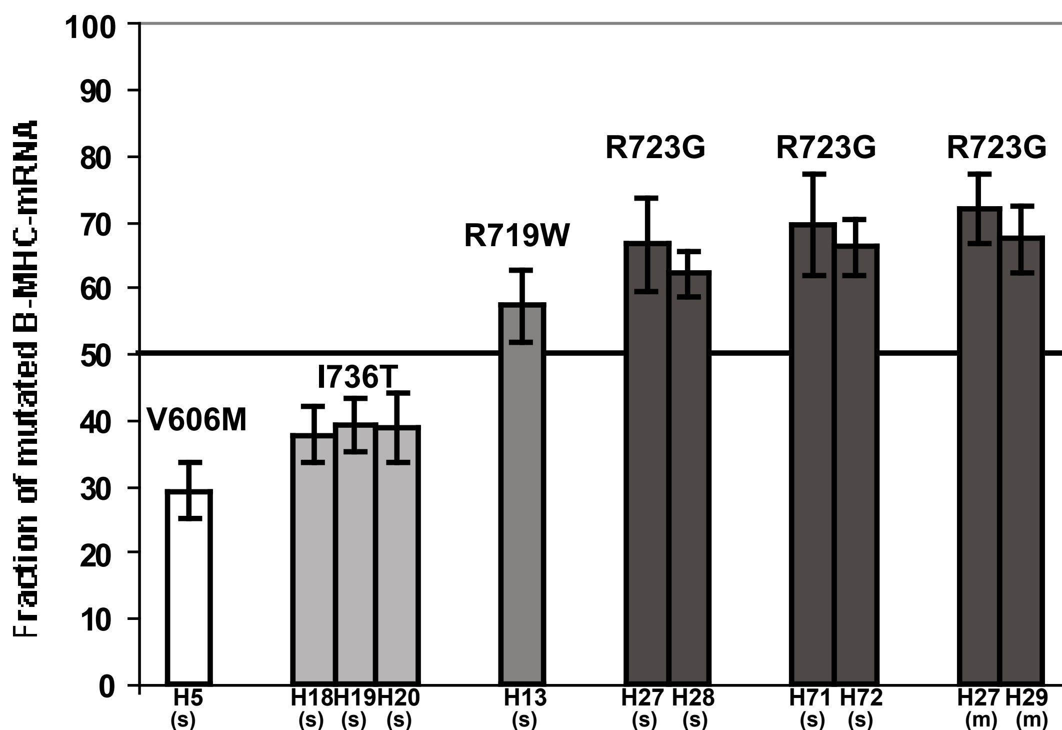


Fig. 12. Fraction of mutated β-MHC-mRNA for different mutations in M. soleus (s) and myocardium (m) of different patients (H). All the mutations show a deviation from 50:50 in the ratio of mutated:wild type β-MHC-mRNA, although it might not be a significant deviation for R719W. Note that mutations I736T and R723G exhibit an intra- and/or inter-familial similarity in expression of mutated β-MHC-mRNA: all three patients with mutation I736T are siblings, patients with mutation R723G are either siblings (H27 and H28) or from the same family (H27, H28 and H71) or from different families.

The data for all patients and samples are summarized in Fig. 12. This shows that the ratio of wild type and mutated β-MHC-mRNA is, in general, quite variable between different mutations. It deviates in all patients from the expected 50:50 ratio and is similar between family members as exemplified by mutations R723G and I736T.

4.5 Fraction of mutated β-MHC-mRNA at single fiber level

To address the question whether the deviation from a 50:50 ratio of mutated:wild type β-MHC-mRNA is due to variable expression in individual muscle fibers, relative quantification of the mutant:wild type ratio in single *soleus* muscle fibers was attempted.

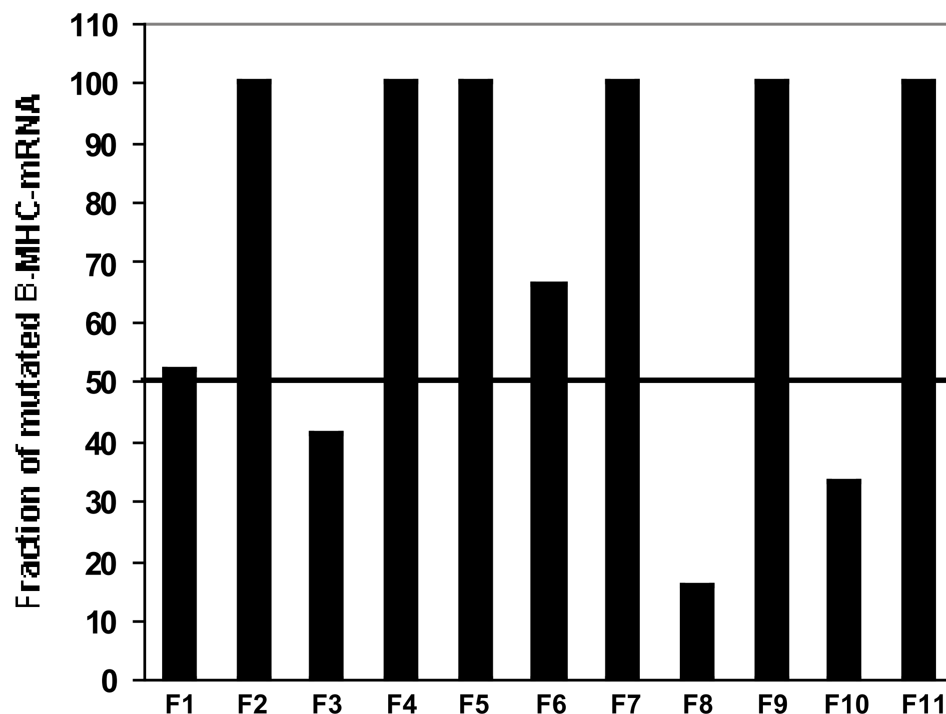


Fig. 13. Preliminary experiments on single fibers (F1-F11) of M. soleus from patient H27, carrying the mutation R723G, for quantification of the fraction of mutated β-MHC-mRNA. The fraction of mutated β-MHC-mRNA varies among individual fibers, in some fibers, the mutated mRNA is not expressed, in other fibers both the mutated and wild type β-MHC-mRNAs are expressed to variable extents. For each fiber n=1. Only single analyses were possible with the small amount of cDNA obtained from each fiber. The number of fibers studied here is too small to be representative of the whole muscle tissue.

In preliminary experiments on a *soleus* muscle biopsy of patient H27 with mutation R723G, eleven single fibers were analyzed in order to determine the fraction of mutated β-MHC-mRNA in each of these fibers. It was observed that there is a variable expression of the mutated β-MHC-mRNA among individual fibers. Out of the 11 fibers that were analyzed

fibers 2, 4, 5, 7, 9 and 11 expressed 100% wild type β-MHC-mRNA. In the remaining five fibers the fraction of mutated β-MHC-mRNA varied considerably.

Fibers 1, 3, 6, 8 and 10 had 52%, 41%, 66%, 16% and 33%, respectively, of the mutated β-MHC-mRNA (Fig. 13). Yet, not enough fibers were studied until now for the result to represent the average of the whole muscle. Since the amount of mRNA extracted from each fiber was sufficient only for one experiment, data from repetitions were not obtained. These data seem to indicate a variation in expression of mutated β-MHC-mRNA in individual fibers.

4.6 Pyrosequencing Experiments

A pyrosequencing method was established for relative quantification of the mutated:wild type β-MHC-mRNA ratios in much smaller samples, possibly even in single cardiomyocytes.

Control experiments with plasmids

To test the pyrosequencing method, 281bp MYH7 wild type (C allele) and mutant (G allele) cDNA sequence including the site of mutation R723G (caused by a c/g transversion) were cloned into plasmids. Several defined mixtures with wild type:mutant ratios 100:0, 90:10, 75:25, 50:50, 25:75, 10:90 and 0:100, respectively, were generated with these plasmids. Fig. 14A (i-iii) illustrate the pyrograms for the plasmid mixtures of 100:0, 50:50 and 0:100 wild type:mutant composition.

Fig. 14A(i). Plasmid mixture: 100% WT 0% Mut

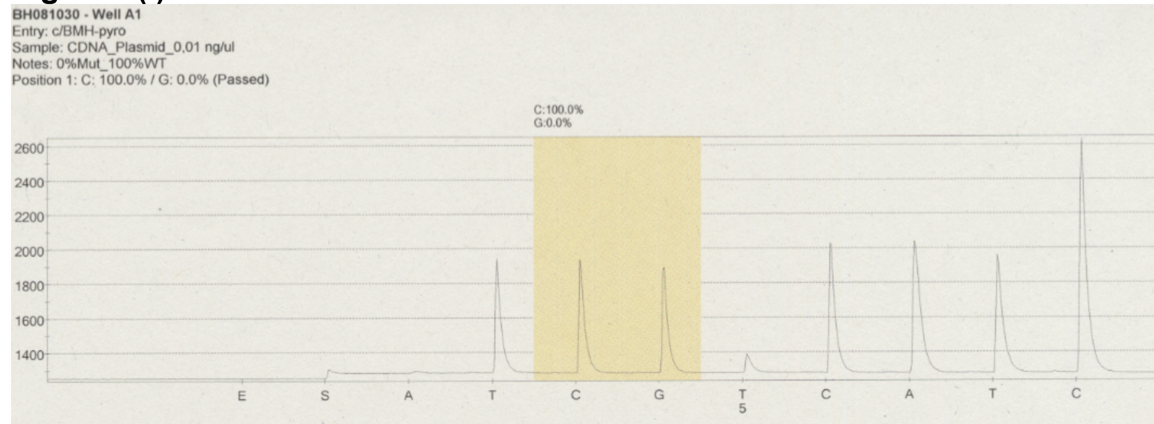


Fig. 14A(ii). Plasmid mixture: 50% WT 50% Mut

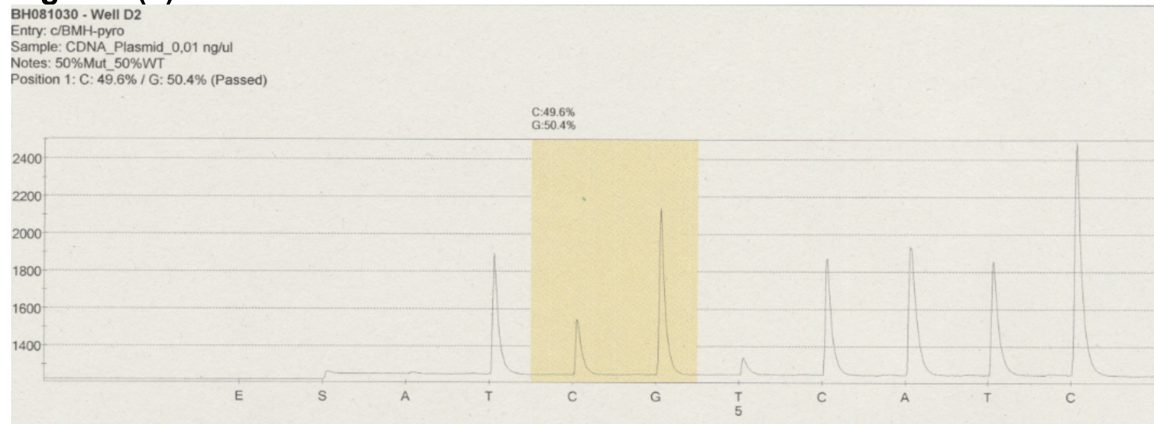
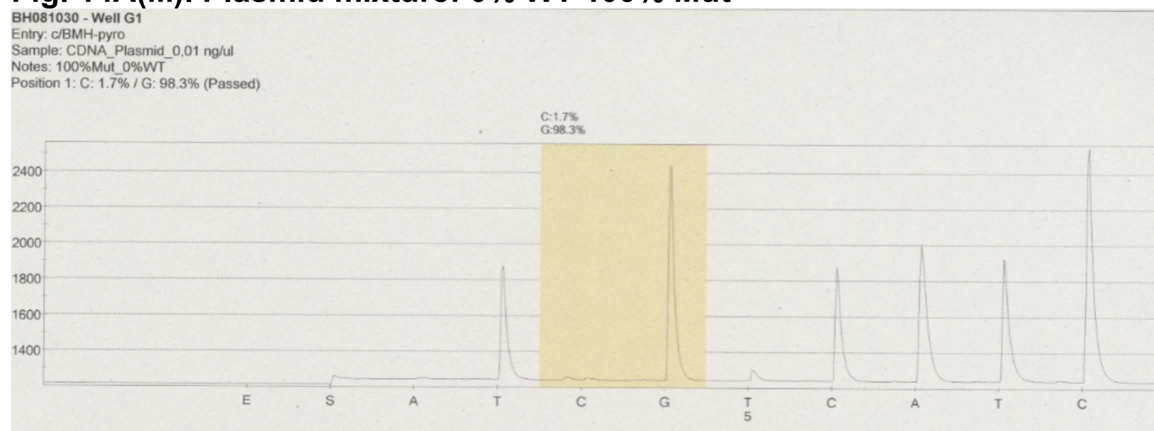


Fig. 14A(iii). Plasmid mixture: 0% WT 100% Mut



14B

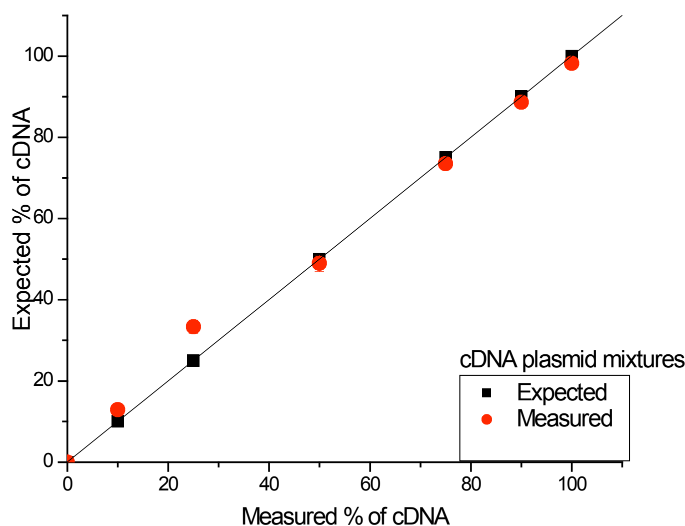


Fig. 14. (A) Pyrosequencing experiment with mixtures of plasmids carrying 281bp R723G wild type and mutant cDNA inserts. Pyrograms generated from defined mixtures of plasmids containing as inserts the 281bp wild type and mutant sequences for mutation R723G. On the y-axis is the peak height (amount of light generated), which is proportional to the amount of nucleotides incorporated into the growing DNA strand. The x-axis shows the nucleotide dispensation order. The peak (G) in the SNP detecting yellow area of the pyrogram of 100% wild type and 0% mutant in Fig. 14A(i) is not due to the mutation but due to another G, which is adjacent to the position of the SNP. The concentrations of both the wild type and mutant plasmids in the stock solution was 0.01ng/µl. (B) Plot of the expected vs. measured fractions of mutated β-MHC cDNA in plasmid mixtures (n=2).

Fig. 14A(i) shows a 100:0 wild type vs. mutant pyrogram. Whereas, the peak for the C allele denotes 100% wild type, the peak of a similar height for the G allele comes from a G nucleotide towards the 3' end of the SNP position. Note how in the 50:50 pyrogram (shown as example in Fig. 14Aii), the peak height of the C allele decreases with the reduction in the fraction of the wild type plasmid and the G allele peak height increases due to the increase in the fraction of mutant plasmid in the plasmid mixture. The results of two such experiments are summed up in Fig. 14B, which gives an estimate of the error that could arise due to

pipetting or other experimental procedures. Plasmids differing by a single nucleotide (c/g) in their 275bp genomic DNA MYH7 inserts encompassing the site of the R723G mutation were also used for further control experiments.

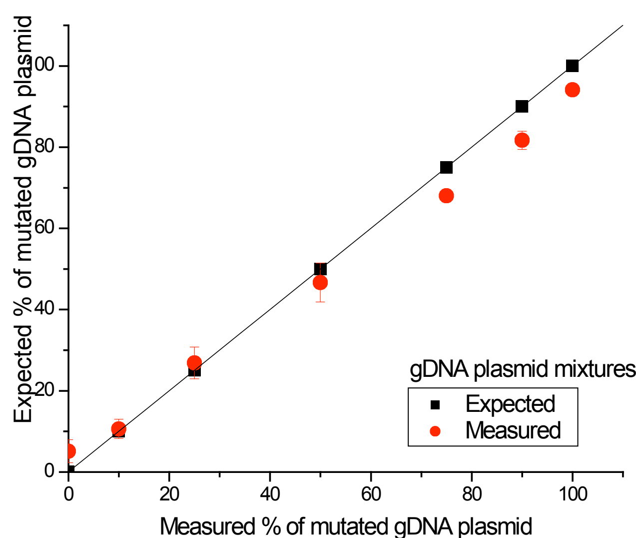


Fig. 15. Pyrosequencing experiments with mixtures of plasmids carrying 275bp MYH7 genomic wild type and mutant sequences including the site of mutation R723G. Plot of expected vs. measured mutant insert in mixtures of plasmid containing the genomic sequence fragments including the site of mutation R723G. The plasmids were mixed in a wild type:mutant ratios of 100:0, 90:10, 75:25, 50:50, 25:75, 10:90 and 0:100%, respectively. The concentrations of the mutant as well as the wild type plasmids in the stock solution was 0.0001ng/µl (n=4).

The genomic wild type and mutant plasmids were also mixed in 100:0, 90:10, 75:25, 50:50, 25:75, 10:90 and 0:100, respectively, and were subjected to pyrosequencing. The results of 4 experiments are presented in Fig. 15. The genomic controls were necessary because we wanted to test whether the patient samples start deviating from 50:50 at the genomic level.

Pyrosequencing experiments with genomic DNA and cDNA

Fig. 16(A). Pyrogram from healthy control (S.T.)

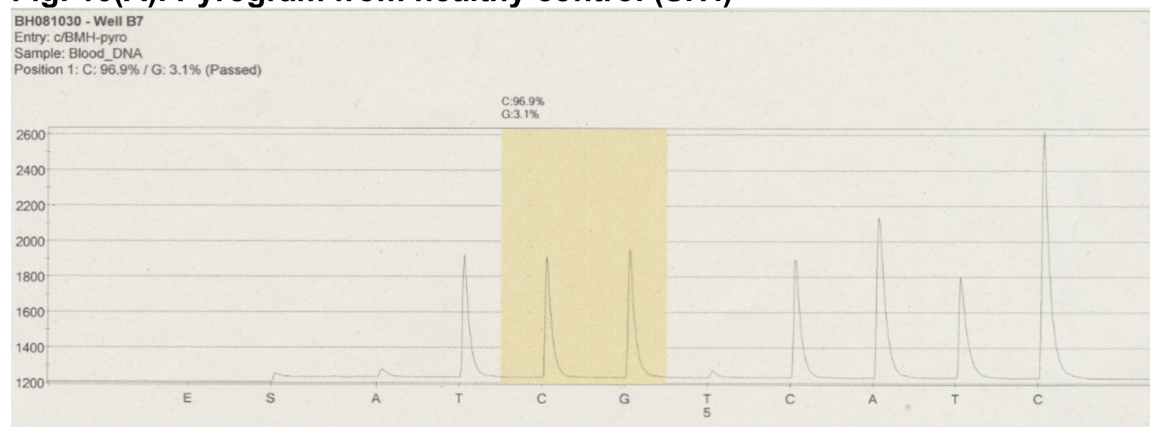


Fig. 16(B). Pyrogram from H27

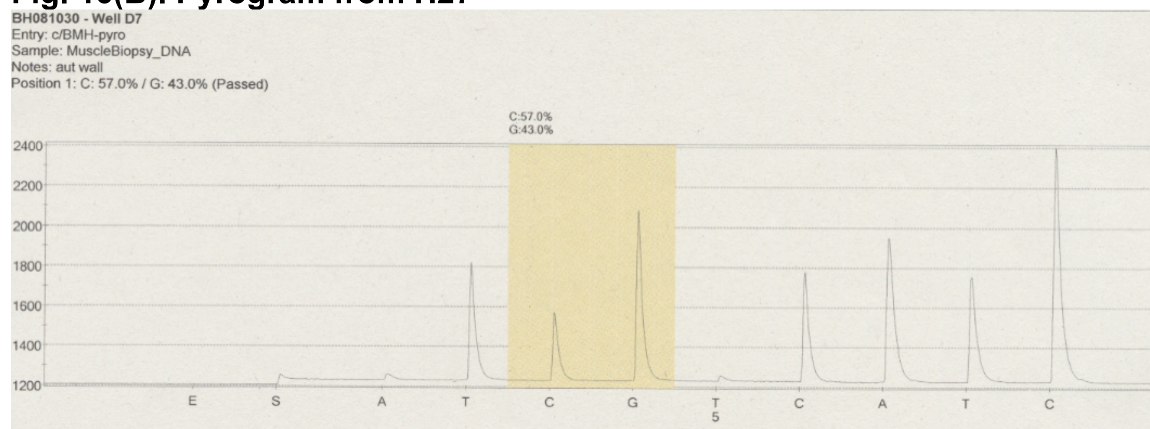


Fig. 16. (A) Pyrosequencing experiments with genomic DNA of human samples. Pyrosequencing results of PCR products obtained from the genomic DNA extracted from whole blood of a healthy control. The wild type allele was 96.9% in this analysis. (B) Pyrogram generated from the PCR product obtained from genomic DNA in the myocardium muscle piece of H27, carrying the R723G mutation. The fractions of wild type and mutated alleles, respectively, were 57% and 43%.

Fig. 17(A). Pyrogram from healthy control H22

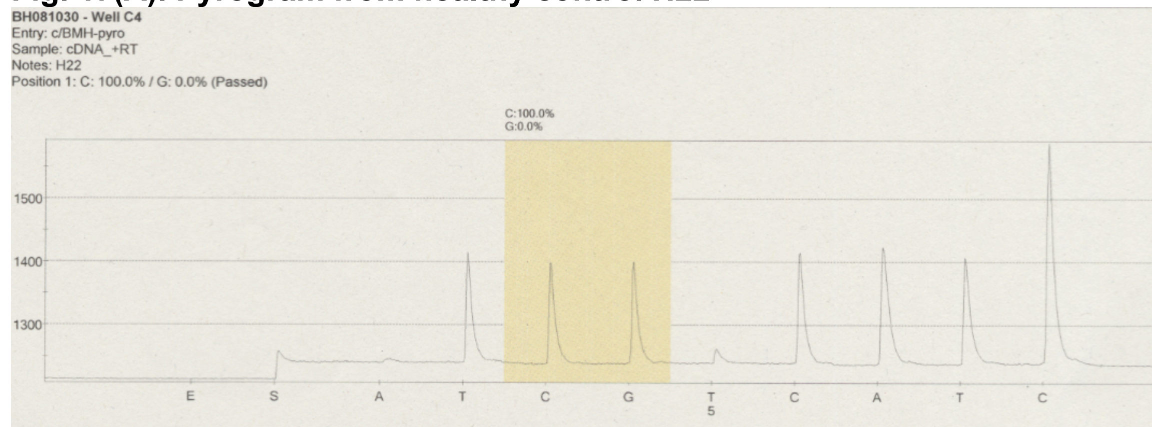


Fig. 17(B). Pyrogram from healthy control H32

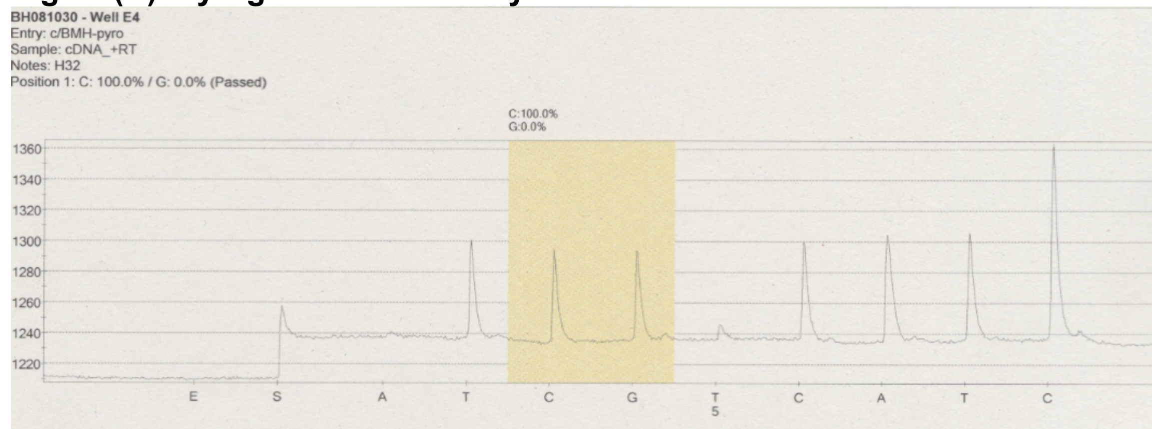


Fig. 17. Pyrosequencing experiments with control cDNA. (A) cDNA from the soleus biopsy of a healthy individual H22 and (B) cDNA from a myocardial sample of a control individual, H32, was subjected to PCR with the pyro cDNA primers listed in Table 3 and subsequently pyrosequenced. In both cases we found 100% wild type (C) and 0% mutant (G) allele.

Pyrosequencing was performed on the PCR amplified genomic DNA from the myocardium of patient H27. We found a fraction of the wild type (C) allele of 57% and of the mutant (G) allele of 43% (Fig. 16B). Another pyrosequencing control experiment was carried out on genomic DNA of control individuals where DNA was extracted from whole blood and amplified with genomic primers listed in Table 3. As expected, the wild type (C) allele was 96.9%, which is very close to 100%. Since more experiments were not performed on these

control samples, the estimation of error was not possible. Until now, relative quantification of cDNA from HCM patient samples with the pyrosequencing method has not been carried out successfully. Experimental conditions still need to be optimized. Instead, pyrosequencing experiments were successfully performed with the cDNAs from the *soleus* and myocardium, respectively, of two healthy controls H22 (Fig. 17A) and H32 (Fig. 17B). As expected the wild type (C) β-MHC-mRNA for both individuals was 100%.

5 Discussion

In this study we found a variable expression of the allelic messages for different myosin head domain missense mutations in HCM. Autosomal dominant inheritance of HCM, when it is caused by single missense mutations in the cardiac myosin isoform, a priori implies that both mutated and wild type mRNA and protein are present at a 50:50 ratio in the muscle tissue of the patients heterozygous for the mutation. Yet, our results show that for four different β-MHC head domain missense mutations, there is a deviation from the expected ratio in the β-MHC-mRNA expression. The deviation from the expected ratio could arise due to several factors.

Method of quantification.

Our assays employ co-amplification of wild type and mutated β-MHC-cDNAs in the same reaction followed by digestion of the PCR product with a restriction enzyme to differentiate between the wild type and the mutant PCR products. It is important to note that in the muscle tissues used for this study the mutated and the wild type β-MHC-mRNAs are co-expressed and would therefore have been subjected to the same experimental insults. Therefore, it is rather unlikely that the two species differing from one another by only a single nucleotide are differentially affected by the experimental protocols. For each mutation the mutant and wild type sequences were reverse-transcribed in a single tube, respectively, using a universal β-MHC specific primer. Likewise, for each mutation the mutated and the wild type sequences were amplified using the same primer set (Table 2). Also, the restriction endonuclease used to differentiate between the mutated and wild type PCR products for a particular mutation was the same.

One problem that would arise with this quantification method is the heteroduplex formation at high PCR cycle numbers. Heteroduplexes would yield faulty quantification results because a chimeric DNA molecule (consisting of one wild type strand and one mutant strand) makes a restriction site refractory to the restriction enzyme, causing a fragment of the digest (125bp, Fig. 8A) from the heteroduplexes to co-migrate with the fragment of the digest of the same size (125bp) from the homoduplex mutant DNA molecule. On the other hand sufficient PCR product is necessary for quantification of restriction digest products on the agarose gels. To circumvent the risk of erroneous quantification due to heteroduplex formation we established a reconditioning PCR method where PCR products from lower cycle numbers were pooled

for restriction digestion and gel analysis. Yet, even with the reconditioning PCR method (Thompson et al., 2002) to avoid heteroduplex formation, the deviation from 50:50 ratio was observed.

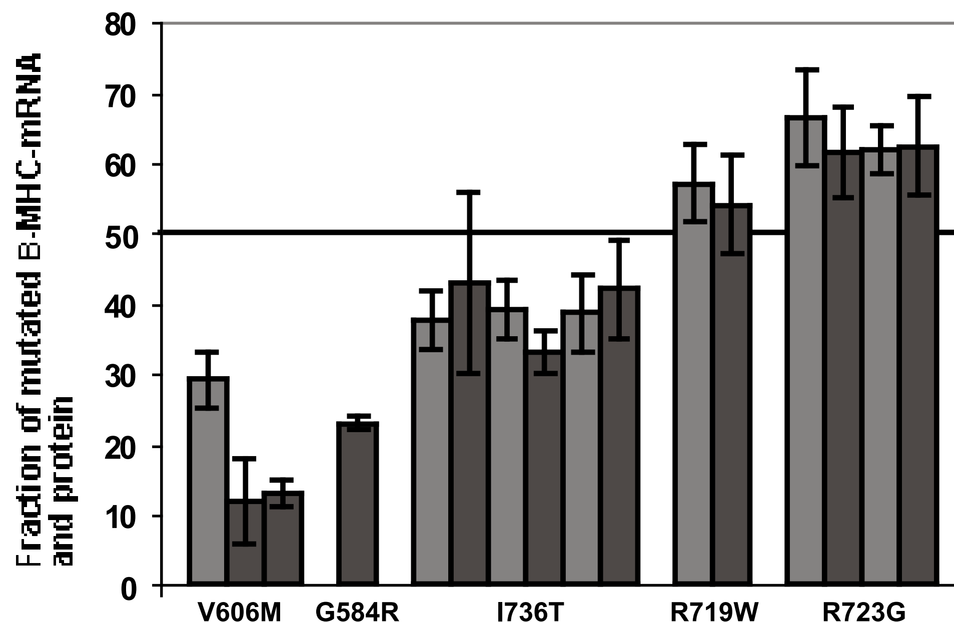


Fig. 18. Relative quantification of the fraction of mutated β-MHC-mRNA (grey) and mutated β-myosin (black) that is incorporated into the sarcomeres. The deviation from 50:50 occurs at both the protein and mRNA levels. The data also show that there is a close correlation between the expression of mutated β-MHC-mRNA and mutated β-myosin for a given mutation. One exception is V606M where the mRNA analyses was performed on a tissue prepared for histology and therefore the data are maybe not as reliable as the other mutations. The protein quantification data have been obtained by different people in our group and have mostly been published in the meantime.

Quite importantly, our mRNA data are extensively reinforced by data of relative quantification of mutated vs. wild type myosin in the biopsies from the same patients, which was carried out in our laboratory on the same samples. It was observed that similar deviations from 50:50 also existed at the protein level. Fig. 18 shows a comparison of mRNA and protein data. For mutation V606M, the fraction of mutated myosin in the British patient was 12±6% (Nier et al., 1999) and for another unrelated patient of American origin it was 13±2%

(unpublished data). Furthermore, the amount of mutated myosin in the muscle biopsy of the patient with mutation R719W was 54±7% (Kirschner et al., 2005) and that in patients with mutation I736T was 43±13%, 33±3% and 42±7% respectively (Becker et al., 2007). Protein quantifications for patients H27 and H28 with mutation R723G have shown 61.6±6.5 and 62.4±6.9% of mutated myosin in the *soleus* biopsies of these patients (Becker et al., 2007), mutated myosin in the remaining biopsies has not been quantified yet. In all relative protein quantifications, the protein incorporated into the sarcomeres, which actually is functionally relevant, was determined. The fact that for nearly all mutations a very similar deviation from the 50:50 ratio was also found for β-MHC-mRNA and for myosin incorporated into the sarcomeres (Fig. 18), strongly supports that the deviation most likely is not due to heteroduplex formation or another experimental error.

Inter- and intra-familial comparison of the mutated vs. wild type β-MHC-mRNA ratio

Among the mutations studied, mutations I736T and R723G allowed us to analyze the intra-familial expression of the mutated and the wild type alleles. For mutation I736T, the three patients H18, H19 and H20 siblings of the same Kyrgyz family have very similar fractions of mutated β-MHC-mRNA (average=38.4%) and of mutated myosin (average=39.3%). Patients H27, H28 and H71 belong to the same Catalan family and have similar fractions of mutated β-MHC-mRNA (average=66%) and mutated myosin at least for patients H27 and H28 (average=62%). Protein quantification has not been done for H71, H72 and H29. For mutation R723G, it was also possible to analyze the inter-familial similarity in expression of the mutated β-MHC-mRNA. Patient H29, belongs to a second family and has approximately 67% mutated β-MHC-mRNA in the myocardium. Patient H72 hailing from a third Catalan family has on an average 66% mutated β-MHC-mRNA in the *soleus* muscle (Fig. 12). For mutation V606M, two patients, one from a family in the UK and the other from an American family, without any known relationship were analyzed for the fraction of mutated myosin. The fraction of mutated protein in the sarcomeres of both these patients was very similar (12% and 13%, respectively, Fig. 18).

The inter- and intra-familial similarity in expression of the mutated β-MHC-mRNA suggests that a specific pattern of expression for a given mutation is an inherent property of the

mutation itself, which would support the idea that the expression is regulated by an epigenetic mechanism. The similar expression levels of mutated β-MHC-mRNA in patients of different generations (H27 and H28 vs. H71 and H72 is in some contrast to the idea that at young age 50% of the myocytes express wild type and 50% mutated protein, which then due to cell death, results in a deviation from 50:50. Our hypothesis is that the deviation from 50:50 is characteristic for each mutation and is most likely present already at very young age.

Correlation of disease severity and the fraction of mutated β-MHC mRNA and β-myosin

HCM is characterized by marked heterogeneity in age of onset, genetic heterogeneity and also in disease manifestation (Rai et al., 2008; al., Bos et al., 2007, Arad et al., 2002). The heterogeneity is usually observed across families, but nevertheless intra-familial heterogeneity is also found (Keller et al., 2008). However, in our present study on the different β-myosin head domain mutations, although the number of patients studied was limited, a correlation is observed: the larger the fraction of mutated β-MHC-mRNA, the more severe is the disease expression. Therefore, the specific fraction of mutated β-MHC-mRNA and protein that is incorporated into the sarcomeres of these patients, for each mutation, may be a cause rather than an effect of the disease.

Genotype-phenotype correlations have shown that mutation R719W is associated with a high risk of sudden death for the individuals carrying this mutation (Anan et al., 1994; Ackerman et al., 2002). We had one female British patient with mutation R719W. The fraction of mutated β-MHC-mRNA in her slow *soleus* muscle fibers was approximately 57%. This patient was only 22 years old when she had a cardiac arrest and received an ICD. Among the chief pathological features was the inter-ventricular septum thickness of 26 mm. Most likely both, the fraction of mutated myosin and the specific location and type of mutation contribute to the malignancy of the disease in this patient. For V606M, however, the situation is not so definitive. Patients from several kindreds with this mutation have a variable extent of ventricular hypertrophy but nearly normal survival; therefore it was considered a benign mutation (Watkins et al., 1992; Marian et al., 1995). However, there are also other reports for single kindreds where V606M mutation was found to cause a malignant HCM phenotype (Fananapazir et al., 1994, Semsarian et al., 1997; Havndrup et al., 2001). For the cases where the V606M mutation was malignant, it is not excluded that the fraction of mutated β-MHC-mRNA may be greater than 50%. Quantification of mutated vs. wild type ratio at protein and mRNA level in these families would be interesting to test this idea. Our hypothesis is that the expression level is characteristic for a given mutation, which would be a low level for mutation V606M. An alternative explanation for a malignant

phenotype with mutation V606M would be that there might have been an additional, as yet undetected mutation contributing to the severity of the disease. For mutation G584R where relative quantification was done only on protein level (Fig. 18), the fraction of mutated molecules is significantly less than 50%. The disease is manifested by mild symptoms. The three patients harboring I736T in the converter domain of the β-MHC show variable clinical symptoms with, most likely, a normal life expectancy. They were diagnosed with moderate left ventricular hypertrophy with pathological ECG and maximal septal wall thickness of 14, 18 and 17 mm, respectively for H18, H19 and H20. These patients for whom the mutated β-MHC-mRNA (38.4% on an average) and protein level (39.3% on an average) were less than 50%, were categorized as NYHA II group (Perrot et al., 2005).

One of the most malignant mutations of this study is R723G. The fraction of mutated β-MHC-mRNA for all the five patients (Fig. 12) is on an average 66.3%. The three male patients, H27, H28 and H71 belong to the same family. H27 and H28 had received an ICD after syncopal episodes. Subsequently, both H27 and H28 underwent heart transplantation. The female patient, H29, from another family had also received a heart transplant. From a third family, H72 had received an ICD. All five patients H27, H28, H29, H71 and H72, at the time of biopsy were classified as NYHA classes III, II, IV, I and I respectively (Enjuto et al., 2000). It has to be mentioned though, that such severe heart failure is not the common patho-mechanism in HCM and is found only for some mutations.

Nevertheless, based on our data we propose that it is not only the type and location of the mutation in the β-myosin and the functional changes they cause which affects the prognosis of HCM, but also the fraction of mutated β-MHC-mRNA and β-myosin may be an indicator/determinant of prognosis and survival.

Possible mechanisms for deviation from 50:50

Regarding the disease causing mechanism in HCM it has been demonstrated in different studies that the missense mutation carrying β-MHC is incorporated into the sarcomeres (Cuda et al., 1993; Nier et al., 1999) of cardiac and slow-twitch skeletal muscles and does not cause alterations in the structure and assembly of the thick filaments (Köhler et al., 2002). Thus, the unequal expression of mutated β-MHC vs. wild type does not cause haploinsufficiency. The mutations studied here apparently act in a dominant negative manner, inducing functional changes of the acto-myosin interactions and therefore, of the whole sarcomere.

Sequence analyses of the β-MHC gene with respect to the mutations described in this study have shown that for all the mutations, save V606M, cytosine is involved. Methylation of cytosines in the CpG dinucleotides plays a crucial role in the regulation of gene expression. There exists evidence that methylation of cytosines in the CpG dinucleotides in the open reading frames or coding sequences of a gene may also cause repression of expression through chromatin remodeling (Komura et al., 1995; Hisano et al., 2003). It is also possible that CpG methylation in the intragenic regions downstream of the promoter cause closed chromatin structures that result in a reduction in RNA Pol II elongation efficiency (Lorincz et al., 2004). However, the change in chromatin conformation alone, due to a single base substitution seems to be an unlikely cause of the observed deviations. It is possible that other cis- or trans-acting factors, in conjunction with the chromatin remodeling cause such deviations.

So far mostly frameshift and nonsense mutations that result in premature termination codon have been described that affect the mRNA turnover by activating pathways that cause a decrease in the mutated mRNAs. It has been demonstrated for the hERG gene in the human Long-QT syndrome (Gong et al., 2007) as well as as for the β-globin gene (Kugler et al., 1995) that such mutations indeed cause a reduction in the mutated mRNA by nonsense-mediated mRNA decay. The cell has evolved different strategies to recognize and decay faulty mRNA. Mechanisms like mRNA surveillance causing the degradation of aberrant mRNAs have been described (Wilusz et al., 2001; Hilleren et al., 1999). However, for the mutations studied here, it is not clear how the mutated and wild type transcripts are differentiated and how missense mutations in the coding region of the β-MHC gene affect the stabilities of the two transcripts, especially when the mutations do not affect a splice site.

One possibility to explain varying levels of mutated β-MHC-mRNA for the different mutations is a variable number of muscle fibers or myocytes containing the mutation. We have evidence from mechanical studies on individual *soleus* muscle fibers from HCM patients with mutations R723G (H27), I736T (H19) and R719W (H13) that a much larger functional variability vs. control exists for a given mutation from one fiber to another i.e., some fibers have Ca^{++} sensitivity like control fibers, whereas others show highly significant differences (Kirschner et al., 2005). Yet it is unclear how or why in each cell only one allele would be activated.

Variable expression of β-myosin from myocyte to myocyte

The functional variability from fiber to fiber (Kirschner et al., 2005), however, suggests a variable expression and incorporation of the mutated β-MHC into the sarcomeres of the individual muscle fibers. Consistent with the mechanical studies, preliminary quantifications of the mutated vs. wild type β-MHC-mRNA ratios in single fibers with mutation R723G of patient H27 have shown a large variability in the expression of mutated β-MHC-mRNA from fiber to fiber (Fig. 13). However, since the *soleus* muscle fibers have multiple nuclei, it remains unclear whether both alleles are transcribed in each nucleus or whether wild type and mutant alleles are transcribed independently in different nuclei to cause the observed deviations in expression.

Therefore, to address the question of variable expression of mutated vs. wild type β-MHC from myocyte to myocyte, relative quantification of β-MHC-mRNA in single cardiomyocytes, each of which is uninucleate, is necessary. As a first step towards relative quantification of the fraction of mutated β-MHC-mRNA in individual cardiomyocytes we have already established a pyrosequencing method. This was necessary because pyrosequencing requires much less cDNA and there is a chance that it can be applied even to single myocytes (Salk et al., 2006). However, also in pyrosequencing great care must be taken to ensure that non-specific PCR products, which could affect the relative quantification, are not generated.

Hypothesis for development of myocyte disarray

We propose that a variable incorporation of mutated β-MHC in the sarcomeres of each cardiomyocyte would cause a variable force generation in each myocyte, thereby resulting in functional imbalances, particularly among myocytes arranged in series, in the myocardium (Fig. 19). This might trigger development of myocyte disarray, which is characteristic for the HCM phenotype in many cases (Elliot and McKenna, 2004; Varnava et al, 2000).

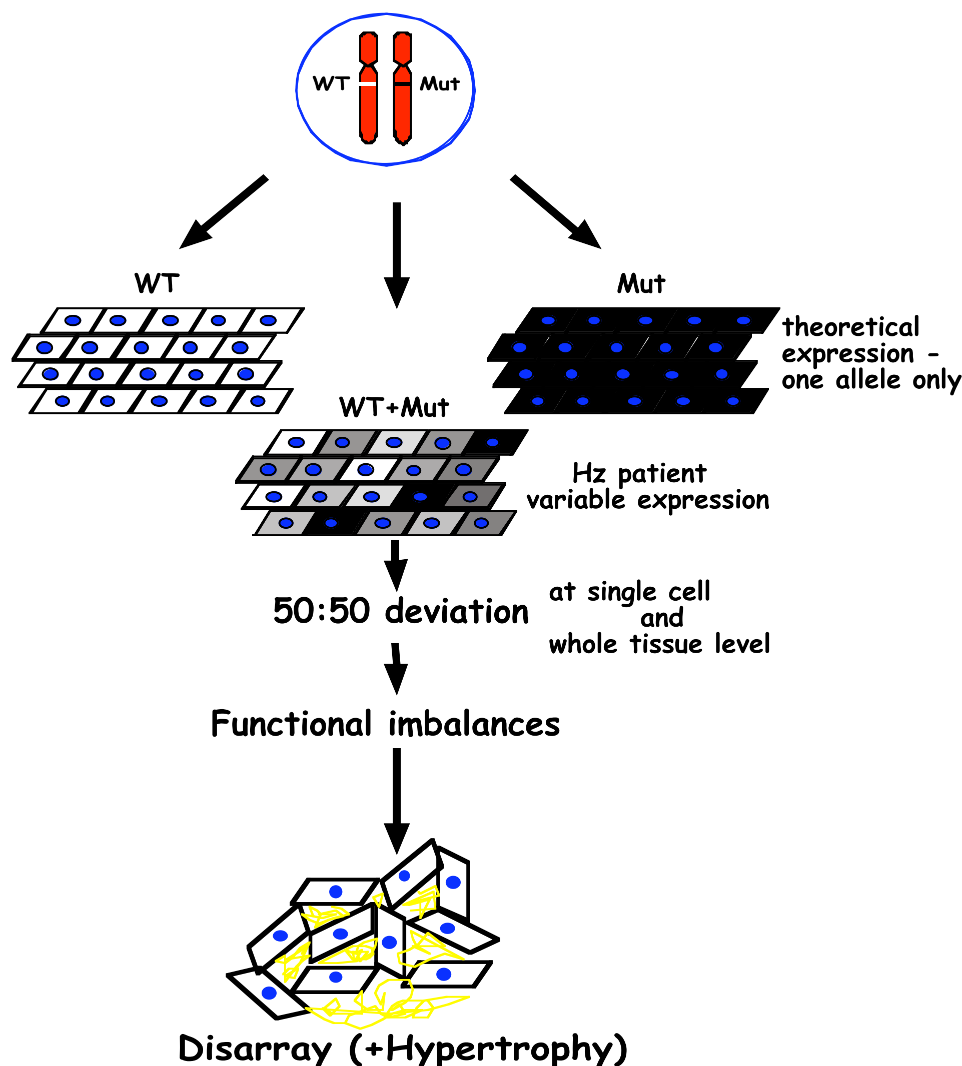


Fig. 19. Hypothesis for the development of disarray and possibly hypertrophy observed in HCM caused by myosin mutations. In the uninucleate (blue) cardiomyocytes of heterozygous (Hz) patients, it is possible that either the wild type (WT; white), or mutated (Mut; black) or both alleles (grey) transcribe the β-MHC mRNA and would therefore possibly result in varying amounts of the wild type and mutated β-MHC in the contractile apparatus of individual cardiomyocytes.

The model in Fig. 19 not only is able to explain why a deviation from 50:50 is observed in the expression of wild type vs. mutated β-MHC-mRNA, it also might provide a unifying mechanism at least for the development of typical HCM-symptoms such as disarray and maybe even hypertrophy in HCM caused by β-myosin mutations. When individual

cardiomyocytes contain varying amounts of the mutated β-myosin, which causes functional changes of the sarcomere (Köhler et al., 2002; Kirschner et al., 2005; Lankford et al., 1995), then this may result in imbalances of force generation and relaxation among the individual myocytes. Such functional imbalances among individual myocytes particularly when they are arranged in series could have at least two consequences: (1) it may disrupt the orderly alignment and cause myocyte disarray and apoptosis of weaker cells with connective tissue deposition (yellow) around the cells which is the key feature of HCM. (2) Variable force generation of neighboring myocytes might cause excessive stretch on some cells (while others may hyper-contract). Increased stretch could activate signaling cascades leading to myocyte hypertrophy, which according to current models can be induced by a stretch sensing mechanism located in the Z-line (Knöll et al., 2002; Pyle et al., 2004) or in transmembrane protein complexes (Brancaccio et al., 2003) of the cardiomyocytes.

It would be of great interest for future studies to find out whether this as yet hypothetical mechanism would even provide a unifying mechanism for development of at least some aspects of the HCM phenotype also when HCM is caused by mutations in other sarcomeric proteins.

References

Ackerman MJ VS, Ommen SR, Will ML, Nishimura RA, Tajik AJ, Gersh BJ,. 2002. Prevalence and age-dependence of malignant mutations in the beta-myosin heavy chain and troponin T genes in hypertrophic cardiomyopathy. A comprehensive outpatient perspective. Journal of the American College of Cardiology 39(12):2042-8.

Ahmad F, Seidman JG, Seidman CE. 2005. The genetic basis for cardiac remodeling. Annu. Rev. Genomics Hum. Genet. 6:185-216

Anan R, Greve G, Thierfelder L, Watkins H, McKenna WJ, Solomon S, Vecchio C, Shono H, Nakao S, Tanaka H and others. 1994. Prognostic implications of novel beta cardiac myosin heavy chain gene mutations that cause familial hypertrophic cardiomyopathy. J Clin Invest 93(1):280-5.

Arad M, Seidman JG, Seidman CE. 2002. Phenotypic diversity in hypertrophic cardiomyopathy. Hum Mol Genet. 11(20):2499-506.

Becker-Andre M, Hahlbrock K. 1989. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). Nucleic Acids Res. 17(22): 9437-9446.

Becker E, Navarro-Lopez F, Francino A, Brenner B, Kraft T. 2007. Quantification of mutant versus wild-type myosin in human muscle biopsies using nano-LC/ESI-MS. Anal Chem. 79(24):9531-8.

Becker KD, Gottshall KR, Hickey R, Perriard JC, Chien KR. 1997. Point mutations in human beta cardiac myosin heavy chain have differential effects on sarcomeric structure and assembly: an ATP binding site change disrupts both thick and thin filaments, whereas hypertrophic cardiomyopathy mutations display normal assembly. J Cell Biol 137(1):131-40.

Belus A, Piroddi N, Scellini B, Tesi C, Amati GD, Girolami F, Yacoub M, Cecchi F, Olivotto I, Poggesi C. 2008. The familial hypertrophic cardiomyopathy-associated myosin mutation R403Q accelerates tension generation and relaxation of human cardiac myofibrils. J Physiol. 586(Pt 15):3639-44. Epub 2008 Jun 19.

Bonne G, Carrier L, Bercovici J, Cruaud C, Richard P, Hainque B, Gautel M, Labeit S, James M, Beckmann J, Weissenbach J, Vosberg HP, Fiszman M, Komaida M, Schwartz K. 1995. Cardiac myosin Binding protein-C gene splice acceptor site mutation is associated with familial hypertrophic cardiomyopathy. Nat. Genet. 11(4): 438-440

Bonne G, Carrier L, Richard P, Hainque B, Schwartz K. 1998. Familial hypertrophic cardiomyopathy: from mutations to functional defects. Circ Res 83(6):580-93.

Bos JM, Ommen SR, Ackerman MJ. 2007. Genetics of hypertrophic cardiomyopathy: one, two, or more diseases? Curr Opin Cardiol. 22: 193-9.

Bottinelli R, Coviello DA, Redwood CS, Pellegrino MA, Maron BJ, Spirito P, Watkins H, Reggiani C. 1998. A mutant tropomyosin that causes hypertrophic cardiomyopathy is expressed in vivo and associated with an increased calcium sensitivity. Circ Res 82(1):106-15.

Brancaccio M, Fratta L, Notte A, Hirsch E, Poulet R, Guazzone S, De Acetis M, Vecchione C, Marino G, Altruda F and others. 2003. Melusin, a muscle-specific integrin beta1-interacting protein, is required to prevent cardiac failure in response to chronic pressure overload. Nat Med. 9(1):68-75.

Braunwald E, Lambrew CT, Rockoff SD, Ross J, Jr., Morrow AG. 1964. Idiopathic Hypertrophic Subaortic Stenosis. I. A Description of the Disease Based Upon an Analysis of 64 Patients. Circulation. 30:SUPPL(4):3-119.

Brock R. 1957. Functional obstruction of the left ventricle; acquired aortic subvalvar stenosis. Guys Hosp Rep. 106(4):221-38.

Brown T, Kennard O, Kneale G, Rabinovich D. 1985. High-resolution structure of a DNA helix containing mismatched base pairs. Nature. 315(6020):604-6.

Charron P, Dubourg O, Desnos M, Bennaceur M, Carrier L, Camproux AC, Isnard R, Hagege A, Langlard JM, Bonne G and others. 1998. Clinical features and prognostic implications of familial hypertrophic cardiomyopathy related to the cardiac myosin- binding protein C gene. Circulation. 97(22):2230-6.

Chung MW, Tsoutsman T, Semsarian C. 2003. Hypertrophic cardiomyopathy: from gene defect to clinical disease. Cell Res. 13(1):9-20.

Cuda G, Fananapazir L, Zhu WS, Sellers JR, Epstein ND. 1993. Skeletal muscle expression and abnormal function of beta-myosin in hypertrophic cardiomyopathy. J Clin Invest 91(6):2861-5.

Dalloz F, Osinska H, Robbins J. 2001. Manipulating the contractile apparatus: genetically defined animal models of cardiovascular disease. J Mol Cell Cardiol. 33(1):9-25.

Diggle MA, Clarke SC. 2004. Pyrosequencing: sequence typing at the speed of light. Mol Biotechnol. 28(2):129-37.

Elliott P, McKenna WJ. 2004. Hypertrophic cardiomyopathy. Lancet 363(9424):1881-91.

Enjuto M, Francino A, Navarro-Lopez F, Viles D, Pare JC, Ballesta AM. 2000. Malignant hypertrophic cardiomyopathy caused by the Arg723Gly mutation in beta- myosin heavy chain gene. J Mol Cell Cardiol 32(12):2307-13.

Epstein ND, Cohn GM, Cyran F, Fananapazir L. 1992. Differences in clinical expression of hypertrophic cardiomyopathy associated with two distinct mutations in the beta- myosin

heavy chain gene. A 908Leu----Val mutation and a 403Arg----Gln mutation. Circulation 86(2):345-52.

Fananapazir L, Epstein ND. 1994. Genotype-phenotype correlations in hypertrophic cardiomyopathy. Insights provided by comparisons of kindreds with distinct and identical beta-myosin heavy chain gene mutations. Circulation 89(1):22-32.

Fokstuen S, Lyle R, Munoz A, Gehrig C, Lerch R, Perrot A, Osterziel KJ, Geier C, Beghetti M, Mach F and others. 2008. A DNA resequencing array for pathogenic mutation detection in hypertrophic cardiomyopathy. Hum Mutat. 29(6):879-85.

Geeves MA, Holmes KC. 1999. Structural mechanism of muscle contraction. Annu Rev Biochem. 68: 687-728

Geisterfer-Lowrance AA, Christe M, Conner DA, Ingwall JS, Schoen FJ, Seidman CE, Seidman JG. 1996. A mouse model of familial hypertrophic cardiomyopathy. Science 272(5262):731-4.

Geisterfer-Lowrance AA, Kass S, Tanigawa G, Vosberg HP, McKenna W, Seidman CE, Seidman JG. 1990. A molecular basis for familial hypertrophic cardiomyopathy: a beta cardiac myosin heavy chain gene missense mutation. Cell 62(5):999-1006.

Gong Q, Zhang L, Vincent GM, Horne BD, Zhou Z. 2007. Nonsense mutations in hERG cause a decrease in mutant mRNA transcripts by nonsense-mediated mRNA decay in human long-QT syndrome. Circulation. 116(1):17-24. Epub 2007 Jun 18.

Greaves SC, Roche AH, Neutze JM, Whitlock RM, Veale AM. 1987. Inheritance of hypertrophic cardiomyopathy: a cross sectional and M mode echocardiographic study of 50 families. Br Heart J. 58(3):259-66.

Guyton and Hall. 1998. A textbook of medical physiology. 9th edition

Havndrup O BH, Andersen PS, Larsen LA, Vuust J, Kjeldsen K, Christiansen M. 2001. The val606 met mutation in the cardiac beta-myosin heavy chain gene in patients with familial hypertrophic cardiomyopathy is associated with a high risk of sudden death at young age. Excerta Medica, The American Journal of Cardiology 87:1315-1317.

Hayashi T, Arimura T, Ueda K, Shibata H, Hohda S, Takahashi M, Hori H, Koga Y, Oka N, Imaizumi T and others. 2004. Identification and functional analysis of a caveolin-3 mutation associated with familial hypertrophic cardiomyopathy. Biochem Biophys Res Commun. 313(1):178-84.

Hilleren P, Parker R. 1999. Mechanisms of mRNA surveillance in eukaryotes. Annu. Rev. Genet. 33: 229-260.

Hisano M, Ohta H, Nishimune Y, Nozaki M. 2003. Methylation of CpG dinucleotides in the open reading frame of a testicular germ cell-specific intronless gene, Tact1/Actl7b, represses its expression in somatic cells. Nucleic Acids Res. 31(16):4797-804.

Hughes SE. 2004. The pathology of hypertrophic cardiomyopathy. Histopathology. 44(5):412-27.

Jaenicke T, Diederich KW, Haas W, Schleich J, Lichter P, Pfordt M, Bach A, Vosberg HP. 1990. The complete sequence of the human beta-myosin heavy chain gene and a comparative analysis of its product. Genomics. 8(2):194-206.

Jones WK, Grupp IL, Doetschman T, Grupp G, Osinska H, Hewett TE, Boivin G, Gulick J, Ng WA, Robbins J. 1996. Ablation of the murine alpha myosin heavy chain gene leads to dosage effects and functional deficits in the heart. J Clin Invest 98(8):1906-17.

Kavinsky CJ, Umeda PK, Levin JE, Sinha AM, Nigro JM, Jakovcic S, Rabinowitz M. 1984. Analysis of cloned mRNA sequences encoding subfragment 2 and part of subfragment 1 of alpha- and beta-myosin heavy chains of rabbit heart. J Biol Chem. 259(5):2775-81.

Keller DI, Schwitter J, Valsangiacomo ER, Landolt P, Attenhofer Jost CH. 2008. Hypertrophic cardiomyopathy due to beta-myosin heavy chain mutation with extreme phenotypic variability within a family. Int J Cardiol 27:27.

Kirschner SE, Becker E, Antognozzi M, Kubis HP, Francino A, Navarro-Lopez F, Bit-Avragim N, Perrot A, Mirrakhimov MM, Osterziel KJ and others. 2005. Hypertrophic cardiomyopathy-related beta-myosin mutations cause highly variable calcium sensitivity with functional imbalances among individual muscle cells. Am J Physiol Heart Circ Physiol. 288(3):H1242-51.

Knöll R, Hoshijima M, Hoffman HM, Person V, Lorenzen-Schmidt I, Bang ML, Hayashi T, Shiga N, Yasukawa H, Schaper W and others. 2002. The cardiac mechanical stretch sensor machinery involves a Z disc complex that is defective in a subset of human dilated cardiomyopathy. Cell 111(7):943-55.

Köhler J, Winkler G, Schulte I, Scholz T, McKenna W, Brenner B, Kraft T. 2002. Mutation of the myosin converter domain alters cross-bridge elasticity. Proc Natl Acad Sci U S A 99(6):3557-62.

Komura J, Okada T, Ono T. 1995. Repression of transient expression by DNA methylation in transcribed regions of reporter genes introduced into cultured human cells. Biochim Biophys Acta. 1260(1):73-8.

Kugler W, Enssle J, Hentze MW, Kulozik AE. 1995. Nuclear degradation of nonsense mutated beta-globin mRNA: a post transcriptional mechanism to protect heterozygotes from severe clinical manifestations of beta-thalassemia? Nucleic Acids Res. 23(3): 413-418.

Lankford EB, Epstein ND, Fananapazir L, Sweeney HL. 1995. Abnormal contractile properties of muscle fibers expressing beta-myosin heavy chain gene mutations in patients with hypertrophic cardiomyopathy. J Clin Invest 95(3):1409-14.

Liew CC, Sole MJ, Takihara KY, Kellam B, Anderson DH, Lin LP, Liew JC. 1990. Complete sequence and organization of the human cardiac beta-myosin heavy chain gene. Nucleic Acids Res. 18(12): 3647-3651.

London B. 2001. Cardiac Arrhythmias: From (Transgenic) Mice to Men. J Cardiovasc Electrophysiol. 12(9): 1089-1091.

Lorincz MC, Dickerson DR, Schmitt M, Groudine M. 2004. Intragenic DNA methylation alters chromatin structure and elongation efficiency in mammalian cells. Nat Struct Mol Biol. 11(11):1068-75. Epub 2004 Oct 3.

Lowey S, Lesko LM, Rovner AS, Hodges AR, White SL, Low RB, Rincon M, Gulick J, Robbins J. 2008. Functional effects of the hypertrophic cardiomyopathy R403Q mutation are different in an α- or β-myosin heavy chain background. J Biol Chem. 283(29): 20579-20589.

Malinchik S, Cuda G, Podolsky RJ, Horowits R. 1997. Isometric tension and mutant myosin heavy chain content in single skeletal myofibers from hypertrophic cardiomyopathy patients. J Mol Cell Cardiol 29(2):667-76.

Marian AJ. 2002. Modifier genes for hypertrophic cardiomyopathy. Curr Opin Cardiol 17(3):242-52.

Marian AJ, Roberts R. 1998. Molecular genetic basis of hypertrophic cardiomyopathy: genetic markers for sudden cardiac death. J Cardiovasc Electrophysiol. 9(1): 88-99.

Marian AJ MA, Kelly DP, Yu QT, Abchee AB, Hill R, Roberts R. 1995. Sudden cardiac death in hypertrophic cardiomyopathy. Variability in phenotypic expression of beta- myosin heavy chain mutations. Eur Heart J 16((3)):368-76.

Marian AJ, Roberts R. 1995. Recent advances in the molecular genetics of hypertrophic cardiomyopathy. Circulation. 92(5):1336-47.

Marian AJ, Roberts R. 2001. The molecular genetic basis for hypertrophic cardiomyopathy. J Mol Cell Cardiol. 33(4):655-70.

Marian AJ, Wu Y, Lim DS, McCluggage M, Youker K, Yu QT, Brugada R, DeMayo F, Quinones M, Roberts R. 1999. A transgenic rabbit model for human hypertrophic cardiomyopathy. J Clin Invest 104(12):1683-92.

Maron BJ, Gardin JM, Flack JM, Gidding SS, Kurosaki TT, Bild DE. 1995. Prevalence of hypertrophic cardiomyopathy in a general population of young adults. Echocardiographic analysis of 4111 subjects in the CARDIA Study. Coronary Artery Risk Development in (Young) Adults. Circulation 92(4):785-9.

Maron BJ, Nichols PFd, Pickle LW, Wesley YE, Mulvihill JJ. 1984. Patterns of inheritance in hypertrophic cardiomyopathy: assessment by M-mode and two- dimensional echocardiography. Am J Cardiol 53(8):1087-94.

Matsuoka R, Yoshida MC, Kanda N, Kimura M, Ozasa H, Takao A. 1989. Human cardiac myosin heavy chain gene mapped within chromosome region 14q11.2----q13. Am J Med Genet 32(2):279-84.

McCarrey JR, Berg WM, Paragioudakis SJ, Zhang PL, Dilworth DD, Arnold BL, Rossi JJ. 1992. Differential transcription of Pgk genes during spermatogenesis in the mouse. Dev Biol. 154: 160-168.

McKenna WJ, Sadoul N, Slade AK, Saumarez RC. 1994. The prognostic significance of nonsustained ventricular tachycardia in hypertrophic cardiomyopathy. Circulation. 90(6):3115-7.

Minamisawa S, Sato Y, Tatsuguchi Y, Fujino T, Imamura S, Uetsuka Y, Nakazawa M, Matsuoka R. 2003. Mutation of the phospholamban promoter associated with hypertrophic cardiomyopathy. Biochem Biophys Res Commun. 304(1):1-4.

Mohiddin SA, Begley DA, McLam E, Cardoso JP, Winkler JB, Sellers JR, Fananapazir L. 2003. Utility of genetic screening in hypertrophic cardiomyopathy: prevalence and significance of novel and double (homozygous and heterozygous) beta-myosin mutations. Genet Test. 7(1):21-7.

Moolman JC, Corfield VA, Posen B, Ngumbela K, Seidman C, Brink PA, Watkins H. 1997. Sudden death due to troponin T mutations. J Am Coll Cardiol 29(3):549-55.

Morita H, Seidman J, Seidman CE. 2005. Genetic causes of human heart failure. J Clin Invest. 115(3):518-26.

Nagueh SF, Chen S, Patel R, Tsybouleva N, Lutucuta S, Kopelen HA, Zoghbi WA, Quinones MA, Roberts R, Marian AJ. 2004. Evolution of expression of cardiac phenotypes over a 4-year period in the beta-myosin heavy chain-Q403 transgenic rabbit model of human hypertrophic cardiomyopathy. J Mol Cell Cardiol. 36(5):663-73.

Nier V, Schultz I, Brenner B, Forssmann W, Raida M. 1999. Variability in the ratio of mutant to wildtype myosin heavy chain present in the soleus muscle of patients with familial hypertrophic cardiomyopathy. A new approach for the quantification of mutant to wildtype protein. FEBS Lett 461(3):246-52.

Niimura H, Bachinski LL, Sangwatanaroj S, Watkins H, Chudley AE, McKenna W, Kristinsson A, Roberts R, Sole M, Maron BJ and others. 1998. Mutations in the gene for cardiac myosin-binding protein C and late-onset familial hypertrophic cardiomyopathy. N Engl J Med. 338(18):1248-57.

Nishi H, Kimura A, Harada H, Adachi K, Koga Y, Sasazuki T, Toshima H. 1994. Possible gene dose effect of a mutant cardiac β-myosin heavy chain gene on the clinical expression of familial hypertrophic cardiomyopathy. Biochem Biophys Res Commun. 200(1): 549-556.

Nishii K, Morimoto S, Minakami R, Miyano Y, Hashizume K, Ohta M, Zhan DY, Lu QW, Shibata Y. 2008. Targeted disruption of the cardiac troponin T gene causes sarcomere disassembly and defects in heartbeat within the early mouse embryo. Dev Biol. 322(1):65-73. Epub 2008 Jul 16.

Palmiter KA, Tyska MJ, Haeberle JR, Alpert NR, Fananapazir L, Warshaw DM. 2000. R403Q and L908V mutant beta-cardiac myosin from patients with familial hypertrophic cardiomyopathy exhibit enhanced mechanical performance at the single molecule level. J Muscle Res Cell Motil. 21(7):609-20.

Perrot A, Schmidt-Traub H, Hoffmann B, Prager M, Bit-Avragim N, Rudenko RI, Usupbaeva DA, Kabaeva Z, Imanov B, Mirrakhimov MM and others. 2005. Prevalence of cardiac beta-myosin heavy chain gene mutations in patients with hypertrophic cardiomyopathy. J Mol Med. 83(6):468-77.

Peters NS, Wit AL. 1998. Myocardial architecture and ventricular arrhythmogenesis. Circulation. 97(17):1746-54.

Pyle WG, Solaro RJ. 2004. At the crossroads of myocardial signaling: the role of Z-discs in intracellular signaling and cardiac function. Circ Res 94(3):296-305.

Rai TS, Ahmad S, Bahl A, Ahuja M, Ahluwalia TS, Singh B, Talwar KK, Khullar M. 2008. Genotype phenotype correlations of cardiac beta-myosin heavy chain mutations in Indian patients with hypertrophic and dilated cardiomyopathy. Mol Cell Biochem 25:25.

Rayment I, Holden HM, Sellers JR, Fananapazir L, Epstein ND. 1995. Structural interpretation of the mutations in the beta-cardiac myosin that have been implicated in familial hypertrophic cardiomyopathy. Proc Natl Acad Sci U S A. 92(9):3864-8.

Rayment I, Rypniewski WR, Schmidt-Base K, Smith R, Tomchick DR, Benning MM, Winkelmann DA, Wesenberg G, Holden HM. 1993. Three-dimensional structure of myosin subfragment-1: a molecular motor. Science 261(5117):50-8.

Redwood CS, Moolman-Smook JC, Watkins H. 1999. Properties of mutant contractile proteins that cause hypertrophic cardiomyopathy. Cardiovasc Res 44(1):20-36.

Richardson P, McKenna W, Bristow M, Maisch B, Mautner B, O'Connell J, Olsen E, Thiene G, Goodwin J, Gyarfas I and others. 1996. Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of cardiomyopathies. Circulation. 93(5):841-2.

Rottbauer W, Gautel M, Zehelein J, Labeit S, Franz WM, Fischer C, Vollrath B, Mall G, Dietz R, Kübler W, Katus HA. 1997. Novel splice donor site mutation in the cardiac myosin-

binding protein-C gene in familial hypertrophic cardiomyopathy. Characterization of cardiac transcript and protein. J Clin. Invest. 100(2): 475-482.

Seidman CE, Seidman JG. 1998. Molecular genetic studies of familial hypertrophic cardiomyopathy. Basic Res Cardiol. 93: SUPPL 3, 13-16

Salk JJ, Sanchez JA, Pierce KE, Rice JE, Soares KC, Wangh LJ. 2006. Direct amplification of single stranded DANN for pyrosequencing using linear-after-the-exponential (LATE)-PCR. Anal. Chem. 353(1): 124-132.

Schulte I. 2001. Variabilität des Schweregrads bei Familiärer Hypertropher Kardiomyopathie: Quantifizierung von Wildtyp und Mutante auf mRNA-und Proteinebene. Dissertation.

Semsarian C, Yu B, Rice C, Lawrence C, Washington H, Trent RJ. 1997. Sudden cardiac death in familial hypertrophic cardiomyopathy: are "benign" mutations really benign? Pathology 29(3):305-8.

Sweeney HL, Straceski AJ, Leinwand LA, Tikunov BA, Faust L. 1994. Heterologous expression of a cardiomyopathic myosin that is defective in its actin interaction. J Biol Chem 269(3):1603-5.

Swynghedauw B. 1986. Developmental and functional adaptation of contractile proteins in cardiac and skeletal muscles. Physiol Rev. 66(3):710-71.

Tajsharghi H, Fyhr IM. 2008. Structural effects of the slow/b-cardiac myosin heavy chain R453C mutation in cardiac and skeletal muscle. Scand Cardiovasc J. 42(2):153-6.

Teare D. 1958. Asymmetrical hypertrophy of the heart in young adults. Br Heart J. 20(1):1-8.

Thierfelder L, Watkins H, MacRae C, Lamas R, McKenna W, Vosberg HP, Seidman JG, Seidman CE. 1994. Alpha-tropomyosin and cardiac troponin T mutations cause familial hypertrophic cardiomyopathy: a disease of the sarcomere. Cell 77(5):701-12.

Thompson JR, Marcelino LA, Polz MF. 2002. Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. Nucleic Acids Res. 30(9):2083-8.

Tyska MJ, Hayes E, Giewat M, Seidman CE, Seidman JG, Warshaw DM. 2000. Single-molecule mechanics of R403Q cardiac myosin isolated from the mouse model of familial hypertrophic cardiomyopathy. Circ Res. 86(7): 720-2

Varnava AM, Elliott PM, Mahon N, Davies MJ, McKenna WJ. 2001. Relation between myocyte disarray and outcome in hypertrophic cardiomyopathy. Am J Cardiol. 88(3):275-9.

Vikstrom KL, Factor SM, Leinwand LA. 1996. Mice expressing mutant myosin heavy chains are a model for familial hypertrophic cardiomyopathy. Mol Med 2(5):556-67.

Watkins H. 1994. Multiple disease genes cause hypertrophic cardiomyopathy. Br Heart J. 72(6 Suppl):S4-9.

Watkins H, Rosenzweig A, Hwang DS, Levi T, McKenna W, Seidman CE, Seidman JG. 1992. Characteristics and prognostic implications of myosin missense mutations in familial hypertrophic cardiomyopathy. N Engl J Med 326(17):1108-14.

Watkins H, Seidman CE, MacRae C, Seidman JG, McKenna W. 1992. Progress in familial hypertrophic cardiomyopathy: molecular genetic analyses in the original family studied by Teare. Br Heart J. 67(1):34-8.

Weiss A, Leinwand LA. 1996. The mammalian myosin heavy chain gene family. Annu Rev Cell Dev Biol. 12:417-39.

Wilusz CJ, Wormington M, Peltz SW. 2001. The cap-to-tail guide to mRNA turnover. Nat Rev. Mol Cell Biol. 2: 237-246.

Own Publications

Tripathi, S., E. Becker, F. Navarro-Lopez, B. Brenner, and T. Kraft. 2006. Cardiomyopathy mutation Arg723Gly in β-myosin: Quantification of mutant protein and β-MHC-mRNA in soleus muscle samples. J Muscle Res Cell Motil. 27: p538.

Tripathi, S., I. Schulte, A. Francino, F. Navarro-Lopez, N. Bit-Avragim, A. Perrot, C. Özcelik, W. McKenna, and T. Kraft. Highly variable expression of wildtype and mutant β-MHC mRNA for different myosin mutations in Familial Hypertrophic Cardiomyopathy. Manuscript in preparation.

Erklärung zur Dissertation

Hierdurch erkläre ich, dass die Dissertation

Variable expression of wildtype and mutant β-myosin-mRNA for different myosin mutations in Familial Hypertrophic Cardiomyopathy

selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den

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