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Mutation Screening in Familial Cardiovascular Diseases
Mutační screening u familiárních kardiovaskulárních onemocnění

Dissertation Thesis

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Prague, 2011

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 29.04.2011 RNDr. Pavel Čapek

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Acknowledgement

I would like to thank to my Ph.D. supervisor, Prof. MUDr. Radim Brdička, DrSc. and to both of my advisors, RNDr. Jiří Škvor, CSc. and Jiří Vondrášek, Ph.D. for their valuable consultations and help.

My Ph.D. project was supported by grant LN00B107 of the Ministry of Education, Youth and Sports of the Czech Republic to the European Centre for Medical Informatics, Statistics and Epidemiology (EuroMISE Centre – Cardio), Institute of Computer Science, Academy of Sciences of the Czech Republic.

1. Index to abbreviations

- β-MHC: β-myosin heavy chain
- HCM: hypertrophic cardiomyopathy
- FHC: familial hypertrophic cardiomyopathy
- TNNT2: troponin T type 2
- uPAR: urokinase plasminogen activator receptor
- uPA: urokinase plasminogen activator
- PSGL-1: P-selectin glycoprotein ligand 1
- MYH7: a gene encoding a beta myosin heavy chain isoform expressed primarily in the heart
- Essentials MLC/ELC, eMLC: essential myosin light chain
- Regulatory MLC/RLC, rMLC: regulatory myosin light chain
- cMyBP-C: cardiac myosin-binding protein C
- SCD: sudden cardiac death
- α-Tm: α-tropomyosin
- PDB: protein data bank
- EMBOSS: the European Molecular Biology Open Software Suite
- BLOSUM62: blocks of amino acid substitution matrix
- MOE: Molecular operating environment
- RMSD: root mean square deviation
- IQ motif: an amino acid sequence motif. The term "IQ" refers to the first two amino acids of the motif: isoleucine and glutamine.
- PEP-FOLD: peptide structure prediction server
- MD simulation: molecular dynamics simulation
- EF hand: helix-loop-helix structural domain

2. Abstract

Introduction: Hypertrophic cardiomyopathy is a congenital cardiac disease with autosomal dominant pattern of inheritance and incomplete penetrance. With the knowledge of the responsible genes, the ability to detect the underlying genetic change and with the study of functional analysis of defected protein, we might be able to determine whether specific genotypes lead to different phenotypes.

Aims of Study: To comprehensively analyze the mechanism of genesis of hypertrophic cardiomyopathy in Czech patients afflicted with this disorder from molecular genetic point of view (*MYH7*, *TNNT2* gene) to functional analysis of the 3D molecular model of defected β-myosin heavy chain protein *in silico*. Beside these aims of the study, the reduction of production of inflammatory aggregates in the cardiovascular system was studied in patients with type 2 diabetes mellitus. The reason of this study was to look into possibilities of therapeutical effect on selected cardiovascular risks in patients with hypertrophic cardiomyopathy simultaneously suffering from type 2 diabetes mellitus. Both of these groups of patients have substantially increased risk of cardiovascular diseases due to development of premature atherosclerosis.

Material and Methods: A total of 170 probands were enrolled in this study of *MYH7* gene. DNA samples were analyzed (PCR, sequence analysis) for mutations in the specific functional regions of *MYH7*. The 3D model of human β-MHC was built using the X-ray structure of nucleotide-free scallop myosin S1 as the structural template. *De novo* structure prediction of two peptides (mutant and wild type variant) spanning the 769–788 region of the β-MHC were performed. A total of 181 probands were enrolled in the study of *TNNT2* gene. DNA samples were genotyped (PCR, sequence analysis) for mutations in the specific binding regions of *TNNT2* gene. The study with rosiglitazone included 33 patients with type 2 diabetes mellitus and 32 normal controls. The expression of leukocyte markers was measured by an immunofluorescence method using single-step staining with monoclonal antibodies. The fluorescence was quantified by the flow cytometry.

Results: The $Asp^{778}Val$ amino acid alteration was found in patient with severe form of hypertrophic cardiomyopathy. This variation was chosen for subsequent 3D molecular modeling *in silico*. The mutation of the Asp by Val not only changes the character of the interaction pattern with other amino acids or ions but Val being a small hydrophobic amino acid can completely change the stability of the region. We hypothesize that it can change the dynamics and flexibility of the long helical part or it can modify its interaction property. In the study with diabetic patients leukocyte expression of uPAR and PSGL-1 was significantly higher in patients than in controls. Leukocyte-platelet aggregates and uPAR and PSGL-1 expression significantly decreased after rosiglitazone treatment.

Conclusion: The mutation location in the *MYH7/TNNT2* genes and therefore changes in amino acid composition may have crucial negative impact on the disease outcome in patients with hypertrophic cardiomyopathy. In addition, a mutation that changes the charge of the amino acid is more likely to affect protein function than a conservative mutation. In the rosiglitazone study we observed substantial lowering of the expression of thrombogenic markers on leukocytes after the treatment, suggesting that rosiglitazone leads to the reduction of atherothrombotic complications.

2. Abstrakt

Úvod: Hypertrofická kardiomyopatie je vrozené srdeční onemocnění s autozomálně dominantním typem dědičnosti a neúplnou penetrancí. Se znalostí odpovědných genů, schopností detekovat genetické změny, které jsou podkladem vzniku tohoto onemocnění, a studiem funkční analýzy defektních proteinů, můžeme stanovit, zda konkrétní genotypy vedou ke vzniku odlišných fenotypových projevů hypertrofické kardiomyopatie.

Cíle práce: Analyzovat mechanismy vzniku hypertrofické kardiomyopatie u českých pacientů postižených tímto onemocněním z molekulárně genetického hlediska (geny *MYH7, TNNT2*), až po funkční analýzu 3D molekulárního modelu defektního proteinu těžkého řetězce β-myosinu *in silico*. Dalším cílem práce bylo studium možnosti snížení tvorby zánětlivých agregátů v kardiovaskulárním systému u pacientů s diabetem mellitem 2. typu. Studie prověřovala možnosti terapeutického ovlivnění vybraných kardiovaskulárních rizik u pacientů s hypertrofickou kardiomyopatií, současně trpících diabetem mellitem 2. typu. Obě tyto skupiny pacientů jsou ohroženy podstatně vyšším rizikem vzniku kardiovaskulárních onemocnění vzhledem k vývoji předčasné ateroskleróz.

Materiál a metody: Do studie genu *MYH7* bylo zařazeno 170 probandů. Vzorky DNA těchto jedinců byly podrobeny mutačnímu screeningu (PCR, sekvenční analýza) specifických funkčních oblastí genu *MYH7*, které jsou spojovány se vznikem hypertrofické kardiomyopatie. Pomocí rentgenové struktury myosinu S1 hřebenatky, který sloužil jako strukturální templát, byl následně vytvořen 3D model lidského těžkého řetězce β-myosinu. Byla vytvořena *de novo* predikce struktury dvou krátkých peptidů (mutantní a divoké varianty) úseku těžkého řetězce β-myosinu. Do studie genu *TNNT2* bylo zařazeno 181 probandů. DNA vzorky těchto jedinců byly genotypovány (PCR, sekvenční analýza) ve specifických vazebných oblastech genu *TNNT2*, které mohou mít vliv na vývoj hypertrofické kardiomyopatie. Soubor použitý pro testování účinků rosiglitazonu byl tvořen 33 pacienty s diabetem mellitem 2. typu a 32 zdravými kontrolami. Exprese leukocytárních markerů byla měřena imunofluorescenční metodou pomocí jednostupňového barvení monoklonálními protilátkami. Fluorescence byla kvantifikována průtokovou cytometrií.

Výsledky: Mutačním screeningem specifických vazebných a funkčních oblastí genů *MYH7* a *TNNT2* byly nalezeny tři sekvenční záměny u třech nepříbuzných pacientů. Mutace $Asp^{778}Val$ byla objevena u pacienta s těžkou formou hypertrofické kardiomyopatie. Tato změna byla vybrána pro následnou 3D molekulární modelaci *in silico*. $Asp^{778}Val$ nemění pouze charakter interakčních vlastností s ostatními aminokyselinami nebo ionty, ale valin, coby malá hydrofobní aminokyselina, může zcela pozměnit stabilitu celé oblasti. Tato mutace může změnit dynamiku a flexibilitu dlouhé helikální části těžkého řetězce β-myosinu. Ve studii s diabetickými pacienty byla významně zvýšena leukocytární exprese uPAR a PSGL-1 ve srovnání s kontrolním souborem. Leukocyto-trombocytární agregáty, exprese uPAR a PSGL-1 po léčbě rosiglitazonem významně poklesly.

Závěr: Umístění mutací v genech *MYH7/TNNT2* a tedy následné změny ve složení sekvence aminokyselin může mít zásadní negativní dopad na mechanismus vývoje hypertrofické kardiomyopatie. Mutace, které mají za následek změnu vlastností (náboje) aminokyseliny, mají pravděpodobně větší vliv na funkci proteinů než konzervativní mutace. Ve studii s rosiglitazonem jsme po léčbě pozorovali významný pokles exprese trombogenních markerů na leukocytech, předpokládáme tedy, že rosiglitazon vede ke snížení rizika aterotrombotických komplikací.

3. Introduction

Clinical insight

Hypertrophic cardiomyopathy (HCM) is a complex inheritable cardiac disease that is highly clinically and genetically heterogeneous. Clinical leading features of HCM are characterized macroscopically by left and/or right ventricular hypertrophy which is in most cases asymmetric with involvement of the intraventricular septum in absence of other causes of hypertrophy (e.g. valvar stenosis and hypertension), however, the symmetrical form of HCM accounts for over one third of cases and is characterized by concentric thickening of the left ventricle with a small ventricular cavity dimension (McKenna 1996, Davies and McKenna 1995).

The prevalence of the HCM in the general population is believed to be 0.2 % according to the echocardiographic criteria (Maron et al. 1995). However, according to Abchee and Roberts (1996) this may not be an accurate reflection of the true prevalence for several reasons:

- Firstly, HCM/familial hypertrophic cardiomyopathy (FHC) may be asymptomatic and never detected except incidentally.
- Secondly, the presence of concomitant diseases such as hypertension or valvular heart diseases may confound the diagnosis.
- Thirdly, the phenotypic expression of the disease (i.e. development of hypertrophy) is age dependent and may not be detected at the time of the evaluation.
- Fourthly, the penetrance of the gene in some families is very low.

The echocardiographic and clinical features that increase the probability of the development of familial hypertrophic cardiomyopathy include:

- Family history of hypertrophic cardiomyopathy or early sudden death.
- Significant regional differences in hypertrophy.

- Diastolic dysfunction.
- Abnormal ultrasonic myocardial reflectivity.
- Absence of deconditioning – induced regression of hypertrophy.
- Abnormalities in coronary flow reserve.

There are also many risk factors associated with HCM worsening that have to be mentioned. These include high cholesterol levels, high blood pressure, diabetes mellitus and a number of pathological abnormalities of the platelet function and of coagulation factors (Capek et al. 2011). These risk factors markedly contribute to development of premature atherosclerosis. Platelets play central role in the development of acute atherothrombotic events (Fuster et al. 2005). They produce molecules which modulate leukocyte recruitment into atherosclerotic lesions (Stratmann and Tschoepe 2005). The activated platelets also interact with the circulating leukocytes to form leukocyte-platelet aggregates which contribute to the development of atherosclerotic lesions as well as plaque disruption and thrombosis (Sarma et al. 2002, Furman et al. 1998).

The formation of leukocyte-platelet aggregates is primarily mediated through binding of platelet P-selectin to its ligand PSGL-1 in leukocytes (Sarma et al. 2002). uPAR is a plasma membrane receptor for the urokinase-type plasminogen activator (uPA). Serving as an anchor for uPA, the uPAR localizes the cell-surface associated activation of plasmin; that in turn leads to fibrinolysis, activation of matrix metalloproteinases and degradation of extracellular matrix (Ragno 2006).

Therefore, long term attention should be paid to testing of new pharmaceuticals that reduce production of inflammatory aggregates/atherosclerotic plaques (Capek et al. 2011). The correction of these aberrations might translate into the reduction of cardiovascular risk in patients with HCM simultaneously suffering from type 2 diabetes mellitus (Davidson et al. 2010, Dimitrow et al. 2008, Cambronero et al. 2009, Capek et al. 2011).

It is important to mention, that in different patients hypertrophy varies markedly in extend and distribution as well as the severity of clinical symptoms, the age of onset, and the natural course of the disease not only among families, but within the same family carrying the same mutation. In some families the onset of the disease is late in adulthood, the hypertrophy is minimal and they have a normal lifespan, while other families have a very early onset, massive hypertrophy associated with severe symptoms and a very short lifespan due to sudden cardiac death. Now, with the knowledge of the responsible genes and the ability to detect the underlying genetic defect, we are able to determine whether specific genotypes lead to different phenotypes (Stroumpoulis et al. 2010, Perrot et al. 2005, Davies and McKenna 1995, McKenna et al. 1981, Watkins et al 1992, Klaassen et al. 2008, Karam et al. 2008).

Molecular genetic basis of hypertrophic cardiomyopathy

From the genetic point of view hypertrophic cardiomyopathy is a congenital heart disease with autosomal dominant pattern of inheritance and incomplete penetrance (Watkins et al. 2008). Hypertrophic cardiomyopathy affects around 1 in 500 people and is the leading cause of sudden cardiac death in youth (Maron et al. 1995). According to Marian and Roberts (2001) approximately two-thirds of patients have a family history of HCM. The rest of the cases are sporadic, which is due to mutations that arise *de novo*. However, these patients can presumably transmit the disease to their offspring.

Hypertrophic cardiomyopathy is defined as a disease of the sarcomere because majority of the genes that are associated with HCM development encode for cardiac sarcomeric proteins, however, other disease causing genes are likely to be found (Bonne et al. 1998, Kaski et al. 2009, Olivotto et al. 2008, Maron et al. 2003). Hypertrophic cardiomyopathy is mostly due to many different mutations in at least sixteen genes that have been identified so far (Kelly and Semsarian 2009, Morimoto 2008, Capek 2005, Capek and Skvor 2006, Capek et al. 2011, Fatkin and Graham 2002, Fung et al. 1999).

Mutations have been found in four genes that encode components of the thick filament: β-MHC (Capek 2005, Capek et al. 2011, Tanjore et al. 2010), essential MLC (Poetter et al. 1996), regulatory MLC (Poetter et al. 1996), and cMyBP-C (Bonne et al. 1995, Watkins et al. 1995, Van Dijk et al. 2009); in five genes that encode thin filament proteins: cardiac actin (Olson et al. 2000), cardiac troponin T (Capek and Skvor 2006, Hershberger et al. 2009), cardiac troponin C (Hoffmann et al. 2001), cardiac troponin I (Kimura et al. 1997), and α-tropomyosin (Thierfelder et al. 1994); and in the sarcomeric cytoskeletal protein titin (Satoh et al. 1999, Millat et al. 2010, Brouwer et al. 2010).

It is not currently possible to establish correlation between the presence of the mutation in one of the sarcomeric proteins and particular phenotype (Arad et al. 2002). The same mutation can be found in individuals with a different clinical manifestation (Van Driest et al. 2002). In genotyped individuals the prognosis varies markedly between different mutations in the same gene.

The age of onset is variable and partly dependent upon the underlying mutation. The diagnosis of familial form of hypertrophic cardiomyopathy depends on molecular identification and analysis of the candidate genes and of the abnormal gene product. It was reported that in the cases of FHC, around 60–70 % have been attributed to a causal mutations (Arad et al. 2005, Watkins et al. 2008).

It is already known, that different mutant proteins cause similar functional abnormalities, which sequentially initialize the same disease pathways, although they are members of the same functional group and have very different properties and roles (Redwood et al. 1999). Some of them have enzymatic and force generating roles (e.g. myosin heavy chain), while others play structural roles (e.g. myosin binding protein C) or have regulatory functions (e.g. troponin T, I and α-tropomyosin) (Redwood et al. 1999).

For each disease gene, a variety of different mutations have been reported. Single nucleotide substitutions „missense mutations“ and deletion or insertion of nucleotides have been identified. In some cases, the encoded protein is of normal size. In other cases, the mutation may

result in a premature termination codon or cause a shift of the reading frame with truncation of the encoded protein. Mutation located at intron – exon boundaries can result in abnormal splicing. Generally, individuals with HCM causing mutations are heterozygous at the disease locus, i.e., one copy (allele) of the gene is mutated and the other allele has the normal DNA sequence (Fatkin and Graham 2002).

Since the majority of HCM disease genes encode protein components of the sarcomere, it has been widely proposed that left ventricular hypertrophy is not a primary manifestation but develops as compensatory response to sarcomere dysfunction. Characterization of the fundamental deficit resulting from HCM causing gene mutations has been a major focus of research over the last decade. A variety of techniques have been used to examine the effects of mutations on sarcomere structure and function, ranging from *in vivo* studies of myocardial performance in genetically engineered mouse models to *in vitro* studies of interactions between single actin and myosin molecules (Fatkin and Graham 2002). Since both the technology and techniques used in the field of genetics and proteomics are becoming more sophisticated, *in silico* functional 3D analysis of defected proteins might help to understand the process of HCM development from the elemental molecular level and therefore illustrate presumptive genesis of the disease (Capek et al. 2011). Investigators have sought to answer questions such as whether the various sarcomere protein mutations cause similar or diverse effects on sarcomere structure and function and whether sarcomere protein mutations act by a dominant negative mechanism or alter function by causing haploinsufficiency. In the dominant negative model, both wild type and mutant proteins are present in equivalent proportions; the mutant peptide is stably incorporated into the sarcomere but acts as „a poison polypeptide” and perturbs wild type protein function. Alternatively, mutations may result in null alleles or cause a reduction in the amount of wild type protein, leading to an imbalance of sarcomere protein stoichiometry. Mutations that truncate the encoded protein are thought to act by haploinsufficiency. Understanding the consequences of sarcomere protein mutations is an essential prerequisite for determining the stimulus for hypertrophy in HCM (Fatkin and Graham 2002).

Mutations in *MYH7* and *TNNT2* genes are associated with HCM development

Cardiac β-myosin heavy chain and its role in hypertrophic cardiomyopathy development

MYH7 gene was the first gene identified as a disease causing gene in hypertrophic cardiomyopathy. Most mutations found in this gene are related to distinct functional and structural domains of the β-myosin heavy chain. These defects are clustered at specific regions in the globular head of the myosin molecule (subfragment S1), that are:

- Firstly, associated with the actin binding site.
- Secondly, near nucleotide binding site (ATP binding).
- Thirdly, adjacent to the region that connects two reactive cystein residues.
- Fourthly, at the myosin light chain binding interface, and lastly, at the head rod junction.

The primary genetic defect appears to be impaired contractility, which triggers the release of growth factors that result in compensatory hypertrophy and fibroblast proliferation (Marian 2000).

Despite the limitations of existing phenotype-genotype correlation studies (e.g. influence of non genetic factors on the phenotypic expression of HCM), it is generally agreed that mutations affect the phenotypic expression of HCM, in particular the magnitude of cardiac hypertrophy and the risk of sudden cardiac death (SCD) (Marian 2005).

Mutations that are associated with a high incidence of SCD and premature death often exhibit high penetrance and an early age of onset. In contrast, the mutations associated with a benign prognosis often exhibit low penetrance, late onset of disease, and milder left ventricular hypertrophy. A few cases of homozygosity for causal mutations and compound mutations have been described. These mutations lead to a more severe morphological phenotype and a higher incidence of SCD (Marian and Roberts 2001). Such a gene defects in *MYH7* and other HCM causing genes have been designated in the literature as either „benign“ or „malignant“. It has also been suggested that charge-changing amino acid

substitutions may be associated with more severe disease (Van Driest et al. 2004). It has, however, become clear, that intrafamilial variation is also marked, particularly with regard to the morphological features of the disease (Redwood et al. 1999).

It has been wildly accepted that patients with mutations that changed the charge of the altered amino acid (e.g. $Arg^{403}Gln$, $Arg^{453}Cys$, $Arg^{719}Trp$) had a significantly shorter life expectancy, whereas patients with mutations that did not produce change in charge (e.g. $Val^{606}Met$, $Phe^{513}Cys$) had nearly normal survival. Each of these benign mutations is a charge-conservative mutation, suggesting that the lack of charge change may in part account for the good prognosis associated with these mutations (Watkins et al. 1992, Woo et al. 2003).

The vast majority of disease related *MYH7* mutations are missense alterations that result in single amino acid substitution. It has been proposed that the „degree of malignancy“ of the *MYH7* mutation relates to the change in residue charge impaired by amino acid substitution. Theoretically, because amino acid differs in terms of structure and side chain charge or polarity, the substitution of an amino acid may lead to the destabilization of the protein structure and function. This effect may be even more emphasized if the substitution occurs in critical sites, such as the areas involved in ATP hydrolysis and in interaction with thin filaments, leading to the production of a myocardial substrate more vulnerable to mechanisms of sudden death (Sorajja et al. 2000).

Most HCM causing mutations in contractile protein genes are relatively „subtle mutations“ changing just one nucleotide and resulting in the substitution of just one amino acid in the particular protein. This raises the question of the mechanism by which such seemingly minor changes result in disease. It appears that most, and perhaps all, mutations that cause HCM do so by a dominant-negative action. Proof of the dominant negative mode of action of HCM causing mutations has important implications. If, instead of following the dominant-negative model, HCM had resulted from an imbalance in stoichiometry of components needed for self-assembly of the sarcomere, there would be no merit in *in vitro* analyses of mutated proteins implicated in hypertrophic

cardiomyopathy. However, in the light of the dominant-negative pathogenesis, the starting point for an understanding of this disease (and its relevance to other causes of hypertrophy) must be a careful biochemical, biophysical and physiological analysis of the mutant proteins and the deficits they cause. The β-MHC studies have shown that the HCM mutations in general result in myosin which generates less force and these data have led to the „hypocontractile“ hypothesis by which the decreased force provides the stimulus for compensatory hypertrophy. In contrast to this, the α-Tm HCM mutants do not appear to cause a depression of maximum force. These proteins cause an increase in the Ca^{2+} sensitivity of force production and hence give an increase in force at submaximal Ca^{2+} concentrations. Mutations in this gene, and possibly those in regulatory MLC or essential MLC, may cause hypertrophy by a more direct “hypercontractile” mechanism. In addition, increased Ca^{2+} sensitivity might produce abnormalities of relaxation. Although the cardiac troponin T mutants give an increased velocity in the *in vitro* motility assay and an elevated unloaded shortening velocity in skinned myotubes, they have also been shown to result in reduced maximum force and hence they may act via a hypocontractile route. Clearly, both the increased energy demands and altered contractility could contribute to the disease progression; potentially the mechanical deficits underlie the compensatory hypertrophy, while the metabolic deficits implicate the propensity to ischemia and arrhythmia, and hence sudden death (Redwood et al. 1999).

According to Van Dries et al. (2004), mutations in the *MYH7* gene are associated with greater hypertrophy and younger age at diagnosis, which may assist in targeted gene screening. Identification of the pathogenic mutation will aid in preclinical diagnosis and genetic counseling, however, the *MYH7* mutations status should not be considered a primary risk factor for sudden cardiac death. Family studies appear to indicate that mutations in the *MYH7* gene generally result in early onset of the disease, usually in the first two decades of life (Tsoutsman et al. 2006).

Taken together, we do not yet have the understanding of HCM necessary to determine which mutation, combination of mutations or combination of mutation and environmental factors portend an ominous clinical outcome (Ackerman et al. 2002).

Cardiac Troponin T and its role in hypertrophic cardiomyopathy development

The localization of the human *TNNT2* gene is on the long arm of the chromosome 1 (1q32.1). *TNNT2* is comprised of 17 kb of genomic DNA and has 17 exons. The principal isoform in the adult heart consists of 288 amino acids and has two major domains: an NH2 terminal domain that interacts with tropomyosin and a COOH terminal domain that binds to tropomyosin, troponin C, and troponin I (Fatkin and Graham 2002).

In general, mutations in the *TNNT2* gene are associated with mild left ventricular hypertrophy, but have a relatively poor prognosis. Despite the significance of the casual mutations, none of the clinical or the echocardiographic manifestations of HCM are specific to a certain mutation or gene. It is also clear that the casual mutations do not fully explain the degree of variability in the phenotype of HCM; there is a significant variability in the phenotype of HCM among individuals with the same mutation. This indicates that other genetic factors (modifier genes) and environmental factors play important roles in modifying HCM phenotypes (Marian 2002).

The greatest amount of mutations is located between the residues 79 and 179 in the region that is known for its binding to the C terminal tropomyosin domain. Regions tested in the presenting research of the *TNNT2* gene play crucial role in the binding ability of cardiac troponin T to α-tropomyosin and therefore these regions are under direct spotlight as one of the probable „HCM causing regions”. Another mutation hotspot is located between residues 92 and 110 of the *TNNT2* gene. Changes in the region cause less effective binding of tropomyosin to actin (Palm et al. 2001). The penetrance and clinical presentation of mutations in *TNNT2* gene differ greatly. Whereas some mutations result in subclinical hypertrophy associated with high sudden cardiac death, others are completely penetrant but without a high risk of arrhythmical events

(Sehnert et al. 2002). To be able to understand how mutations in different genes especially those that encode for contractile proteins cause hypertrophic cardiomyopathy, it will be necessary to understand the functional consequences of the mutations at a molecular level (Redwood et al. 1999, Fatkin and Graham 2002, Capek et al. 2011).

4. Aims of Study

1. To analyze specific binding and functional regions of “HCM causing genes” that are commonly associated with severe forms of hypertrophic cardiomyopathy.
2. To build a 3D model of human β-MHC (mutant and the wild type variant) based on the results of the molecular genetic part of the study.
3. To perform structure prediction and functional analysis of the 3D molecular model of β-myosin heavy chain protein (mutant and the wild type variant of myosin fragment based on the results of the molecular genetic part of the study) *in silico*.
4. To perform a study of the selected cardiovascular risk factors (e.g. number of prothrombotic abnormalities and diabetes mellitus) that may play a crucial role in the HCM worsening.

5. Material and Methods

Molecular genetic analysis of specific binding and functional regions of "HCM causing genes"

170 probands from different parts of the Czech Republic were enrolled in the study of *MYH7* gene. The DNA bank of the Department of Anthropology and Human Genetics, Faculty of Science, Charles University in Prague comprises all the samples tested and all procedures were carried out in line with the institutional ethical guidelines to meet requisite criteria.

This cohort can be divided into three major subgroups: 1) patients with sporadic form of HCM where HCM has been clinically proved by echocardiography and in this case no family history of HCM was reported, 2) patients with familial form of HCM where positive HCM occurrence have been found in at least one of the family member – 24 families with FHC diagnosis have been identified to meet this criteria. 3) third subgroup included family members of patients suffering from HCM. These probands did not have any signs of heart disorder at the moment of the study, however were enrolled in the genetic screening for possible sequence alteration in the *MYH7/TNNT2* specific binding and functional regions.

DNA was extracted from peripheral blood leukocytes by the use of phenol-chloroform extraction. Using forward and reverse primers polymerase chain reaction was performed for amplification of specific regions of the genomic DNA. Oligonucleotides for the amplification of tested *MYH7* gene regions were synthesized with 394 DNA/RNA Synthesizer (Applied Biosystems, USA). Sequencing primers were purified with OPC (Oligonucleotide Purification Cartridge, CPG Inc., USA). Concentration of synthesized oligonucleotides was measured with spectrophotometer UV/VIS (Beckman, USA). The oligonucleotides were diluted and concentration of 0.1 mM was used in the experiments. Optimal temperature reaction profile and $MgCl_2$ ions concentration were tested with gradient thermocycler Peltier Thermal Cycler DNA Engine DyadTM (MJ Research, USA). MinElute PCR Purification Kit (Qiagen, USA) was used to purify PCR products. The sequencing reactions were performed

using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with automatic sequencer ABI PRISM® 3100 Avant (Applied Biosystems, USA).

Material and methods, including results (retested sequencing raw data) used in the research of selected functional regions of *MYH7* gene were part of the full underlying mutation report that was submitted to the mutation database (Capek 2005, Sarcomere Protein Gene Mutation Database: http://genepath.med.harvard.edu/~seidman/cg3/index.html). The Genetic analysis of *TNNT2* gene is described in detail in the paper: Hypertrophic Cardiomyopathy: Molecular Genetic Analysis of Exons 9 and 11 of the *TNNT2* Gene in Czech Patients (Capek and Skvor 2006).

Homology model of the human β-MHC protein

The 3D model of human β-myosin heavy chain was built using the X-ray structure of nucleotide-free scallop myosin S1 (PDB accession code 1KK8) as the structural template. Based on global pair-wise alignment of the human (UniProt id P12883) and scallop myosin (UniProt id P24733) performed by EMBOSS Pairwise Alignment Algorithms (http://www.ebi.ac.uk/Tools/emboss/align/) with BLOSUM62 matrix, replacement of the structurally conserved regions and rebuilding of the variable regions was done with the homology module of program MOE (Chemical Computing group Inc., Canada). RMSD of the framework (Cα) was about 2.4 Å in the energy optimized model of human β-myosin compared to scallop myosin. It is necessary to say that only the region spanning amino acids 1 to 835 of the β-myosin heavy chain was modeled. The reason is that the mutation in question – $D^{778}V$ was present in this part of the structure (Capek et al. 2011).

Short peptides mapping the 769–788 region

De novo structure prediction of two peptides spanning the 769–788 region of the human β-myosin heavy chain was performed. The first peptide sequence contained the D amino acid at position 778 (LLGLLEEMRDERLSRIITRI), the second peptide was $D^{778}V$ mutant variant of the wild type myosin fragment (LLGLLEEMRVERLSRIITRI)

(Capek et al. 2011). The web based prediction PEP-FOLD server (http://bioserv.rpbs.univ-paris-diderot.fr/PEP-FOLD/) was utilized to obtain 3D models of the peptides. The PEP-FOLD method is based on structural alphabet (SA) and utilizing a greedy algorithm and a coarse-grained force field to predict a structure (Maupetit et al. 2009).

Reduction of expression of thrombogenic markers on leukocytes

The study with rosiglitazone included 33 patients with type 2 diabetes mellitus and 32 normal controls. The patients were examined at baseline and after 5 month of treatment.

The expression of leukocyte markers was measured by an immunofluorescence method using single-step staining with monoclonal antibodies. The fluorescence was quantified by the flow cytometry (FACSCalibur, Becton Dickinson, USA); lymphocytes, monocytes and neutrophils were identified according to their light-scattering properties and were analyzed separately.

A specific fluorescence was used as a measure of antigen expression which was calculated as the difference between the fluorescence of cells labeled with the specific antibody and the non-specific fluorescence of cells labeled with the control antibody. Material and methods used in the study of possible reduction of cardiovascular risk factors are described in detail in the paper: The effect of Rosiglitazone on the Expression of Thrombogenic Markers on Leukocytes in Type 2 diabetes mellitus (Svobodova et al. 2009).

6. Results

There were three sequence alterations found in three unrelated patients afflicted with the severe form of hypertrophic cardiomyopathy. ΔGlu160 was localized in the *TNNT2* gene (Capek and Skvor 2006). The $Arg^{870}His$ mutation was observed in the exon 22 of the *MYH7* gene. The last one, $Asp^{778}Val$ – reported for the first time in a European patient with hypertrophic cardiomyopathy was detected in the encoding sequence of exon 21 of the *MYH7* gene (Capek 2005). There was no other sequence alteration found in the regions of *MYH7/TNNT2* genes that were tested. The $Asp^{778}Val$ amino acid alteration was chosen for subsequent molecular modeling followed by structure prediction and functional analysis *in silico*, since such a significant change can play crucial role in the molecule behavior.

Homology model of the β-MHC protein

Homology model of the β-MHC N-terminal, motor domain and EF hand binding site (1–75, 76–779, 780–830) was created using scallop myosin as a template (PDB 1KK8). The full length alignment of both sequences of length 1958 amino acids shows a relatively good agreement to build a homology based model. The identity of both chains was 55.8 % (1092/1958) and the similarity was 74.6% (1460/1958). Two structures of β-MHC were obtained representing two variants of the proteins – the wild type and the $D^{778}V$ mutant. There is no difference between these two models at general molecular level (Fig. 1). Even the $D^{778}V$ mutant shows the same helicity spanning the region 761–830 (Capek et al. 2011).

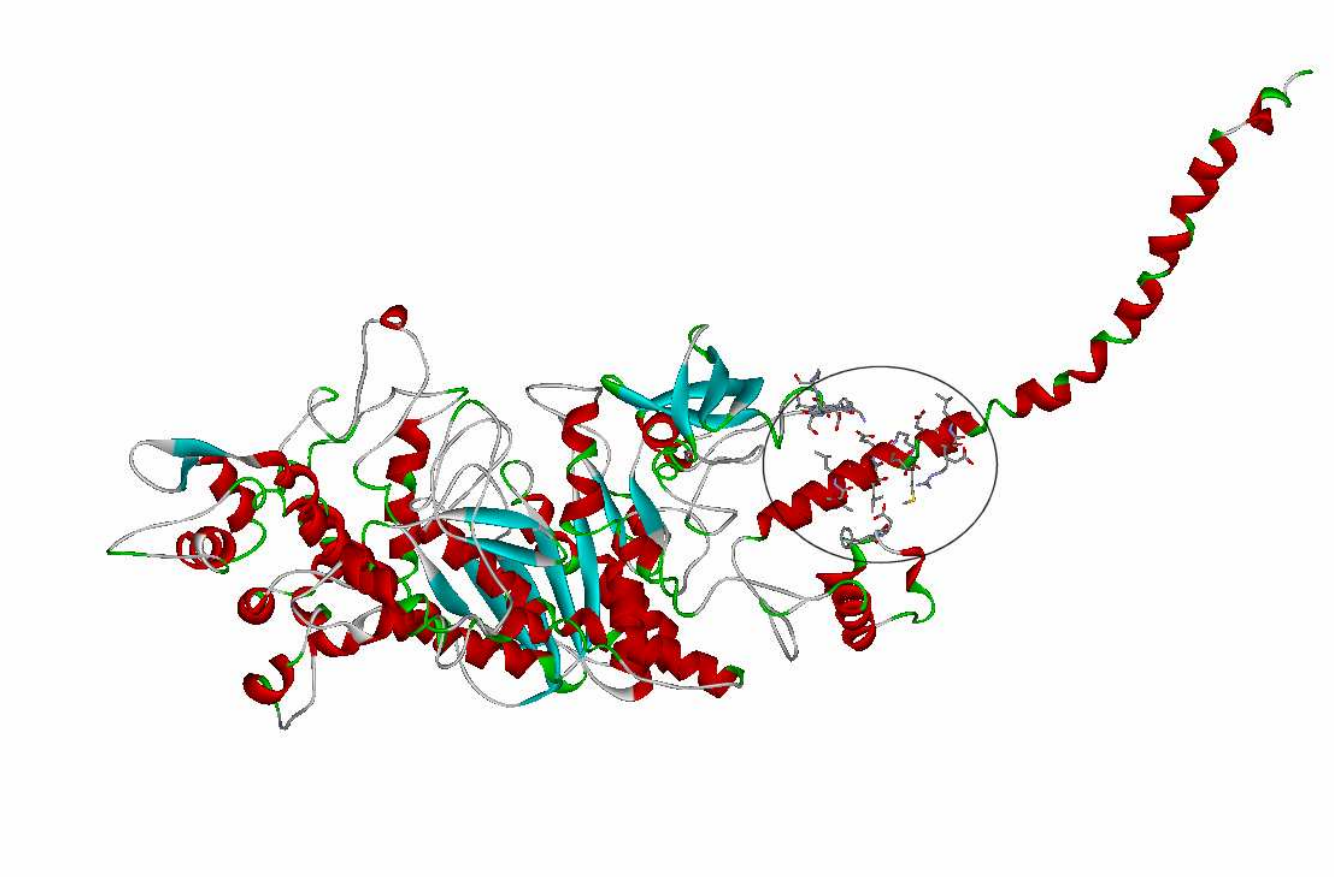

Fig. 1

Short peptides mapping the 769–788 region

The basic idea behind the model of short peptides mapping 769–788 region was to localize the differences in predicted peptide structures suggesting how a property of one amino acid can change the quality or dynamics of the short sequence in question. As follows from the predicted structures both regions are helical but there is one very important difference. The D778 is stabilized by interaction with R777 and E 779 is stabilized by interaction with R780 (Fig. 2A) (Capek et al. 2011).

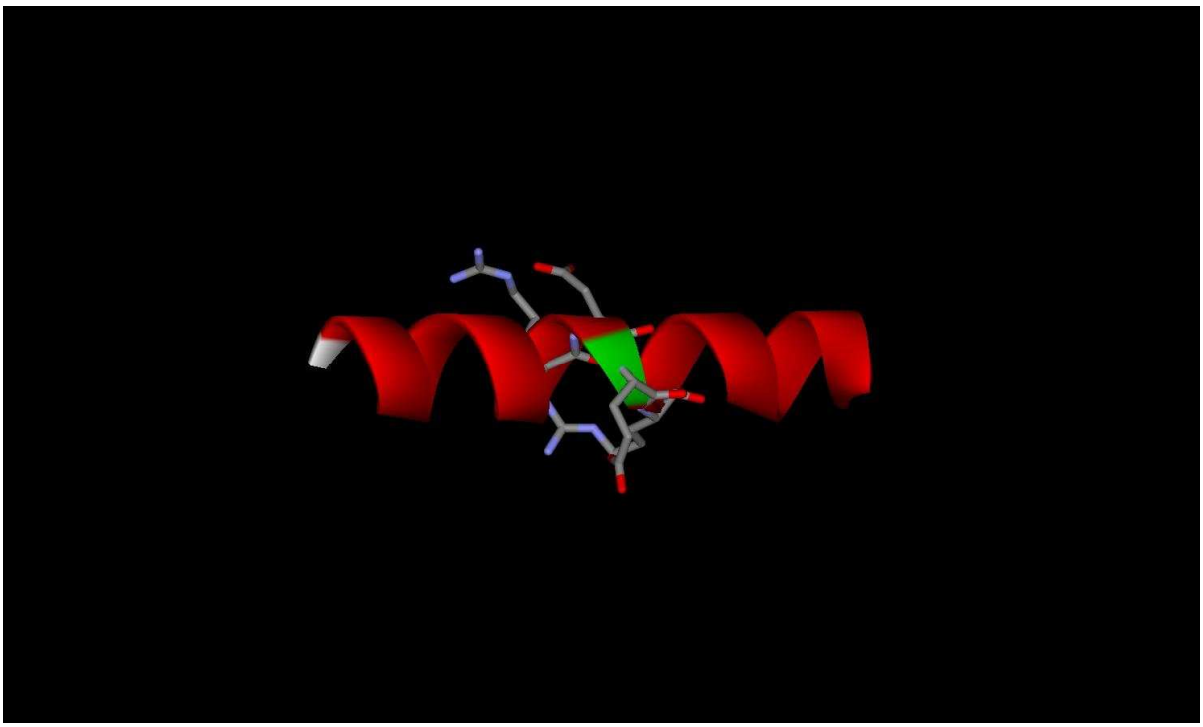

Fig. 2A

This introduces a tension in the helix and indeed, the predicted structure shows the measurable difference from ideal helicity. Contrary to D, the V in position 778 does not destabilize the structure of the helix (Fig. 2B).

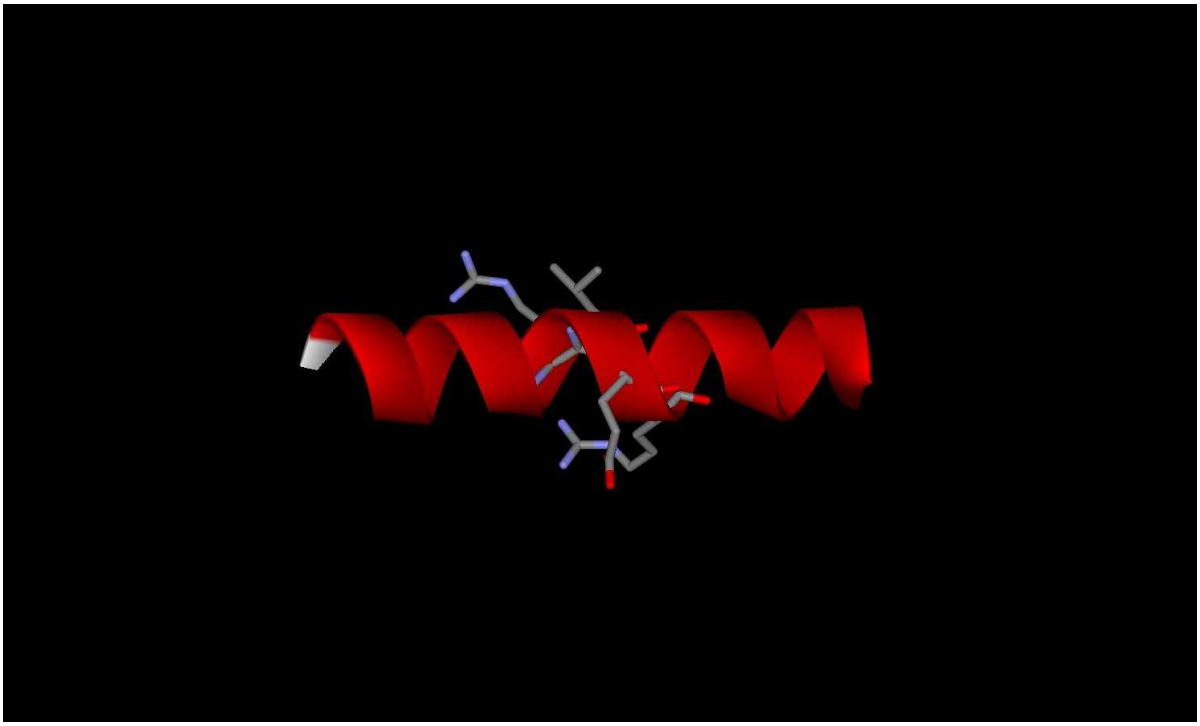

Fig. 2B

No information about dynamical behavior of this part of myosin was studied before this analysis. According to our study, dynamical behavior of both peptides is quite different. Short MD simulation (10ns) in explicit solvent revealed that rigidity of the $D^{778}V$ mutant of the peptide is 5–6 times higher than the D778 variant of the peptide. This can be very important factor in dynamics of the myosin taking also other factors into account – Ca^{2+} affinity, EF hand binding properties and solvation of the region (Capek et al. 2011).

Reduction of expression of thrombogenic markers on leukocytes

Increased expression of PSGL-1 in all leukocyte subpopulations and of uPAR in monocytes was found in comparison to controls. All these parameters were normalized after rosiglitazone treatment. The differences were not significant for uPAR in neutrophils and uPAR expression was not detectable in lymphocytes.

There was no significant difference in leukocyte-platelet aggregates in patients compared to controls; the aggregates were reduced significantly after the treatment. The treatment effect was more pronounced for total platelet fluorescence than for the percentage of platelet-positive cells, suggesting that rosiglitazone not only decreased the number of platelet-positive cells, but also the number of platelets per one leukocyte.

7. Discussion and Conclusion

This comprehensive study represents not only an analysis of critical regions of HCM causing genes commonly reported as regions with high mutation clustering (Buvoli et al. 2008) followed by molecular 3D modeling of defected cardiac β-myosin heavy chain protein, structure prediction and functional analysis of the 3D molecular model of β-myosin heavy chain protein *in silico* but also focuses on the reduction of selected cardiovascular risk in patients with hypertrophic cardiomyopathy.

The regions analyzed in this study are associated with severe forms of hypertrophic cardiomyopathy and therefore with very poor prognosis (Wang et al. 2008, Woo et al. 2003). Mutation location in the *MYH7/TNNT2* genes and therefore changes in the amino acid composition may have crucial negative impact on the disease outcome in HCM affected patients. Due to the different properties between the globular head domain (S1), the neck or hinge region (S2) and the tail (light meromyosin) domain of the *MYH7* gene, mutations may have diverse effects depending on their location (Miroshnichenko et al. 2000). In addition, as a mutation can lead to a change in the amino acid sequence, the structure and interactive properties of the mutant protein may also be altered. Therefore, the positioning of the mutations along the gene and protein may offer insights into the mechanism by which normal protein function is impaired. It is hypothesized that a change in the amino acid charge may affect the severity of the phenotype (Walsh et al. 2010).

Genetic results from this study show unusually low frequency of mutations in *MYH7/TNNT2* genes in the regions tested in patients with HCM in the Czech population in contrast to other studies published elsewhere (Van Driest et al. 2002, Walsh et al. 2010, Zheng et al. 2010, Millat et al. 2010). However, in comparison to these results, Roncarati et al. (2011) recently published a study showing similar low frequency of mutations in the *MYH7* gene on large cohort of Italian HCM patients. This supports the idea that there is a need for more inclusive investigative approaches in order to fully understand the development of this disease. In the research of *TNNT2* gene ΔGlu160 was localized in the overlapping region which is responsible for binding of troponin T to α-Tm, suggesting

that this deletion causes important negative changes in binding these two molecules together. This deletion possibly causes an increasing affinity to the whole molecule of cardiac troponin T to tropomyosin and therefore might have crucial negative impact on the disease (Capek and Skvor 2006). The Asp^{778}Val amino acid alteration was situated in the region that is highly conserved inside of the known sequence of the cardiac β-myosin heavy chain (where head of the gene extends from exon 3 to part way through exon 21 and the neck from part way through exon 21 to part way through exon 25), thus indicating that the alteration in this region of this gene may have crucial structural and functional impact (Capek et al. 2011).

Homology model of the β-MHC protein was built. This model was created using scallop myosin as a template (PDB 1KK8). The full length alignment of both sequences of length 1958 amino acids shows relatively good agreement to build a homology based model. The identity of both chains was 55.8 % (1092/1958) and the similarity was 74.6% (1460/1958). Two structures of β-MHC were obtained representing two variants of the proteins – the wild type and the D^{778}V mutant. There is no difference between these two models at general molecular level. Even the D^{778}V mutant shows the same helicity spanning the region 761–830. There are other models of β-MHC in databases – 1KK2 in the PDB database and model at Protein Model Portal (http://www.proteinmodelportal.org/query/uniprot/P12883) based on the alignment of human β-MHC with sequence of *Gallus gallus* myosin whose structure was recently published (PDB ID 2MYS, sequence identity 79 %). The structural alignment of this model with both obtained models showed similar RMSD about 14Å. In both cases, the majority of the differences are caused by distortion of the C terminal helix containing the studied mutations D^{778}V. Moreover the part around residue 778 is in 1KK2 model significantly non helical, whereas both other models provide good agreement in this part.

The model shows that the aspartic acid at position 778 is located at the beginning of the long helix (starting Thr 761) in charge rich environment – Glu, Asp, Arg. One can assume extensive solvation takes

place in this region or the region is important for its interaction dependent on Ca^{2+}. The hypothesis that this is quite unique environment is further supported by the finding that 780–810 region is predicted to be an IQ motif – the EF hand binding site. Mutation of the Asp by Val not only changes the character of the interaction pattern with other amino acids or ions but Val being a small hydrophobic amino acid can completely change the stability of the region. It is hypothesized that it can change the dynamics and flexibility of the long helical part or it can modify its interaction property.

Beside the homology model of the β-MHC protein, short peptides mapping the 769–788 region were built as well. The basic idea behind the model of short peptides mapping 769–788 region was to localize differences in predicted peptide structures suggesting how a property of one amino acid can change the quality or dynamics of the short sequence in question. As follows from the predicted structures, both regions are helical but there is one very important difference. The D778 is stabilized by interaction with R777 and E779 is stabilized by interaction with R780. This introduces a tension in the helix and indeed, the predicted structure shows a measurable difference from the ideal helicity. Contrary to D, the V in position 778 does not destabilize the structure of the helix and seems to be important stabilizing element of this part of the structure.

No information about dynamical behavior of this part of myosin was known before this analysis. According to our study, dynamical behavior of both peptides is quite different. Short MD simulation (10ns) in explicit solvent revealed that the rigidity of the D778V mutant of the peptide is 5–6 times higher than the D778 variant of the peptide. This can be a very important factor in dynamics of the myosin taking also other factors into account – Ca^{2+} affinity, EF hand binding properties and solvation of the region.

As follows from the homology model as well as from the modeled peptides, there are 3 principal aspects which can alter the function of this domain dramatically. The first is the intramolecular stabilization pattern dependent on D778 interaction with R777 which on the other hand can destabilize the helicity and make this part more dynamic. The second

aspect is connected with interaction pattern of the original as well as the mutated variant of the 778 region. The fact that 780–810 is IQ interaction pattern – a partner for EF hand suggest that D can play a very important role in maintaining proper interaction properties for such process. Last but not least the dynamics of the D778 and $D^{778}V$ is different suggesting that for proper function of the myosin the flexibility or probably structure stability D is important and V in this position increase rigidity which seems to be counterproductive for proper β-MHC function. In addition, a mutation that changes the charge of the amino acid is considered more likely to affect protein function than a conservative mutation as it was hypothesized earlier by Ng and Henikoff (2006).

From the perspective of possible cardiovascular risks, the clinical and pathological characteristics of hypertrophic cardiomyopathy could involve a number of diverse mechanisms that include inflammation, endothelial dysfunction, fibrosis and extracellular matrix degradation, as well as coagulation and platelet activation. Also diabetes mellitus is associated with the overexpression of thrombotic and hemostatic factors (Svobodova et al. 2009). Our data are in agreement with this fact; we observed an increase of uPAR and PSGL-1 expression on leukocytes in diabetic patients contrary to controls. Leukocyte-platelet aggregates are mostly considered as markers of platelet activation. HCM patients have high platelet aggregation (Cambronero et al. 2009). Its increase was furthermore observed in acute coronary syndromes and stable coronary heart disease (Furman et al. 1998). It is important to mention that thromboembolic events are frequent and potentially serious causes of mortality and morbidity amongst HCM patients (Cambronero et al. 2009). According to Dimitrow et al. (2008) the platelet (e.g. P-selectin) and coagulation markers are significantly higher in the HCM patients compared to controls. Formation of leukocyte-platelet aggregates is primarily mediated through binding of platelet P-selectin to PSGL-1 in leukocytes. Our results suggest that interactions with other molecules are also involved in this process (Svobodova et al. 2009).

Expression of uPAR, PSGL-1, and leukocyte-platelet aggregates was significantly decreased after the rosiglitazone treatment, suggesting its antithrombotic effects (Svobodova et al. 2009).

Patients with HCM simultaneously suffering with type 2 diabetes mellitus have substantially increased risk of cardiovascular diseases due to development of premature atherosclerosis that could be positively affected by modification of the abnormalities of thrombotic and haemostatic factors. Therefore, long term attention should be paid to testing of new pharmaceuticals that reduce formation of inflammatory aggregates/atherosclerotic plaques (Capek et al. 2011).

This Ph.D. research was designed to use molecular genetic, proteomic and biochemical techniques to investigate specific binding and functional regions of “HCM causing genes”. Exactly these domains may play crucial role in proper protein/sarcomere function in patients with hypertrophic cardiomyopathy. This was the case of ΔGlu160 mutation in the *TNNT2* gene, the $Arg^{870}His$ mutation and the $D^{778}V$ alteration in *MYH7* gene that were found in three unrelated patients afflicted with severe forms of hypertrophic cardiomyopathy.

The major principle of this complex study was to obtain as much relevant information from the analysis that would help in patients diagnosis and in the following personalized treatment. The future is, in my view, in utilization of new sophisticated methods of genetics, proteomics and biochemistry, precisely these methods that were used in the presented research. These biomedical applications will be needed in the new era of personalized medicine. I am convinced, that 3D molecular models and structure predictions of defected proteins involved in the HCM development followed by functional analysis of these “disease causing” biomolecules *in silico* will be essential in the research of HCM in the very near future.

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