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cMyBP-C was decreased via KLHL3-mediated proteasomal degradation in congenital heart diseases

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ABSTRACT

Cardiac myosin binding protein C (cMyBP-C) is a cardiac structural and regulatory protein; mutations of cMyBP-C are frequently associated with hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM). Cardiac special transcription factors may regulate the expression of cMyBP-C. However, the role of cMyBP-C in congenital heart diseases (CHD) remains poorly understood. In the current study, western blotting and the MRM approach showed that cMyBP-C expression was significantly reduced in fetuses with CHD compared to those without. Furthermore, we found that cMyBP-C interacted with KLHL3 by immunoprecipitation and immunofluorescence, and the degradation of cMyBP-C was caused by KLHL3-mediated ubiquitination. In addition, homocysteine (Hcy, a risk factor of CHD) treatment caused a decrease in cMyBP-C and an increase in KLHL3 expression, and the proteasome inhibitor MG132 reversed the Hcy-induced reduction of cMyBP-C expression. Finally, we verified that reduced cMyBP-C by Hcy promoted apoptosis in cardiomyocytes. These results demonstrate that Hcy decreases the expression of cMyBP-C through a KLHL3-mediated ubiquitin–proteasome pathway, and thereby influences heart development.

1. Introduction

Congenital heart diseases (CHD) are the most prevalent type of birth defects and the main cause of infant mortality. With the improvement of diagnostic methods of birth defects including deformity of internal organs, the incidence of prenatal CHD is on the rise. In China, CHD accounted for 28.82% of all congenital birth defects [1]. CHD can be divided into non-syndromic phenotypes including gene mutations, and syndromic phenotypes including chromosomal abnormalities, such as Down syndrome, Edward syndrome and Patau syndrome [2]. The causes of CHD are complicated, and environmental and genetic factors play significant roles. Recent studies about environmental factors suggest that folic acid deficiency is associated with CHD, and folic acid supplementation can effectively reduce the incidence of fetal CHD. Homocysteine (Hcy), an intermediate metabolite of the methionine cycle, is influenced by folic acid metabolism. Early experiments revealed that 23% of surviving embryos showed ventricular septal defects that could be attributed to treatment with Hcy [3]. In addition, Hcy was able to inhibit cardiac neural crest cell formation, morphogenesis [4] and differentiation [5], induce apoptosis of rat ventricular myocytes [6], and suppress hemodynamic parameters of early embryonic heart function [7]. However, due to the complexity of CHD, its pathogenesis remains poorly understood.

Cardiac myosin binding protein C (cMyBP-C), exclusively expressed in the heart during human and mouse development, is a constitutive component of thick filaments and plays an important role in both sarcomere formation and regulation of contraction [8]. The N-terminus of cMyBP-C can stabilize the ON status of the thin filaments and the OFF status of the thick filaments, leading to a model for the control of heart muscle contraction in which both filament systems are coordinated by cMyBP-C [9]. This suggests that cMyBP-C regulates the positioning of myosin and actin for their interaction, and specifically constrains the actin-myosin interaction, which limits loaded shortening velocity and ultimately the power output. cMyBP-C mutations have been identified as being the underlying cause of hypertrophic cardiomyopathy (HCM) and other cardiomyopathies, such as dilated cardiomyopathy and left ventricular non-compaction. This genetic variant is present in 4% of the population in South Asia and is associated with a 7-fold higher risk of developing heart failure [10]. Together, these studies reveal that cMyBP-C is one of the major mutated genes in cardiomyopathies and heart failure. In animal models, loss of cMyBP-C protein contributes to pericardial effusion, dilatation of the atrium,

Abbreviations: cMyBP-C, Cardiac myosin binding protein C; HCM, hypertrophic cardiomyopathy; CHD, congenital heart diseases; Hcy, homocysteine; MYH6, myosin heavy chain 6; AF, amniotic fluid; KLHL3, Kelch like protein 3

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heart failure [11] and structural rearrangements [10]. In Down syndrome with frequent CHD, cardiomyopathy has been shown to occur [12,13]. In addition, cardiac transcription factors Tbx5, Gata4 and Myocd have been reported to specifically activate the expression of Mybpc3 gene [14]. Mutations in both MyBPC3 and cardiac myosin heavy chain 6 (MYH6) can cause HCM; however, MYH6 has not only been shown to be a downstream transcriptional target of GATA4 and TBX5, but is also associated with human atrial septal defects [15]. It is known that CHD is mainly related to abnormal fetal cardiac structure and function. Therefore, we hypothesized that the cardiac structural and regulatory protein - cMyBP-C is associated with CHD and may play a prominent role in the pathogenesis of abnormal heart development.

In addition to alterations in the protein content of cMyBP-C leading to HCM, the function of cMyBP-C is also regulated by an ever-growing spectrum of posttranslational modifications, namely phosphorylation, acetylation, and citrullination, and so on. cMyBP-C is a highly phosphorylated protein in normal cardiac tissue, but a decrease in cMyBP-C phosphorylation was detected in both end-stage heart failure and HCM [16,17]. In addition, cMyBP-C undergoes severe degradation during myocardial infarction [18]. Protein ubiquitination is a key regulatory process essential to cellular metabolism. Udin Bahrudin et al. [19] provided evidence that the proteasome inhibitor MG132 reversed the decrease of the mutated cMyBP-C protein, indicating that cMyBP-C can be degraded by the ubiquitin-proteasome, and that the mutated cMyBP-C protein importantly increased the pro-apoptotic/anti-apoptotic protein ratio as well as inducing apoptosis in neonatal rat cardiac myocytes. Giulia Mearini et al. [20] found that Atrogin1 interacted with both mutant and wild-type cMyBP-C, but only degraded truncated mutant cMyBP-C, while MuRF1 indirectly reduced cMyBP-C levels. It is known that cMyBP-C protein expression can be degraded via the ubiquitin–proteasome pathway, but the ubiquitin E3 ligases directly involved in ubiquitination of cMyBP-C have not been identified, and this therefore requires further investigation. Kelch like protein 3 (KLHL3) belongs to the Kelch-like gene family, which is mainly involved in cell morphology and organization, the degradation of proteins, gene expression and signal transduction [21]. KLHL3 is a component of an E3 ubiquitin ligase complex, and could interact with WNK4 and claudin-8 to regulate their ubiquitination and degradation [22,23]. Both KLHL3 and cMyBP-C have been shown to bind to actin, so as to suspect that KLHL3 is involved in the control of cMyBP-C ubiquitination.

In the present study, we aimed to investigate the potential pathway by which Hcy modulates cMyBP-C expression. We identified an alteration of cMyBP-C in amniotic fluid (AF) obtained from CHD compared with normal controls, and found that cMyBP-C expression was lower in CHD. We then used Hcy (as a risk factor of CHD) for administering to embryonic rat cardiomyocytes (H9C2), and found that Hcy inhibited cMyBP-C expression. Importantly, we showed that the reduced expression of cMyBP-C protein was due to the KLHL3-mediated ubiquitin–proteasome pathway.

2. Materials and methods

2.1. Amniotic fluid collection

AF samples were obtained from pregnant women who underwent amniocentesis during the 18th to 22nd week of gestation. All pregnant females with fetuses were detected by routine ultrasound and karyotype analysis of cultured amniotic cells. Fetuses with heart malformations were selected as a CHD group (n=36) including CHD, trisomy 21 and trisomy 18, and fetuses with normal ultrasound and karyotype were normal control group (n=18). Ethical approval and informed consent were obtained from the ethics committee of Shengjing hospital and all individuals (ethical approval code: 2015PS66K), respectively. The AF supernatant was collected by centrifugation by 10 min at 3000rmp at room temperature.

2.2. LC-MS/MS

All samples were depleted of albumin and IgG with a protein A/G column (Bio-Rad, Hercules, USA). The samples were then denatured using 8 M urea, reduced with 200Mm dithiothreitol at 50 °C, and alkylated in 500 mM iodoacetamide at room temperature in a dark room. The samples were desalted with a fresh Amicon 10-kDa cutoff centrifugal ultrafiltration device. The samples were then adjusted to equal total protein amounts (200 μg) before digestion with trypsin (Promega, Madison, USA) in the supplied trypsin buffer (1: 50 trypsin: protein concentration; 120 μl 50 mM ammonium bicarbonate, 100 μl methanol, 150 μl H2O) overnight at 37 °C. The samples were lyophilized to dryness and resuspended before analysis. The samples were quantified using API 3200 Q-trap liquid chromatography-tandem mass spectrometry system (LC-MS/MS System, Applied Biosystems, Foster City, CA), and separated on a reverse-phase symmetry C18 column (3.5 μm, 2.1 μm*150 mm; Waters Associates, Milford, MA) at a flow rate of 0.2 mL/min using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) (0–2 min 5% B, 2–25 min 5–25% B, 25–27 min 25–80% B, 27–28 min 80–5% B, 28–30 min 5% B). The effluent was ionized using positive ion electrospray and quantified by multiple reaction monitoring (MRM). The peptide of cMyBP-C protein is LNFDLIQELSHEAR. The peak area was obtained as the originating data. The peptide concentration was calculated according to standard curves generated over a range from 0.125 to 10 μg/mL. We then calculated the originating protein concentration according to the measured peptide concentration.

2.3. Cell culture and treatment

Embryonic rat cardiomyocytes H9C2 were cultured in Dulbecco's modified Eagle's medium (Biological Industries, Kibbutz Beit-Haemek, Israel) containing 10% fetal bovine serum (Biological), 100 U/mL penicillin, 100 g/mL streptomycin at 37 °C in 5% CO2. Hcy (Sigma-Aldrich, Saint Louis, USA) treatment were performed at 100 μM, 200 μM, 300 μM, 400 μM, 500 μM for 24 h. Cells were treated with proteasome inhibitor MG132 (Sigma-Aldrich) at 1 μM, 5 μM, 10 μM, 20 μM for 24 h.

2.4. Western blotting analysis

Total protein was extracted for western blotting. Firstly, cells were homogenized in ice-cold lysis buffer containing protease inhibitor at 4 °C for 30 min. Then homogenates were centrifugated by 10 min at 12,000 rmp at 4 °C. And the protein concentrations were determined. Proteins were separated by 10%SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (PVDF, Millipore, Lake Placid, USA). Whereafter, the membranes were blocked with 5% nonfat dry milk in TBS-T solution (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) at room temperature for 2 h, and incubated with primary antibody specific for cMyBP-C (Abcam, Cambridge, UK), KLHL3 (Proteintech Group Inc., Chicago, USA), ubiquitin (Cell Signaling Technology, Danvers, USA), GAPDH (Proteintech) and β-tubulin (Proteintech) in 1% BSA overnight at 4 °C. The membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. The final detection reactions were visualized using ECL plus chemiluminescence reagent (Biotool , Houston, USA) and analyzed with ECL chemiluminescence detection system (Bio-Rad).

2.5. Co-immunoprecipitation assay (CoIP)

The total extracts were prepared from H9C2 cells, and the protein concentrations were determined. The total protein samples were pre-incubated with primary antibody by rotating at 4 °C overnight. Then protein–antibody complexes were subsequently recovered by 20 μl