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Heterozygous deletion of AKT1 rescues cardiac contractility, but not hypertrophy, in a mouse model of Noonan Syndrome with Multiple Lentigines

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ABSTRACT

Noonan Syndrome with Multiple Lentigines (NSML) is associated with congenital heart disease in form of pulmonary valve stenosis and hypertrophic cardiomyopathy (HCM). Genetically, NSML is primarily caused by mutations in the non-receptor protein tyrosine phosphatase SHP2. Importantly, certain SHP2 mutations such as Q510E can cause a particularly severe form of HCM with heart failure in infancy. Due to lack of insight into the underlying pathomechanisms, an effective custom-tailored therapy to prevent heart failure in these patients has not yet been found.

SHP2 regulates numerous signaling cascades governing cell growth, differentiation, and survival. Experimental models have shown that NSML mutations in SHP2 cause dysregulation of downstream signaling, in particular involving the protein kinase AKT. AKT, and especially the isoform AKT1, has been shown to be a major regulator of cardiac hypertrophy. We therefore hypothesized that hyperactivation of AKT1 is required for the development of Q510E-SHP2-induced HCM. We previously generated a transgenic mouse model of NSML-associated HCM induced by Q510E-SHP2 expression in cardiomyocytes starting before birth. Mice display neonatal-onset HCM with initially preserved contractile function followed by functional decline around 2 months of age. As a proof-of-principle study, our current goal was to establish to which extent a genetic reduction in AKT1 rescues the Q510E-SHP2-induced cardiac phenotype in vivo. AKT1 deletion mice were crossed with Q510E-SHP2 transgenic mice and the resulting compound mutant offspring analyzed. Homozygous deletion of AKT1 greatly reduced viability in our NSML mouse model, whereas heterozygous deletion of AKT1 in combination with Q510E-SHP2 expression was well tolerated. Despite normalization of pro-hypertrophic signaling downstream of AKT, heterozygous deletion of AKT1 did not ameliorate cardiac hypertrophy induced by Q510E-SHP2. However, the functional decline caused by Q510E-SHP2 expression was effectively prevented by reducing AKT1 protein. This demonstrates that AKT1 plays an important role in the underlying pathomechanism. Furthermore, the functional rescue was associated with an increase in the capillary-to-cardiomyocyte ratio and normalization of capillary density per tissue area in the compound mutant offspring. We therefore speculate that limited oxygen supply to the hypertrophied cardiomyocytes may contribute to the functional decline observed in our mouse model of NSML-associated HCM.

1. Introduction

In most cases of non-syndromic hypertrophic cardiomyopathy (HCM), systolic contractile performance of the left ventricle (LV) is normal or even elevated. Only a small subset of patients with 'classic' forms of HCM develop systolic heart failure in end-stage disease [1,2]. Therefore, treatment strategies for patients with HCM are mostly focused on reducing the risks of arrhythmias and sudden death as well as ameliorating symptoms caused by outflow tract obstruction and diastolic dysfunction [3]. In contrast, HCM associated with Noonan Syndrome with Multiple Lentigines (NSML, formerly termed LEOPARD Syndrome) can lead to serious contractile dysfunction in children. In

Abbreviations: +, transgenic; AKT, protein kinase B; HCM, hypertrophic cardiomyopathy; LV, left ventricle; LVAWd, LV anterior wall thickness at end-diastole; LVIDd, LV inner diameter at end-diastole; LVPWd, LV posterior wall thickness at end-diastole; MHC, myosin heavy chain; mTOR, mechanistic target of rapamycin; NTG, nontransgenic; NSML, Noonan Syndrome with Multiple Lentigines; PHLPP1, PH domain and leucine rich repeat protein phosphatase 1; WGA, wheat germ agglutinin

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particular, the Q510E mutation in the protein tyrosine phosphatase SHP2 (encoded by *PTPN11*) results in a severe biventricular form of HCM and heart failure already in infancy [4–7]. Mortality is very high and heart transplantation is often the only option to improve survival for patients carrying the Q510E-SHP2 mutation.

The reasons why these forms of HCM lead to heart failure are still unknown. Genetically, most non-syndromic forms of HCM are caused by mutations in various sarcomeric proteins [8], whereas mutations in the signaling regulator SHP2 are responsible for the majority of NSML-associated HCM cases. Recent in vitro studies have shown that NSML mutations in SHP2 result in increased activation of focal adhesion kinase, phosphatidylinositol-3-kinase, protein kinase B (AKT) and mechanistic target of rapamycin (mTOR) [9–11]. Supporting a central role of the latter signaling mediator in the pathomechanism of NSML-associated HCM, mouse models of NSML have revealed that inhibition of mTOR with rapamycin can ameliorate cardiomyocyte hypertrophy [12,13]. However, rapamycin failed to rescue the functional decline caused by Q510E-SHP2 expression in the mouse heart [13], suggesting that pharmacological intervention targeted at mTOR may have been too far downstream to completely rescue the phenotype. One step further upstream, AKT controls mTOR activity via tuberous sclerosis factor 2 and Rheb [14,15]. Interestingly, it has been demonstrated that inhibition of mTOR can result in suppression of a negative feedback loop that in turn increases the activity of AKT and other kinases [16]. Consequently, inhibition of AKT instead of mTOR may be more effective as a treatment for HCM induced by Q510E-SHP2 expression.

AKT (and in particular the AKT1 isoform) has been shown to play a critical role in the regulation of cardiac hypertrophy [17]. However, AKT's role in NSML-associated HCM may not be straightforward. Prior studies have provided strong evidence that moderate increases in AKT activity do not result in pathologic alterations in the heart and even have cardioprotective effects. For example, physiological stress such as exercise has been shown to increase AKT activation in normal mice [18]. In addition, deletion of PH domain and leucine rich repeat protein phosphatase 1 (PHLPP1) increased AKT activity without affecting cardiomyocyte size or contractility and was beneficial under pathological stress in form of chronic pressure overload [19]. Furthermore, nuclear targeting of AKT also did not induce cardiac hypertrophy, and induced protective effects after pressure overload [20,21]. Importantly, only prolonged transgenic activation of AKT at high levels led to ventricular dilation and death [22]. These findings would argue against the increase in AKT activity observed in NSML models playing a causal role in the HCM pathomechanism. On the other hand, we recently found that pharmacological AKT inhibition prevents cardiomyocyte hypertrophy induced by Q510E-SHP2 expression in culture [11], which supports the notion that AKT promotes pathologic cardiac hypertrophy in NSML.

To resolve this controversy and evaluate AKT1 as a potential new therapeutic target, the goal of our current study was to examine the role of AKT1 in NSML by conducting a genetic rescue experiment. Based on our prior data, we hypothesized that increased signaling through AKT1 is necessary for the development of cardiac hypertrophy and contractile dysfunction in the context of NSML. To test our hypothesis, we crossed Q510E-SHP2 transgenic mice with AKT1 deletion mice [23] and characterized the phenotype of the compound mutant offspring. Importantly, deletion of one AKT1 allele by itself does not result in overt cardiac pathology in adult mice and does not alter susceptibility to stressors such as transverse aortic banding [24]. Therefore, this approach is well suited for proof-of-principle studies to determine whether AKT1 plays an important role in the pathomechanism of NSML-associated HCM.

2. Methods

2.1. Animals

All animal studies were conducted in accordance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals. All procedures were submitted to and approved by the Animal Care and Use Committee of the University of Missouri. Transgenic mice expressing Q510E-SHP2 driven by the β-MHC promoter were previously generated and described [13,25]. Only the lowest-expressing transgenic line (2.7-fold over endogenous SHP2 levels) was used and crossed from FVB/N into the C57BL/6J strain background for over 14 generations. Subsequently, Q501E-SHP2 transgenic mice were crossed with AKT1 deletion mice (B6.129P2-Akt1^{tm1Mbb}/J, JAX stock number 004912) [23].

2.2. Gravimetry, tissue harvest

All tissue harvests were performed under deep isoflurane inhalation anesthesia. For protein analyses, hearts were rinsed in cold phosphate-buffered saline before weighing and freezing in liquid nitrogen. To arrest hearts in end-diastole for histological studies, hearts were perfused under deep isoflurane anesthesia through the apex with 4% paraformaldehyde in phosphate-buffered saline containing 25 mM KCl and 5% dextrose (cardioplegic buffer).

2.3. Western blotting

Flash-frozen LV tissues were homogenized in lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1% Triton-X, 1 × Halt Protease & Phosphatase Inhibitor Cocktail (Sigma-Aldrich)). Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). For Western blotting, we used anti-SHP2 (SH-PTP2, C-18, rabbit polyclonal, catalog number sc-280, Santa Cruz Biotechnology), anti-phospho-AKT (pS472/pS473, 104A282, mouse monoclonal, catalog number 550747, BD Pharmingen) and the following antibodies obtained from Cell Signaling Technologies: AKT1 (C73H10, rabbit monoclonal, catalog number 2938), GAPDH (D16H11, rabbit monoclonal, catalog number 5174), S6 (54D2, mouse monoclonal, catalog number 2317), and phospho-S6 (S240/244, D68F8, rabbit monoclonal, catalog number 5364). All phospho-specific antibodies were used at 1:500 dilution, all other antibodies at 1:1000. Phosphorylated and total protein bands were quantified using the Bio-Rad ChemDoc imaging system (Bio-Rad). For densitometry (Bio-Rad Quantity One and Image Lab software), rectangular regions of interest of identical size and shape in each lane were drawn tightly around the respective bands. Background signal intensities were determined by placing rectangular regions of interest of identical size and shape in adjacent empty areas in each individual lane. Background intensities were substracted from the respective band intensities measured. Non-specific bands were not included in any of the regions of interest. For all membranes, band intensities were normalized to the GAPDH signal obtained on the same membrane. In addition, Ponceau staining (Sigma-Aldrich) was used to confirm that there were no changes in GAPDH expression compared to total protein loading.

2.4. Echocardiography

Echocardiograms were performed under inhalation anesthesia (1.2–1.8% isoflurane, 0.6 L flow of O_2) using either a GE Vivid-i ultrasound system (GE Healthcare) with a 12 MHz transducer or a Vevo 2100 ultrasound system (Visualsonics) with a 40 MHz transducer. Anesthetized animals were kept warm in supine position for 25 min to ensure hemodynamic stability before image acquisition was started. No data were excluded except for rare cases where hemodynamic instability was evident (such as non-uniform ventricular performance in consecutive beats, heart rate instability, or LV dilation to > 4 mm end-diastolic diameter). M-mode echocardiography was performed using the parasternal short-axis view of the LV. The guidelines of the American Society of Echocardiography were used for measurement of the LV end-diastolic and end-systolic diameters, and anterior and posterior wall thickness. Images were captured digitally and 6 consecutive cardiac cycles were measured and averaged for each animal.