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Human c-MYBPC3 RNA Targeted Therapy, Reversal of Hypertrophic Cardiomyopathy in the Zebrafish Model

Huseyin Yalcin[1], Sahar I. Da'as[1], Iman A. Mohammed[1], Gheyath K. Nasrallah[2], Jasim Al Suwaidi[3], Magdi H Yacoub[1]

[1]Division of Cardiovascular Research, Sidra Medical and Research Center, QA
[2]Qatar University, Biomedical Sciences, QA
[3]Hamad Medical Corporation, QA

Email: hyalcin@sidra.org

Hypertrophic cardiomyopathy (HCM) is a serious heart disease and is defined as abnormal left ventricular (LV) wall thickening with diastolic dysfunction. HCM is an autosomal dominant monogenic disease caused by a mutation in 1 of 13 or more genes encoding protein components of sarcomere (i.e. sarcomere is the subunit for muscle tissue). The myosin binding protein C (MYBPC) encoded by mybpc3 gene, a key constituent of the thick filaments of the sarcomere (Dhandapany et al., 2009). By binding to myosin, titin, and actin, MYBPC contributes to maintaining the structural integrity of the sarcomere and regulates cardiac contractility and relaxation (Harris et al., 2002). Mutations of c-MYBPC3 gene have been demonstrated to be associated with a risk of cardiac hypertrophy and represent one of the common causes of HCM with about more than 20% frequency (Houston & Stevens, 2015). Zebrafish is a widely used animal model for the cardiac genotype – phenotype association since it allows easy genetic manipulation. We have previously identified four disease causing missense mutations of MYBPC3 domain C1 in cardiac patients: Mutation1 (Arg177His), Mutation 2 (Ala216Thr), Mutation 3 (Glu258Lys) and Mutation 4 (Ser217Gly). Previously, it was shown that mybpc3 gene mutations induced a zebrafish embryonic phenotype resembling HCM(Chen et al., 2013). We have recapitulated these mutations in the zebrafish model (Da'as et al., 2014). The efficacy of human RNA injection to zebrafish embryos for rescuing the induced hypertrophic defects was recently suggested as a novel rescue strategy for HCM (Behrens-Gawlik, Mearini, Gedicke-Hornung, Richard, & Carrier, 2014). Previously, we showed that, zebrafish specific cardiac phenotypes resembling the different human mybpc3 mutations were partially reverted upon co-injection of Human c-MYBPC3 mRNA (Da'as et al, 2015). In the current study, we induced hypertrophic condition to zebrafish embryos with morpholino injections to target exon 5 (Mutation 1, 2 and 4) and exon 6 (Mutation 3). We have also analyzed the recovery of these conditions with RNA co-injection.

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Methods

Zebrafish embryos were injected with Morpholino (Genetools) targeting human cardiac MYPBC3 missense mutations. Mutation 1, 2 & 4 located within exon 5 (MO e5i5) and Mutation 3 located within exon 6 (MO e6i6).Human c-MYBPC3 was cloned into pcDNA-DEST47 vector (Life Technologies), to be used to generate wild type human c-mybpc3 mRNA using the T7 polymerase (Ambion).

RT-PCR confirmed Morpholino exon splicing. Total RNA was extracted from zebrafish embryos with Trizol Reagent and further purified with PureLink® RNA Mini Kit (Invitrogen). First-strand cDNA was synthesized from 425 ng total RNA using SuperScript® III (Invitrogen) with MYBPC3 primers flanking exon 4-8 (F: 5′ GGTCAAGCTCAGCAGCTCTC 3′, R: 5′ CTGATCCGCCGACCACCTC 3′) followed by PCR.

Zebrafish embryos were injected in groups:

Group 1: Morpholino sequences designed to target the human cardiac MYPBC3 mutations:

Mutation 1, 2 & 4: Exon 5: MO e5i5: 5′TGTTTTCCTGTGGTCAGACCTTAGT 3′

Mutation 3: Exon 6: MO e6i6: 5′GCCTATGATCTGAGTCTTACCATGT 3′

Group 2: Human wildtype c-MYBPC3 mRNA co-injected with Morpholino targeting exon 5 or exon 6

For the structural and functional analysis, zebrafish cardiac phenotype were first imaged using SteREO Zeiss LUMAR.V12 microscope and Micro-manager software. Recorded time-lapse images were then analyzed using ImageJ software. Time lapse movies of beating ventricles were recorded for 3dpf embryos at 100fps. For the structural analysis, we measured ventricular wall thickness. For this purpose, from the sequential images, still frames of ventricular end-diastole (ED) and ventricular end-systole (ES) images were extracted. At these images, endocardial and myocardial boundaries were traced. Ventricular wall thickness was calculated as average thickness between these two regions. For the functional analysis, we measured heart rate and stroke volumes. Heart rate was calculated by first measuring time duration between two sequential identical timepoints in the cardiac cycle (i.e. ED or ES). 60 divided by this duration gives heart rate. To calculate stroke volume, we first calculated ventricular volumes at ED and ES. For measuring ventricular volume, we assumed that the ventricle is a prolate spheroid and employed the following standard formula to calculate the volume: volume = $4/3\ \pi\ l\ s2$ where l is the long-axis and s the short-axis radius. Stroke volume is the differences in volumes at ED and ES.

Results

Exon5 morpholino injection induces hypertrophy by increasing myocardial thickness at both systole and diastole. Additional RNA injection does not cause a statistically significant change in wall thickness. Exon6 morpholino injection induces hypertrophy and additional RNA injection partly rescues hypertrophy severity (i.e. reduced wall thickness).

Exon 5 morpholino injection decreases heart rate and additional RNA injection does not recover that. Exon 6 morpholino injection decreases heart rate even more and RNA injection does not recover that as well.

Exon 5 morpholino injection decreases stroke volume and additional RNA injection does not recover that. Exon 6 morpholino injection decreases stroke volume even more and RNA injection does not recover that as well.

Conclusion

We successfully induced hypertrophic cardiomyopathy on zebrafish embryos by targeting mybpc3 gene through morpholino injections to exon5 and exon6 sites in the gene. Compared to exon 5 mutant, exon 6 mutant had more severe hypertrophy, with thicker ventricular walls, more drastic decreased heart rate and stroke volume. Additional RNA injection partly rescued phenotype by Exon 6 injection, by restoring myocardial thickness but not heart rate and stroke volume. Additional RNA injection did not cause any difference for Exon 5 mutants. We can conclude that, RNA rescue approach partially recovered the cardiac phenotype and function in exon 5 zebrafish morphants and wasn't enough to modify the severity of the cardiac phenotype of the exon 6) zebrafish morphants.

Morpholino injection and RNA based correction strategies on zebrafish are novel ways to explore genetic causes of disease and rescue strategies.

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