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Basic Science and Experimental Study

Micro-RNA Expression in Hypoplastic Left Heart Syndrome

CARMEN C. SUCHAROV, PhD,[1] JULIANA SUCHAROV,[2] ANIS KARIMPOUR-FARD, PhD,[3] KARIN NUNLEY,[1]
BRIAN L. STAUFFER, MD,[1,4] AND SHELLEY D. MIYAMOTO, MD[5]

Aurora and Denver, Colorado

ABSTRACT

Objective: Micro-RNAs (miRNAs) are important regulators of gene expression through interaction with the 3′UTR of target messenger RNAs (mRNAs). The role of miRNAs has been extensively studied in adult human and nonhuman animal models of heart disease. Hypoplastic left heart syndrome (HLHS) is the most common form of severe congenital heart disease and is an important cause of morbidity and mortality in infants and children. The objective of this work was to analyze the miRNA profile in HLHS patients.
Methods and Results: miRNA profile was determined in the right ventricle with the use of miRNA array, and expression was validated with the use of reverse-transcription polymerase chain reaction (RT-PCR). Based on bioinformatics analysis, targets were selected and their expression analyzed with the use of RT-PCR.We found that the miRNA profile of HLHS is novel, with few similarities between pediatric and adult idiopathic dilated cardiomyopathy. Moreover, our analysis identified putative targets for these miRNAs that are known to be important for cardiac development and disease, and that miRNAs and their putative targets are antithetically regulated. We also found that miRNA expression changes with stage of surgery, suggesting that volume unloading of the ventricle has important consequences for gene expression.
Conclusions: Our data suggest a unique miRNA profile for HLHS that may be associated with defects in cardiac development and disease. (*J Cardiac Fail 2015;21:83–88*)
Key Words: miRNA, HLHS.

Micro-RNAs (miRNAs, miRs) are small noncoding ∼22-nucleotide RNAs capable of modulating the expression of many genes by recognizing a reverse complementary 6–8-nucleotide "seed" sequence, most frequently located within 3′ untranslated regions (3′UTR) of messenger RNAs (mRNAs). The interaction of miRNAs and RNA binding proteins to the 3′UTR can cause translational repression or RNA destabilization. It is estimated that as much as 60% of the genome is subject to their regulation.[1]

Over the past few years, several array-based studies have been published detailing changes in miRNA expression in normal versus failing adult human hearts. The results of these studies have been summarized recently.[1] miRNAs that repeatedly surface as dynamically regulated (up or down) in adult heart failure (HF) include several let-7s, miR-1, miR-133a/b, miR-100, miR-195, miR-199, miR-214, miR-222, miR-23a/b, miR-29a/b, miR-30 family, and miR-320. We have recently shown that pediatric idiopathic dilated cardiomyopathy (IDC) patients display an miRNA profile that is quite different from the adult population, suggesting that pediatric IDC is a unique disease process.[2]

In the present study, we show that expression of a subset of miRNAs is differentially regulated in the right ventricle (RV) of pediatric patients with hypoplastic left heart syndrome (HLHS), a severe form of congenital heart disease. Furthermore, we show that volume unloading of the RV

From the [1]Division of Cardiology, Denver Department of Medicine, University of Colorado, Aurora, Colorado; [2]Pediatric Research Program, Children's Hospital Colorado; [3]Center for Computational Pharmacology, University of Colorado School of Medicine, Aurora, Colorado; [4]Division of Cardiology, Denver Health and Hospital Authority, Denver, Colorado and [5]Department of Pediatrics, University of Colorado School of Medicine, Children's Hospital Colorado, Aurora, Colorado.

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Reprint requests: Carmen C. Sucharov, PhD and Shelley D. Miyamoto, MD, Division of Cardiology, Department of Medicine, University of Colorado, 12700 E 19th Ave, B139, Aurora, CO 80045. Tel: (303) 724-5409; Fax: (303) 724-5450. *E-mail addresses:* kika.sucharov@ucdenver.edu or shelley.miyamoto@childrenscolorado.org

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after surgical palliation normalizes expression of several miRNAs and their putative targets.

Materials and Methods

Tissue Procurement

Human subjects were boys and girls of all races and ethnic background ≤ 13 years of age who donated their hearts to the Institutional Review Board–approved pediatric heart tissue bank at the University of Colorado. Nonfailing (NF) control hearts were obtained from donors whose hearts could not be placed for technical reasons. HLHS RV tissue was obtained from explanted hearts of patients at the time of heart transplantation. All heart tissue was rapidly flash frozen in the operating room immediately after removal from the subject. A detailed description of all of the pediatric patients is presented in Supplemental Table 1. In Supplemental Table 1, RV failure is defined as signs and symptoms of heart failure in the setting of abnormal systolic function on echocardiography, low cardiac output measured at catheterization [cardiac index <2.5 L min^{-1} m^{-2}], and/or diastolic dysfunction of the RV [end-diastolic pressure of the RV >12 mm Hg].[3] Informed consent was obtained from every patient.

Micro-RNA Extraction and Array Analysis

Total RNA was extracted from the RV of 6 NF and 15 HLHS patients. The HLHS patients were divided into 2 groups based on their stage of surgical palliation: (1) stage 3: 5 patients were status post stage 3, the Fontan operation; and (2) stage 1: 10 patients had no previous surgeries performed or were status post stage 1, the Norwood operation. miRNA extraction was performed with the use of the Mirvana kit (Ambion) according to the manufacturer's recommendations. miRNA expression analysis was performed in house with the use of the Taqman array human miRNA card (Invitrogen), which is capable of detecting 754 miRNAs.

Array data were analyzed with the use of Expression Suite Software v1.0 (Invitrogen). Internal control was determined based on an algorithm generated by the software for changes on baseline miRNA values among all samples. In plate A, hsa-miR-361 had the lowest amount of variability among all samples, whereas in plate B hsa-miR-30d* showed the lowest variability in expression.

miRNA and mRNA RT-PCR

Reverse transcription (RT) of miRNAs was performed with the use of the miScript Reverse Transcription Kit (Qiagen) for miRNAs or the iScript Reverse Transcription Kit (Bio-Rad) for mRNAs according to manufacturers' recommendations and essentially as previously described.[2] miRNA expression was normalized to miR-361, and mRNA expression was normalized to 18S. Results obtained for some miRNAs with the use of the miScript kit were confirmed with the use of the Taqman RT–polymerase chain reaction (PCR) kit (Invitrogen): 9 NF as well as 6 stage 3 and 16 stage 1 HLHS patients.

Gene expression primers were BAZ2A: forward (F) 5′ GACGTATTGCTACCCCAGAAG, reverse (R) 5′ TTGCTTCATCCTCTTCCCAC; FOG2: F 5′ AAAGGCTCAGGTCCCAATG, R 5′ ATGGCCTTCGTAGTTGTACAC; CDK6: F 5′ CCGAAGTCTTGCTCCAGTC, R 5′ GAGTCCAATCACGTCCAAGAT; Sox11: F 5′ GCGAACTTCTCCGACCTG, R 5′ ACCATCAACACCACCATCATC; QKI: F 5′ TGTGGAAGATGCTCAGAACAG, R 5′ TGTAGGTGCCATTCAGAATCG; dHAND: F 5′ GCTACATCGCCTACCTCATG, R 5′ CTGCTCACTGTGCTTTTCAAG; GATA6: F 5′ GCTAGACGTCAGCTTGGAG, R 5′ CTGGAAAGGCTCTGGAGTC; and GATA4: F 5′ CTTGCAATGCGGAAAGAGG, R 5′ TGCTGGAGTTGCTGGAAG.

Pathways Analysis

Pathways analysis was done with the use of Diana Lab miRPath v2.0 software (www.microrna.gr/miRPathv2). Target prediction was done with the use of Diana-microT-CDS. *P* values for significant pathways are listed in the tables. Targets of interest were confirmed by Targetscan.

Statistical Analysis

RT-PCR. Statistical analyses were performed with the use of Statview software (SAS Institute, Cary, North Carolina). Two-way analysis of variance (ANOVA) was performed on all outcomes. Statistical significance was set a priori at $P < .05$, and all data are presented as mean + SEM in the figures.

Array. ANOVA with Benjamini-Hochberg correction for false discovery rate was used to determine differential miRNA expression between groups. Significance was set at $q < 0.1$ (adjusted *P* values). Supervised hierarchical clustering was performed on the log-scaled array samples that were significantly different by ANOVA (adjusted *P* value of $<.01$) with the use of the Ward method. The analysis for miRNA expression was performed with the use of R.

Results

Subject Characteristics

All of the patients included in the no surgery group were listed for primary transplantation (based on parent preference). The primary indication for transplant in all of the stage 1 patients and in 4 of the 6 stage 3 patients was RV failure (as defined in Materials and Methods). One of the stage 3 patients had plastic bronchitis in addition to RV failure (with RV failure being the primary indication for transplant) and 2 of the stage 3 patients had protein-losing enteropathy, which was their primary indication for transplant. There were 8 patients on milrinone (0.5 $\mu\text{g kg}^{-1}$ min^{-1}) at the time of transplantation. No other inotropes were being used at the time of transplantation in this cohort. The time from last surgical palliation procedure to transplantation for the stage 1 patients was a mean of 0.7 ± 0.4 years (range 0.4–1.3 years) and for the stage 3 patients a mean of 3.2 ± 1.9 (range 1.8–6.2 years; $P = .02$).

Differential Expression of miRNAs in Pediatric HLHS Patients

We have previously shown that miRNAs are differentially regulated in pediatric and adult IDC patients.[2,4] Because miRNAs play an important role in the regulation of gene expression and have an effect in cardiovascular diseases, we analyzed their expression in the RV of HLHS patients. HLHS is typified by lack of development of all left-side heart structures (including the left ventricle) and only a single RV is present, which is therefore inherently a volume- and pressure-overloaded chamber.