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A Study of Cardiovascular miRNA Biomarkers for Kawasaki Disease

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Abstract

We hypothesized that cardiovascular miRNAs might be diagnostic biomarkers for Kawasaki Disease (KD). We identified dysregulated miRNAs in KD coronary arteries, and tested sera from KD patients and febrile controls for cardiovascular miRNAs using two methods. We did not identify cardiovascular miRNAs diagnostic for KD; our results may help guide future studies of potential miRNA biomarkers for KD.

Keywords

Kawasaki Disease; biomarkers; miRNA

Introduction

Kawasaki Disease (KD) is a unique febrile illness of childhood that can lead to coronary artery aneurysms in up to 30% of untreated patients, with the potential for myocardial infarction and sudden death in a small subset of patients [1]. Other illnesses in the differential diagnosis may share its clinical and laboratory features, complicating diagnosis, but early diagnosis is critical for prompt institution of intravenous gammaglobulin therapy [1]. miRNAs are small noncoding RNA molecules that play an important role in controlling mRNA translation. These molecules show promise as diagnostic markers for many pathogenic states, including certain cardiovascular diseases [2]. We performed a series of experiments to determine whether cardiovascular-related miRNAs could serve as diagnostic biomarkers for KD. First, we determined whether miRNAs that were dysregulated in coronary arteries of

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KD patients were also dysregulated in serum of KD patients. Second, we determined whether any of 84 miRNAs known to be cardiovascular-associated were dysregulated in KD sera.

Materials and Methods

Coronary artery tissues

. Demographic and clinical information on acute (death within 7 weeks after fever onset) KD patients (n=10) and childhood controls (n=10) whose coronary artery tissues were studied are provided in Table S1 [Supplemental Digital Content]. All KD patients had significant coronary arteritis and none received intravenous gammaglobulin, steroid, or infliximab therapies. Table S1 indicates the case numbers in our previously published pathologic study [3], which included the majority of these cases. Coronary arteries from childhood controls were pathologically normal.

KD and febrile childhood control sera

Demographic and clinical information on acute KD patients and febrile children whose sera were used for modified nuclease protection assay and for real-time RT-PCR are available upon request from the authors. Coronary artery abnormalities in KD patients whose sera were used in this study was defined as a Z score >2.5 in the left anterior descending coronary artery or the right coronary artery[1]. The study was approved by the Institutional Review Board of the Ann & Robert H. Lurie Children's Hospital of Chicago.

Modified nuclease protection assay.

To avoid direct RNA isolation procedures on formalin-fixed, paraffin-embedded coronary artery tissues, multiple 8 μ sections of KD epicardial coronary artery aneurysms and childhood control epicardial coronary artery tissues were directly lysed and a modified quantitative nuclease protection assay performed to detect expression of miRNAs (qNPA, High Throughput (HT) genomics [now HTG Molecular], Tucson, AZ [4]). Briefly, the lysed tissue was incubated with biotinylated single-stranded miRNA probes, treated with S1 nuclease to degrade unhybridized single-stranded miRNAs, and hybridized miRNAs treated with alkaline conditions to release the biotinylated probes. These probes were hybridized to an array plate whose wells contained ~650 human complementary miRNAs. Hybridization was detected via use of avidin-horseradish peroxidase conjugate and substrate. A similar method was applied to 10 μl aliquots of KD and febrile control sera, using a custom qNPA array (HT genomics). miRNA expression levels were first $\log_2$-transformed and then subjected to quantile normalization using function quantile.normalize in R library preprocessCore. Differentially expressed genes were identified with two-sample t-tests on $\log_2$-transformed miRNA expression values at alpha=0.05 and >1.5 fold change. For serum miRNA qNPA array, we identified miRNAs with > 1.5 fold change and false discovery rate (FDR)<0.05.

Real-time RT-PCR

RNA was extracted from 100 μl aliquots of KD and febrile childhood control sera using the miRNeasy Serum/Plasma kit (Qiagen, Valencia, CA) and adding miR-39 miRNA mimic

(Qiagen) as an internal spiked-in control. The RNA was then reverse transcribed and amplified using the miScript PreAMP PCR kit (Qiagen). Real-time PCR was performed using SYBR green chemistry on an Applied Biosciences Step One Plus real-time PCR instrument using a commercially available Cardiovascular miRNA array (Qiagen). The plate also included reverse transcriptase controls and PCR controls, and a control for human genomic DNA contamination. For differential expression analysis, we used the comparative C_T method [5], where C_T is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. A two-sample *t*-test was used to compare C_T, where C_T = (C_T gene of interest – C_T internal control). The difference in expression levels of individual genes was determined by comparing C_T values between the KD and control groups. The differential expression was compared using variances estimated by empirical Bayes models. We controlled for FDR to account for multiple comparisons using *q*-values [6].

Results

qNPA reveals a set of miRNAs that are dysregulated in KD coronary arteries

qNPA analyses revealed 26 miRNAs that were upregulated in KD coronary arteries with > 1.5 fold change and p value<0.05 (Table 1, Figure S1[Supplemental Digital Content]). Three miRNAs (miR-1249,-1260, and -195) showed significantly downregulated expression ($p<0.05$) with > 1.5 fold change. Some of the miRNAs that were upregulated in KD coronary arteries (e.g., miR-223 and -150) are known to be highly expressed by white blood cells [7], and their upregulation likely represents inflammatory cells that are present in KD but not control coronary arteries.

Custom qNPA serum assays show similar miRNA expression in KD patients and febrile controls

We designed a custom qNPA assay to determine whether 20 of the miRNAs that were significantly upregulated in KD coronary arteries (Table 1) were serum biomarkers of KD. The array also included miR-22, -16, -24, and -103 as internal controls, as these miRNAs have previously been reported to be detected in normal human sera. When comparing expression levels in 18 KD patients with coronary artery disease to 19 KD patients without coronary artery disease, none of the miRNAs met the significance criteria. When comparing expression of the miRNAs in 37 KD patients (both with and without coronary artery disease) to 15 febrile childhood controls, none met the significance criteria (data available upon request from the authors).

Cardiovascular real-time RT-PCR miRNA array shows no significant miRNA expression differences between sera from KD patients with coronary artery disease and febrile Childhood controls

To determine whether previously identified cardiovascular-associated miRNAs served as KD biomarkers, we performed real-time RT-PCR on sera from 8 KD patients with coronary artery abnormalities, one KD patient with a very mildly dilated left main coronary artery (Z score 2.3), and 11 febrile childhood controls using a commercially available array and a

spiked-in synthetic miRNA mimic as an internal control. Each array included assays for 84 cardiovascular miRNAs; the full list can be obtained upon request from the authors. Expression levels of these 84 miRNAs did not significantly differ in KD and control sera using *q* value of <0.05. Many of the miRNAs on the PCR array had been included on the custom qNPA array, supporting those results (miR-22, -210, -320a, -16, -185, -223, -150, -24, -93, and -103). Pearson correlation for expression of these miRNAs in febrile control sera assayed by both methods showed a correlation of 0.39.

Discussion

A biomarker of cardiovascular disease has the potential to be very useful in KD diagnosis because the inflammatory/infectious diseases in the differential diagnosis of KD are generally not associated with cardiovascular disease [1]. Our study does not rule out the possibility of a cardiovascular miRNA biomarker for KD, but indicates that the miRNAs included in this study are not likely candidates for such a biomarker. These results may prove useful in future investigations of potential miRNA biomarkers for KD diagnosis.

Prior studies of miRNA expression in KD are limited. Shimizu et al [8] used a high throughput sequencing approach to identify miRNAs differentially expressed in acute and convalescent KD peripheral blood, and reported that six miRNAs (miR-143, 199b-5p,-618,-223,-145, and 145*) were differentially expressed. They reported that one of these genes, miR-145, was expressed at high levels in whole blood samples from 16 acute KD patients compared with 14 acute adenovirus-infected controls by real-time RT-PCR. Our study utilized sera rather than whole blood, and did not reveal significant differences in miR-145 expression between KD patients and febrile childhood controls with a variety of infectious/inflammatory conditions by real-time RTPCR. Yun et al [9] recently reported elevated serum levels of miRNA-200c and -371-5p (miRNAs involved in inflammatory responses) in KD patients, but used afebrile controls as the comparison group. We believe that it is critical to use febrile childhood controls with various infectious and non-infectious diseases in acute KD diagnostic biomarker studies, to ensure that a potential diagnostic biomarker is specific to the KD disease process and is not simply a non-specific marker of inflammation.

Our study has several limitations. It was not possible to determine the biologically relevant targets of dysregulated miRNAs in KD coronary arteries in this study. The KD coronary artery tissues used in our study were from patients who died at 2-7 weeks after illness onset, because fatalities in the first two weeks of illness are very rare. However, the miRNAs expressed in KD coronary arteries at 2-7 weeks after onset may differ from those expressed in the first 10 days of illness, when the diagnosis should optimally be established. Both serum and plasma have been widely utilized in miRNA biomarker [10]. Our statistical significance criteria precluded identification of small differences in miRNA expression between KD and febrile control patients, but were based on the premise that a clinically useful miRNA biomarker would be characterized by marked differences in expression between KD patients and febrile controls, exceeding the potential for experimental variances, as previously recommended [10].

As the diagnosis of KD can be difficult, and missed diagnoses have potentially devastating consequences in previously healthy children, the search for diagnostic biomarkers of KD should continue to be a research priority.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

miRNAs significantly upregulated in KD compared with childhood control coronary arteries and with at least 1.5 fold change

gene	ratio KD/ctr	*p*-value	Log2- transformed value+SD in KD	Log2- transformed value+SD in control	Included on serum qNPA array
hsa-miR-210	3.5	0.0001	10.4+1.0	8.5+0.6	X
hsa-miR-663b	3.4	0.0002	12.5+0.8	10.7+0.8	X
hsa-miR-663	4.1	0.0003	12.1+1.2	10.0+0.8	X
hsa-miR-638	2.9	0.0003	14.2+0.7	12.7+0.8	X
hsa-miR-650	2.7	0.0009	9.3+1.0	7.9+0.9	X
hsa-miR-675	3.7	0.0009	10.7+1.2	8.8+1.3	X
hsa-miR-93	2.5	0.0028	9.7+1.0	8.3+0.6	X
hsa-miR-320b	2.2	0.0029	11.7+0.6	10.6+0.8	X
hsa-miR-1291	3.4	0.0034	11.2+1.2	9.4+1.2	X
hsa-miR-1246	1.7	0.0036	8.7+0.5	8.0+0.4	X
hsa-miR-320a	2.3	0.0038	12.2+0.7	11.0+0.9	X
hsa-miR-1274b	2.4	0.0053	9.7+0.9	8.4+0.9	X
hsa-miR-146b-5p	1.7	0.0079	9.2+0.6	8.3+0.6	X
hsa-miR-1285	2.1	0.0113	11.5+0.6	10.4+1.0	X
hsa-miR-566	1.5	0.0159	8.7+0.5	8.2+0.4	X
hsa-miR-423-5p	2.0	0.0164	10.5+0.9	9.5+0.8	X
hsa-miR-181b	1.5	0.0187	8.7+0.5	8.1+0.5	
hsa-miR-185	1.6	0.0241	8.9+0.7	8.2+0.5	X
hsa-miR-1307	1.6	0.0251	9.0+0.6	8.3+0.5	
hsa-miR-223	2.4	0.0284	10.0+1.4	8.7+0.8	X
hsa-miR-654-5p	1.6	0.0294	8.8+0.8	8.2+0.3	X
hsa-miR-150	2.2	0.0354	9.6+1.4	8.4+0.6	X
hsa-miR-1300	1.5	0.0364	8.7+0.7	8.1+0.6	
hsa-miR-548c-5p	1.9	0.0396	9.0+1.2	8.0+0.4	
hsa-miR-127-3p	2.4	0.0437	10.3+1.4	9.1+1.1	
hsa-miR-92a	1.9	0.0442	12.7+1.0	11.8+1.0	