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MicroRNA deep sequencing reveals chamber-specific miR-208 family expression patterns in the human heart☆,☆☆

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ABSTRACT

Background: Heart chamber-specific mRNA expression patterns have been extensively studied, and dynamic changes have been reported in many cardiovascular diseases. MicroRNAs (miRNAs) are also important regulators of normal cardiac development and functions that generally suppress gene expression at the posttranscriptional level. Recent focus has been placed on circulating miRNAs as potential biomarkers for cardiac disorders. However, miRNA expression levels in human normal hearts have not been thoroughly studied, and chamber-specific miRNA expression signatures in particular remain unclear.

Methods and results: We performed miRNA deep sequencing on human paired left atria (LA) and ventricles (LV) under normal physiologic conditions. Among 438 miRNAs, miR-1 was the most abundant in both chambers, representing 21% of the miRNAs in LA and 26% in LV. A total of 25 miRNAs were differentially expressed between LA and LV; 14 were upregulated in LA, and 11 were highly expressed in LV. Notably, the miR-208 family in particular showed prominent chamber specificity; miR-208a-3p and miR-208a-5p were abundant in LA, whereas miR-208b-3p and miR-208b-5p were preferentially expressed in LV. Subsequent real-time polymerase chain reaction analysis validated the predominant expression of miR-208a in LA and miR-208b in LV.

Conclusions: Human atrial and ventricular tissues display characteristic miRNA expression signatures under physiological conditions. Notably, miR-208a and miR-208b show significant chamber-specificity as do their host genes, α-MHC and β-MHC, which are mainly expressed in the atria and ventricles, respectively. These findings might also serve to enhance our understanding of cardiac miRNAs and various heart diseases.

1. Introduction

The human heart consists of four characteristic chambers wherein the atrial and ventricular myocardia are morphologically and functionally distinct. Ventricles play the central role in contraction for pumping out the blood stream to the lungs or body, whereas atria mainly serve as the source and target for neurohumoral signals [1]. Gene expression analyses have shown that some mRNAs are specifically expressed in particular human cardiac chambers, such as contractile protein MLC2 in ventricles and the natriuretic peptide NPPA in atria [2,3]. It is quite important to understand the chamber specific gene expression signatures under physiological conditions to facilitate the diagnoses of pathological changes and to develop treatments of cardiac diseases.

MicroRNAs (miRNAs) are short noncoding RNAs comprising 18 to 24 nucleotides, which generally suppress gene expression at the posttranscriptional level [4]. Over the last decade, it has been discovered that miRNAs widely regulate the cardiovascular system, and could serve as clinical biomarkers for various types of heart failure including acute myocardial infarction, cardiac hypertrophy, and viral myocarditis [5–7]. Although miRNA research has primarily utilized the ventricular tissues from patients with heart failure, recently the atrial tissues from patients with atrial fibrillation have also been analyzed using miRNA microarrays [8,9]. These clinical studies have demonstrated that the expression patterns of some miRNAs differ under severe cardiac stress in vivo; such alterations have been confirmed in animal transgenic experiments. However, the expression patterns of human cardiac miRNAs under physiological conditions have not been thoroughly elucidated.

In contrast to the vulnerability of mRNAs, miRNAs are known to be resistant to rough environments such as extreme changes of pH or temperature [10,11]. We have previously shown that human cardiac miRNAs can be stably detected even in stored postmortem samples [12]. Here, we utilized autoptic tissue samples of paired human atrial and ventricular muscles without cardiac pathology for miRNA deep sequencing. Next generation sequencing can evaluate absolute transcriptional levels, and therefore produces more high-throughput observations than does the microarray technology that was the main screening method used in the last decade [13]. In this study, we aimed

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to reveal the fundamental miRNA expression patterns in normal human heart, and to examine the differences in the atrium and ventricle-specific miRNA expression profiles.

2. Methods

2.1. Tissue sampling

Human cardiac tissue samples were obtained at autopsy. A part of the left atrial appendage (LA) and left ventricular free wall (LV) were immediately immersed in liquid nitrogen and stored at −80 °C until RNA isolation. Other parts of the heart were fixed with 10% formalin for histopathological examination. Formalin-fixed paraffin-embedded tissue was sectioned into 4-μm thick slices and stained with hematoxylin and eosin dyes, as well as with Masson trichromal dyes. No apparent histopathological changes were observed in the cardiac tissues used in this study. A total of 4 paired LA–LV samples were used for miRNA sequencing, and 9 paired tissues were used for subsequent real-time polymerase chain reaction (PCR) analysis. The characteristics of the subjects are summarized in Table 1. The study protocol was approved by the Ethics Committee of Tokai University. Informed consent to allow experimental use of the tissue samples was obtained from the bereaved relatives of all patients.

2.2. RNA extraction

RNase-free water and equipment were used throughout this study. RNA was isolated from approximately 100 mg tissue using the mirVana miRNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. The total RNA concentration and purity were measured with a spectrophotometer, and the integrity of the small RNA was assessed using microcapillary electrophoresis on a 2100 Bioanalyzer using a Small RNA kit (Agilent Technologies, Santa Clara, CA, USA). All RNA samples were stored at −80 °C until further analysis.

2.3. miRNA sequencing and data processing

A small RNA library was prepared from 24 ng miRNA of each LA or LV sample using the Ion Total RNA-seq kit v2 (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Briefly, 3′ and 5′ adapters were hybridized to small RNAs, followed by an overnight ligation. After the reverse transcription reaction, cDNAs were purified and size-selected using magnetic beads. The cDNA samples were then PCR amplified using a common primer set. No barcode was added in our protocol because we sequenced only one library per chip in order to gain the maximum read depth. The amplicons were again purified and size-selected with magnetic beads. Final yields and size distribution of the amplified cDNAs were assessed by a 2100 Bioanalyzer with a High sensitivity DNA kit (Agilent Technologies).

Table 1
Summary of sample characteristics.

Sample ID	Age (years)	Sex	Cause of death	BMI (kg/cm^2)	Heart (g)	Coronary atherosclerosis
1	44	M	Head trauma	22.1	374	Little
2	41	F	Suffocation	23.8	271	No
3	65	F	Pneumonia	14.9	218	Little
4	76	F	Subarachnoid hemorrhage	20.2	312	Little
5	35	M	Drowning	25.4	339	Little
6	49	M	Alcohol cirrhosis	18.9	270	Moderate
7	52	M	Alcohol cirrhosis	24.8	395	Moderate
8	70	M	Blood loss	17.0	397	Moderate
9	65	M	Head trauma	27.6	446	No

Samples 1–4 were used for miRNA sequencing.

Next, 5 μl of each library (100 pmol/l) was sequenced on an Ion 318 chip using the Ion Torrent PGM system (Life Technologies). Sequencing data was transferred to the Torrent Browser, where adapter sequences were removed and low quality reads such as primer dimers were excluded. Filtered reads were then successively mapped to the UCSC human genome hg19 using the TMAP program, and to the human miRNA database miRBase v21 using the SHRiMP2 program with default parameters [14]. The coverage depth data were analyzed on the CLC Genomics Workbench v6.0.1 (QIAGEN, Venlo, the Netherlands). The read counts of each known miRNA were normalized to the library size and presented as reads per million mapped reads (PMMR). Only the miRNAs with normalized read counts over 10 in at least one sample were subjected to further analysis. Differential expression between the LA and LV groups was calculated using Baggerley's test followed by a false discovery rate correlation. An expression alteration with a fold change >2 and P-value <0.05 was considered significant.

2.4. Quantitative reverse transcription-PCR

From among the candidate miRNAs with implied chamber specificity as ascertained by deep sequencing, we selected 6 miRNAs that are expressed abundantly in the heart and are considered to exhibit cardiac functioning to some degree, and performed real-time PCR to validate their chamber-specific expression patterns.

Each cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). Specific primers for hsa-miR-208a-3p (TaqMan 000511), hsa-miR-208b-3p (TaqMan 002290), hsa-miR-204-5p (TaqMan 000508), hsa-miR-29b-3p (TaqMan 000413), hsa-miR-378a-5p (TaqMan 000567), hsa-miR-221-3p (TaqMan 000524), and U6 snRNA (TaqMan 001973) were used, and PCR reactions were performed following the manufacturer's protocol. Serially diluted cDNAs for each RNA sample were used as the template for each primer set, and standard curves were generated. The threshold value was set at 0.1 throughout this study. The ΔΔCq method was used for quantification of the target miRNAs, with U6 snRNA used as an endogenous control [15]. Comparison between LA and LV samples was performed using the paired t-test, and P-values < 0.05 were considered statistically significant.

3. Results

3.1. miRNA expression signatures in human LA and LV tissues

Paired LA and LV samples from 4 patients without cardiac pathogenesis were used for miRNA sequencing. After trimming the adaptor sequences from the miRNA reads, the average read length was 22 bp. Of the total 19,830,155 reads that were generated from the 8 samples, 17,939,353 (90.5%) could be aligned to the human genome sequence (hg19), among which 6,975,989 reads (35.2%) were mapped to known miRNAs (miRBase v21). The summary of the sequencing results is described in the online-only Data supplement Table 1.

From the total sequences, 1134 mature miRNAs were identified, and 438 miRNAs were represented by >10 reads PMMR in at least one library (online-only Data supplement Table 2). The most abundant miRNA throughout the libraries was miR-1, which accounted for 21.3% in LA and 25.5% in LV. The top 20 miRNA species were almost identical between the two chamber groups (Table 2). Principal component analysis also showed that the major component in LA and LV was similar, whereas the second component was obviously different between the two groups (Fig. 1). These results indicate that several miRNAs are specifically expressed in human LA or LV under physiological conditions.

Pairwise comparison of normalized read counts representing >10 reads PMMR revealed that 25 miRNAs were differentially expressed in LA and LV (fold change >2 and P-value <0.05, Fig. 2 and Table 3). Among these, 14 miRNAs were significantly increased in LA, and 11 were significantly elevated in LV. Notably, members of the miR-208