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Exosomal piRNA sequencing reveals differences between heart failure and healthy patients

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Abstract. – OBJECTIVE: Heart failure is a leading cause of cardiovascular mortality in industrialized countries. Increasing evidence has highlighted the relationship between noncoding regulatory RNAs, especially microRNAs, and heart failure. However, few studies address the role of piRNAs in heart failure. Therefore, we compared exosomal piRNAs between heart failure patients and healthy volunteers to investigate the role of piRNAs in heart failure.

PATIENTS AND METHODS: First, exosomes were isolated from the serum of heart failure patients and healthy volunteers. After confirming exosome identity by electron microscopy, nanoparticle analysis, and Western blotting, RNA was extracted from exosomes. The expression of piRNAs was then compared by RNA sequencing and bioinformatics analysis.

RESULTS: Serum exosomes from heart failure patients and healthy volunteers were successfully isolated and identified. Serum exosome presence was increased in heart failure patients compared to healthy volunteers. RNA sequencing and bioinformatics analysis revealed that 585 piRNAs were upregulated in heart failure patients, and 4,623 piRNAs were downregulated. Among these piRNAs, has-piR-020009 and has-piR-006426 were the most downregulated.

CONCLUSIONS: Collectively, a dramatic difference in the expression of piRNAs in serum exosomes of heart failure patients was observed. Altogether, these data suggest that piRNAs are potential biomarkers of heart failure and that serum exosome isolation may provide a clinically relevant source of piRNAs for sequencing analysis.

Key Words:
Heart failure, Exosomes, piRNAs.

Introduction

Heart failure (HF) is the ultimate outcome of many cardiovascular diseases and is one of the leading causes of morbidity and mortality in aging people worldwide[1]. Investigation of the mechanisms that control cardiac gene expression in HF provides information conducive to better understanding HF pathogenesis and could lead to new diagnostic and therapeutic tools[2]. One of the greatest surprises of advanced deep sequencing analysis of the transcriptome has been the discovery of noncoding RNAs (ncRNAs), which are not translated into protein. Recent evidence suggests that the non-protein coding portion of the genome, which was previously thought to be dispensable, may have functional importance. NcRNAs can be divided into two groups by size: small ncRNAs (< 200 nt) and long ncRNAs (lncRNAs) (0.2 to 2 kb) that have a coding potential of less than 100 amino acids[3-6]. Small ncRNAs can be further subdivided into microRNAs (miRNAs), p-element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs), and endogenous small interfering RNAs (siRNAs). The cardiology field has primarily investigated the role of miRNAs and lncRNAs. However, few studies have examined the role of piRNAs in cardiovascular disease. PiRNAs are ncRNAs of 24 to 32 nt length that are found in association with PIWI proteins. PiRNAs have been shown to repress transposable elements in the germline and their expression was originally thought to be limited to germ cells. Therefore, investigation of piRNAs primarily focused on understanding the mechanisms that maintain germ line integrity[7]. More recently, roles for piRNAs in gene regulation have emerged. Several researches have uncovered the hitherto unknown mechanisms of piRNA biogenesis. Recent evidence[8,9] also indicates that piRNAs are expressed in somatic cells of the brain, liver, and heart. In fact, it has been suggested that abundant piRNA expression stimulates cell viability through serine/threonine kinase (AKT) signaling in the cardiac system[10]. Exosomes are

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secreted membrane-bound vesicles with diameters ranging from 30 to 200 nm that carry a multitude of signals, such as DNA, RNA, cytokines, and other proteins[11]. In this study, we investigated the role of serum exosome piRNAs as a novel diagnostic and therapeutic strategy for cardiac remodeling and HF. Serum exosomes from HF patients and healthy volunteers were isolated. After exosome identity was confirmed by transmission electron microscopy (TEM), qNano analysis, and Western blotting, RNA was extracted. Using advanced deep sequencing, the expression of serum exosome piRNAs was compared between HF patients and healthy volunteers.

Patients and Methods

Exosome Isolation

After overnight fasting, exosomes were isolated from male HF patients whose left ventricular ejection fraction was less than 40% and healthy male volunteers with matched ages. All human studies were approved by the Appropriate Institutional Review Boards. After receiving informed written consent, 60 ml of blood were drawn from the ante-cubital vein by butterfly syringe into citrated vaccutainers™ (BD Biosciences, Franklin Lakes, NJ, USA). The first two vaccutainers™ were excluded from the exosome isolation procedure to avoid platelet activation. Exosomes were isolated by differential centrifugation at 1,600 g for 20 min, then 10,000 g for 30 min, and finally three times at 100,000 g for 60 min. All centrifugation steps were performed at 4°C. Human exosome samples were tested for endotoxin contamination using the LAL Chromogenic Endotoxin Quantitation Kit (Pierce, Rockford, IL USA). Endotoxin levels were found to be less than 0.1 EU/ml. When exosomes were added to cells, the endotoxin concentration was less than 0.001 EU/ml, well below the minimum activation threshold of toll-like receptor 4 (TLR4)[12].

Transmission Electron Microscopy Sample Preparation

TEM sample preparation was performed following the approach of Malik et al[13]. Briefly, a drop of purified exosome pellet was settled on a gold-coated grid, blotted and fixed in 1% glutaraldehyde, washed for 2 min in double-distilled water, and incubated in uranyl oxylate for 5 min. Next, the grid was incubated in three separate drops of methyl cellulose with uranyl acetate for 5 min in the first two drops, 10 min in the last drop, and finally removed from the solution. Exosomes were observed by standard TEM with a Philips CM120 microscope.

Nanoparticle Analysis

The quantity of exosomes was measured by qNano analysis (Izon instrument, Cambridge, MA, USA) according to the manufacturer's instructions[14]. The protein concentration of the exosomes was quantified using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Western Blotting

Western blotting was performed to identify the surface markers of exosomes. Briefly, 5 protein-loading buffer was added to exosome samples and heated at 95°C for 5-10 min. Then, exosome proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were run at 120 V for 45 min and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) at 100 mA. Binding of primary antibodies was detected by peroxidase-conjugated secondary antibodies, and bands were quantified by densitometry. The following antibodies were used: anti-CD9, anti-Alix, and anti-CD81 (Abcam, Cambridge, MA, USA). Western blots were normalized to GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RNA Sample Preparation

Serum exosomes were collected and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The study was approved by the Local Ethics Committee. As previously published, libraries were prepared from 1 μg total RNA for each sample[15]. First, small RNAs were isolated by 15% urea-PAGE and subsequent excision from the gel between 18 and 30 nt, which corresponds to mature piRNAs and other small RNA molecules. After gel purification, the adenylated 3' adapter was ligated to the piRNA fragment. Next, the barcoded reverse transcriptase (RT) primer was used to anneal the 3' adenylated adapter to the redundant unligated 3' adenylated adapter. Then, the 5' adapter was ligated, and the RT reaction was performed. After cDNA first strand synthesis, the product was amplified by

15 cycles. A second size selection was performed to isolate 100 to 200 bp fragments from the gel. This step was conducted to purify the polymerase chain reaction (PCR) products and remove any nonspecific products. After gel purification, the PCR yield was quantified by Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA), and samples were pooled to make a single strand DNA circle (ssDNA circle), which constituted the final piRNA library[15].

RNA Sequencing Using BGISEQ-500

DNA nanoballs (DNBs) were generated with the ssDNA circle by rolling circle replication to strengthen the fluorescent signal during the sequencing process, as previously described[16]. The DNBs were loaded into patterned nanoarrays, and single-end reads of 50 bp were sequenced on the BGISEQ-500 platform for the following data analysis study. For this step, the BGISEQ-500 platform combines the DNB-based nanoarrays[16] and stepwise sequencing using polymerase, as previously published[17].

Bioinformatics Analysis

The bioinformatics analysis followed the pipeline outlined in Figure 1. Mapping to the human genome was carried out with Bowtie2 software[18] (one mismatch allowed). The prediction of novel piRNAs was performed using an extended feature set built on Piano software[19]. For GeneTrail2 analysis, all available categories were analyzed, the minimum category size was set to four, and all *p*-values were adjusted using Benjamini-Hochberg adjustment[15].

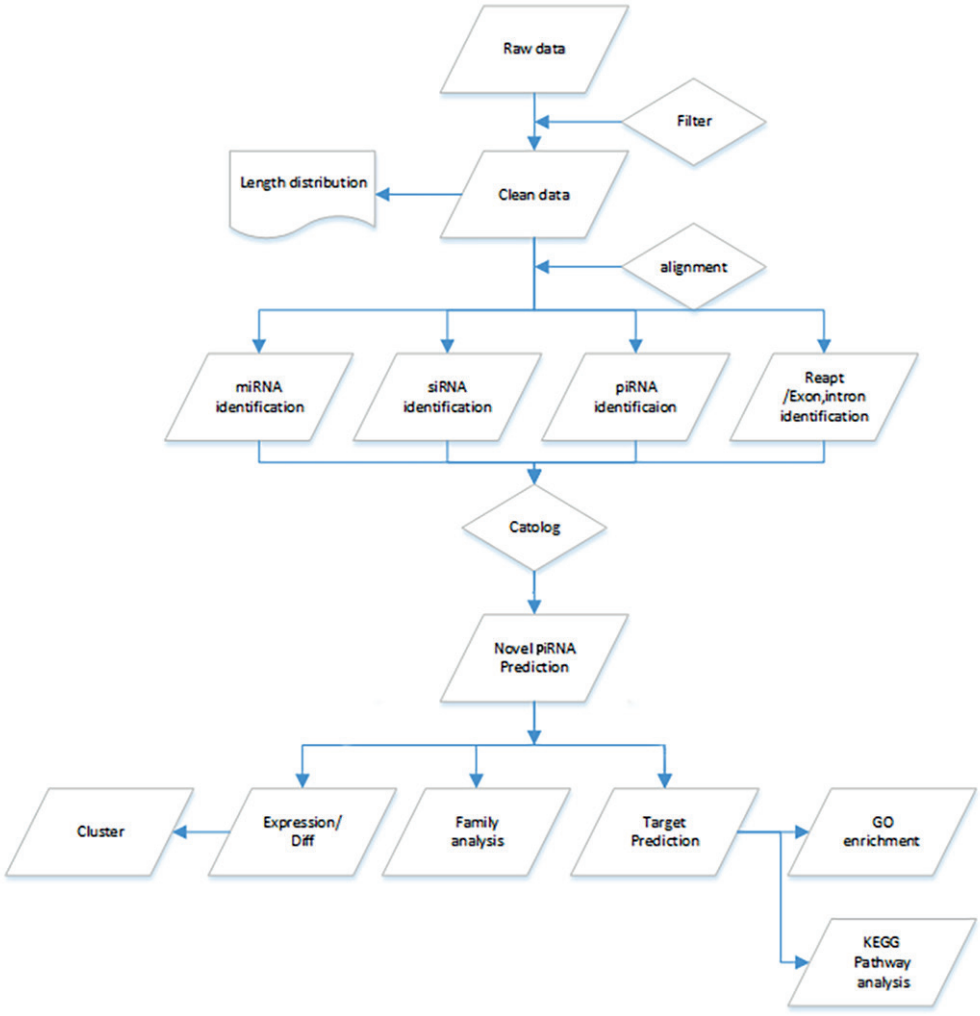

Figure 1. Bioinformatics analysis pipeline.

Results

Serum Exosome Identification and Quantitative Analysis

After exosomes were isolated from serum, we confirmed their identity by a combination of TEM, nanoparticle tracking analysis, and western blotting. Using TEM, we observed that the isolated exosomes appeared to be round or cup-shaped vesicles, which ranged from approximately 30 to 200 nm in diameter (Figure 2A). The quantity of exosomes was detected by qNano analysis, which revealed that the diameter of most exosomes ranged from 50 to 150 nm. Nanoparticle analysis also demonstrated an increased number of serum exosomes in HF patients compared to healthy volunteers (Figure 2B). Western blotting identified the presence of specific exosome surface markers, CD9, CD81, and Alix, and showed that exosomes had been isolated successfully (Figure 2C).

PiRNA Profiling of HF Patients and Healthy Volunteers

After comparing the expression of piRNAs, we found that there was a statistically signifi-

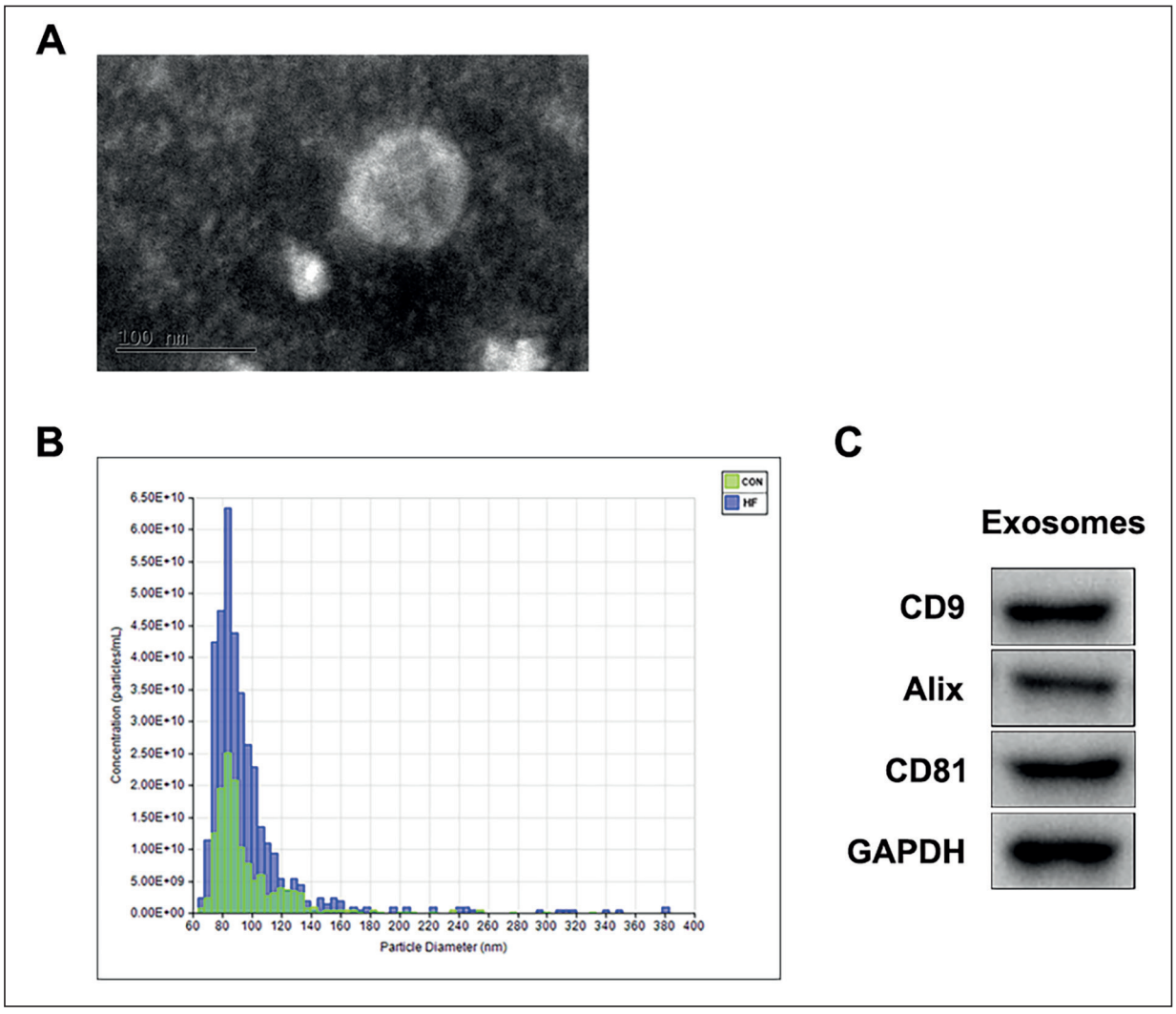

Figure 2. Identification of serum exosomes. ***A.*** Representative electron microscopy image of serum exosomes. Scale bar: 100 nm. ***B.*** Exosome identity was further confirmed by qNano analysis, which measured the diameter of particles with respect to concentration. Most particles ranged from 60 to 140 nm in diameter. The X-axis represents particle diameter (nm), and the Y-axis represents concentration (particles/ml). Green, exosomes from healthy volunteers; blue, exosomes from HF patients. HF: heart failure; CON: control. ***C.*** Western blot showing the expression of exosomal markers CD9, Alix, and CD81. GAPDH was used as a loading control.

cant difference between HF patients and healthy volunteers. In sum, 585 piRNAs were upregulated in HF patients, and 4,623 piRNAs were down-regulated (Figure 3). We then compared individual samples and found that most piRNAs were downregulated in HF patients (Figure 3). To determine the function of the differentially expressed piRNAs between HF patients and healthy volunteers, we identified genes with significantly different expression patterns and performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database analysis[20]. Genes with differential expression patterns were placed into the following KEGG pathway categories: cellular processes, environmental information processing, genetic information processing, human diseases, metabolism, and organismal systems (Figure 4). Using the KEGG analysis, the differentially expressed genes were determined to be enriched globally, regardless of species affiliation. In the functional group of cellular processes, the products of most genes were enriched in cell community and transport and catabolism. Most genes in the environmental information-processing group were related to signal transduction. Under the category of genetic information processing, genes were enriched in folding, sorting, and degradation. Genes that fell under human diseases were broadly related to cancers. In the metabolism category, most genes were enriched in global and overview maps of metabolism. Finally, most genes in the organismal systems group were distributed throughout the immune system. The fold change of piRNA expression between HF patients and healthy volunteers was calculated and illustrated by heat map (Figure 5). The 22 most significantly different piRNAs were included in the heat map. This visualization demonstrated that most piRNAs were downregulated in HF patients.

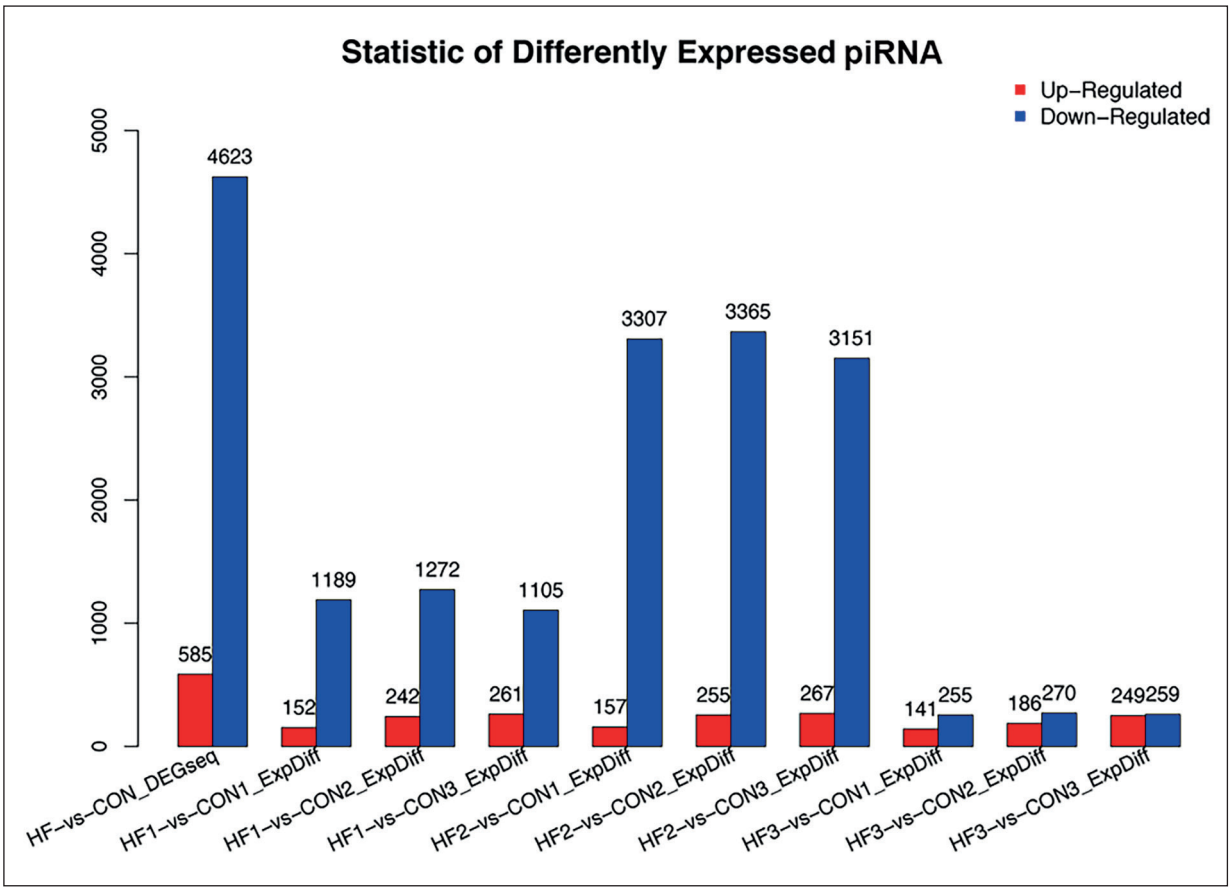

Figure 3. Differential expression of piRNAs. Generally, 585 piRNAs were upregulated in HF patients, and 4,623 piRNAs were down-regulated. When individual samples were compared, most piRNAs were found to be downregulated in HF patients. The X-axis represents comparison groups, and the Y-axis represents the number of piRNAs. Red, upregulated; blue, down-regulated. HF: heart failure; CON: control.

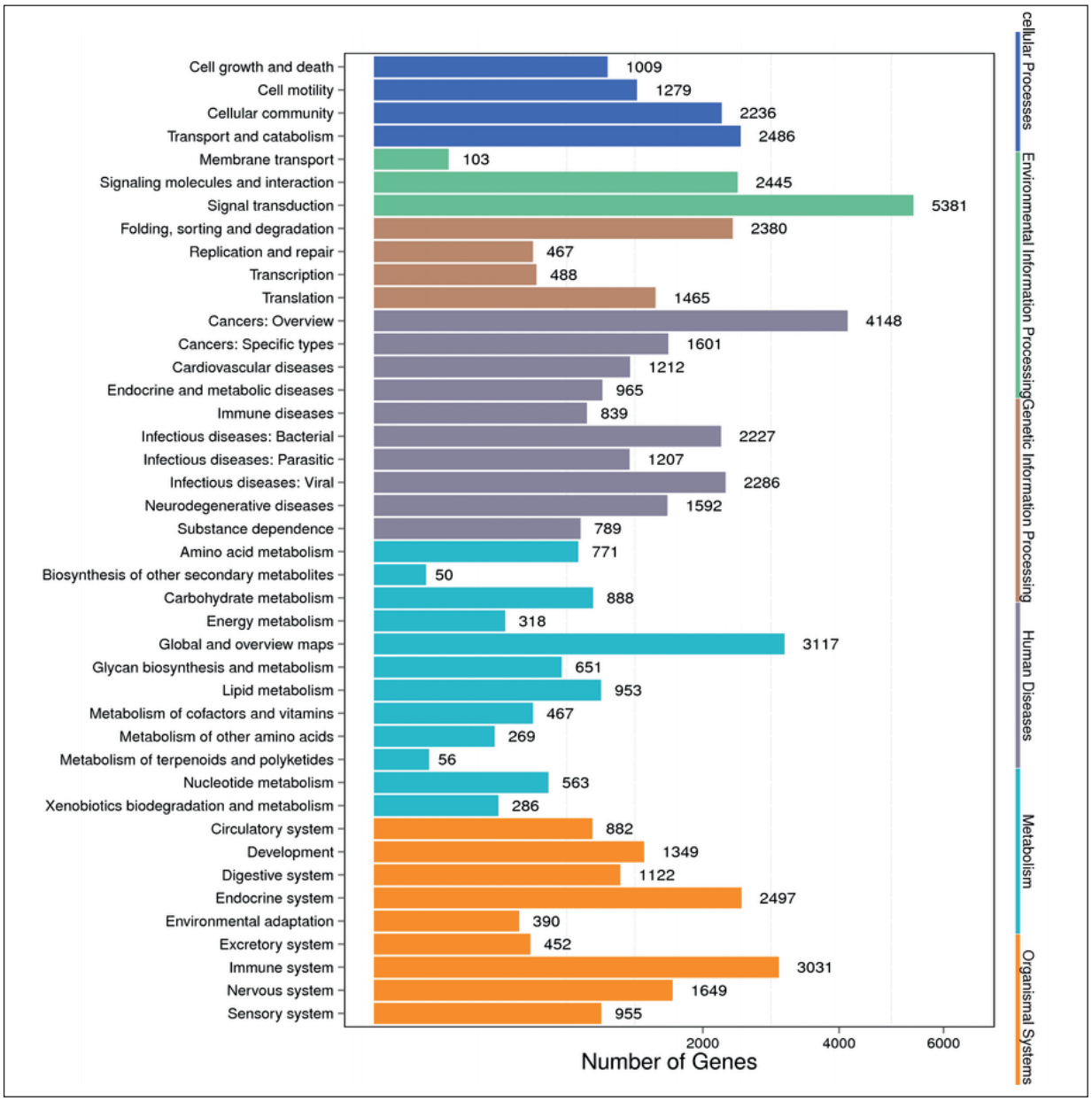

Figure 4. Distribution of KEGG pathway analysis. Distribution of the KEGG pathway analysis of gene-significant differences is shown. The number of gene hits is shown along the X-axis, and the KEGG pathways are shown along the Y-axis. Results of significantly different genes are summarized for six main categories: cellular processes, environmental information processing, genetic information processing, human diseases, metabolism, and organismal systems. $p < 0.05$ was used as the threshold in selecting significant KEGG pathways. The 42 significantly different KEGG terms are shown.

Among these piRNAs, has-piR-020009 and has-piR-006426 were the most downregulated and may be involved in the progression of HF.

Discussion

HF is characterized by adverse ventricular remodeling, which can occur after a series of cardiac injuries. These injuries can include myocardial infarction (MI) and hemodynamic stress, as seen in hypertrophic and dilated cardiomyopathy. In recent decades, improvements in cardiac revascularization therapy have reduced the number of deaths from MI; however, these improvements increased the number of individuals developing HF after MI. HF is one of the leading causes of cardiovascular mor-

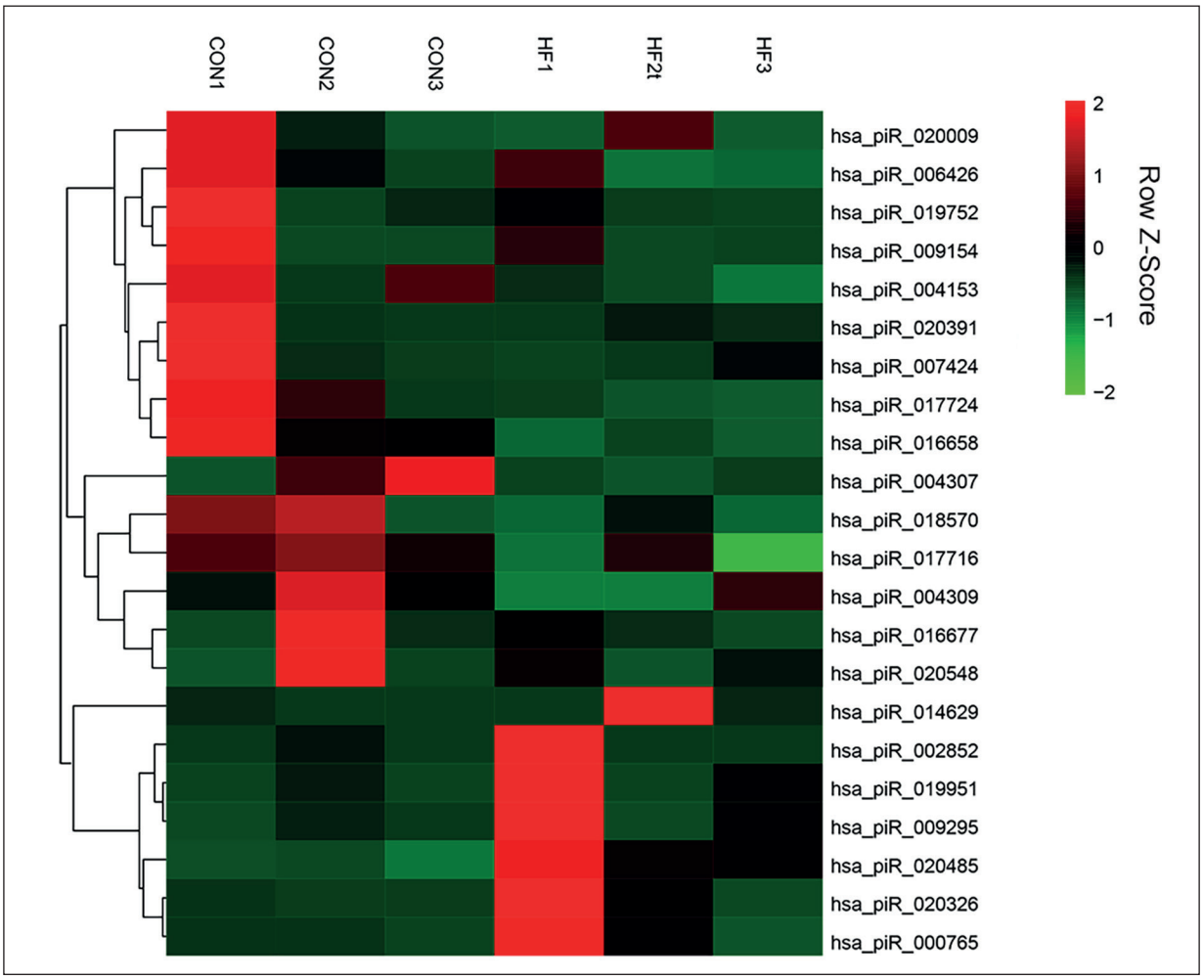

Figure 5. PiRNA expression heat map. Comparative analyses of piRNAs in HF patients and healthy volunteers. Green and red colors indicate down-regulated and up-regulated piRNAs, respectively. PiRNAs were transformed to Z-scores using the inverse of the standard normal cumulative density function. A high Z-score is represented by red. Among these piRNAs, hsa-piR-020009 and hsa-piR-006426 are the most downregulated in HF patients. HF: heart failure; CON: control.

tality in industrialized countries, and alternative therapies to improve cardiac function and reverse remodeling processes are still scarce. Therefore, new approaches for early diagnosis and treatment are of great importance. With the development of next-generation sequencing technologies, we are now able to study the mechanisms that control cardiac gene expression in HF. NcRNAs influence gene expression through various mechanisms, such as RNA interference, at the level of transcription and translation. Not surprisingly, ncRNAs are emerging as important players in many human pathologies, including cardiovascular diseases. In view of the many classes of ncRNAs, whose roles have yet to be explored in pathological conditions, our current knowledge only scratches the surface. Due to advanced deep sequencing and other analysis technologies, the detection of ncRNAs has been simplified, enabling the initiation of in-depth research into the different classes of ncRNAs[3-6]. Indeed, miRNAs, one class of ncRNAs, play key roles in driving gene expression changes of HF, atherosclerosis, and cardiac ischemic/reperfusion injury[21]. The expression of miRNAs can be tissue-specific or cell-specific, and their expression patterns can reflect an underlying pathophysiological condition. Beyond this biological function, their role as potential biomarkers has emerged in recent years[22-24]. Although only a very small percentage of identified lncRNAs

have been explored experimentally, they are known to be implicated in many biological processes such as X chromosome inactivation, cell fate specification, RNA splicing, translational control, and chromatin modification[25]. The role of lncRNAs ranges from the control of pluripotency to lineage specification[26-28]. Many researches have suggested that lncRNAs are important players in heart development[29] and are involved in HF[30]. However, few studies have investigated the role of piRNAs in cardiovascular disease. PiRNAs are another class of ncRNAs that are 24 to 32 nt in length. They are found in association with PIWI proteins, which is where they get their name. It was thought that piRNAs were expressed only in germ cells; however, emerging data has shown that piRNAs are also expressed in somatic cells, like brain, liver, and heart[8,9]. Additionally, functions for piRNAs beyond transposon silencing, e.g. in regulation of mRNA, have been described. Moreover, mechanisms beyond target slicing, including transcriptional regulation and mRNA deadenylation, have been noted, and striking evidence for transgenerational effects of piRNAs has also been documented[7]. In this work, we have begun to uncover the role of piRNAs in HF. Due to rapidly developing technologies, it is now possible to screen thousands of piRNAs under different conditions. This rapid growth in technology has enabled researchers to identify piRNAs that are crucial for the onset and progression of various disease conditions. In this study, we compared the expression of exosomal piRNAs between HF patients and healthy volunteers with a goal of uncovering the mystery of piRNAs in HF. Exosomes are small endosome-derived vesicles ranging in diameter from 40 to 100 nm. Exosomes are actively secreted from cells through exocytosis, a process normally used for receptor discharge and intercellular cross-talk[31]. We isolated exosomes from serum by ultracentrifugation and then confirmed their identity by collective evidence from electron microscopy, nanoparticle analysis, and Western blotting. By electron microscopy, we observed the morphological characteristics and diameter of exosomes. By nanoparticle analysis, we quantitatively compared exosomes of HF patients with healthy volunteers. Western blotting was then used to confirm the identity of exosomes, which are characterized by the presence of the specific surface proteins CD9, CD81, and Alix[32]. Exosomes derived from different types of cardiac cells have been widely investigated; however, few are related to serum exosomes. RNA sequencing and bioinformatics analysis revealed that 585 piRNAs were upregulated in HF patients, and 4,623 piRNAs were down-regulated. Among these piRNAs, has-piR-020009 and has-piR-006426 were the most significantly different. Has-piR-020009 is located on chromosome 7q35, and has-piR-006426 is located on chromosome 17p11.2. These piRNAs have not been previously reported in the cardiovascular system, which highlights the importance of our analysis. Altogether, our data suggest that piRNAs may be involved in the progression of HF, especially has-piR-020009 and has-piR-006426. However, the underlying mechanism still requires further investigation. We found a dramatic difference in piRNA expression in serum exosomes of HF patients, which suggests that piRNAs are potential biomarkers of HF. Additionally, exosomes are secreted as membrane-bound vesicles, which could provide their cargo protection from degradation by cellular RNases in the circulatory system. This ensures that exosomal piRNAs can be detected in blood samples in a surprisingly stable form, in contrast to mRNAs. It also makes piRNAs interesting candidates for biomarkers, providing information with respect to potential pathophysiological conditions, and could influence specific treatment strategies in patients.

Conclusions

Currently, miRNAs are widely accepted as potential therapeutic targets in cardiovascular diseases. It is anticipated that miRNA modulators will be available as therapeutic options in the near future. Moreover, miRNAs are attractive potential biomarkers for various cardiovascular diseases. However, additional investigations and stringent analyses are necessary to confirm and test this potential before miRNAs can be widely used as clinically important biomarkers for cardiovascular diseases. This study has shed light on the role of piRNAs in HF patients, providing novel insight into biomarker selection for HF.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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