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Original Article

Genome-wide screening for circRNAs in epicardial adipose tissue of heart failure patients with preserved ejection fraction

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Abstract: Objective: Heart failure with preserved ejection fraction (HFpEF) is a complex cardiovascular syndrome. Along with pro-inflammatory and metabolic factors, epicardial adipose tissue (EAT) is believed to play a key role in the pathogenesis of HFpEF. Studies have increasingly shown a critical role of circRNAs in the development of cardiovascular diseases; however, their role in the pathogenetic mechanism of HFpEF is not well characterized. The objective of this study was to investigate the expression profiles of circRNAs in EAT of HFpEF patients. Methods: Samples of epicardial adipose tissue were obtained from patients with HFpEF (n=5) and patients without heart failure (non-HF; n=5). CircRNA expression profiles were screened using RNA sequencing method. RNA-sequencing results were confirmed by qRT-PCR analysis. Gene Ontology enrichment and Kyoto Encyclopedia of Genes and Genomes pathway analysis were performed on the differentially expressed circRNAs. Results: A total of 131 circRNAs were differentially expressed between HFpEF and non-HF groups (77 upregulated and 54 downregulated). Among these, hsa_circ_0118464 corresponding to *HECW2* gene which showed the highest fold-change was assessed by qRT-PCR, and the outcome was consistent with RNA-sequencing results. The differentially expressed circRNAs corresponded to genes mainly involved in regulation of cellular and metabolic processes. Conclusion: This study provides the expression profile of circRNAs in EAT of HFpEF patients and the associated molecular mechanism. Our findings may provide insight into diagnostic markers and therapeutic targets in the context of HFpEF.

Keywords: Circular RNAs, epicardial adipose tissue, heart failure, heart failure with preserved ejection fraction

Introduction

Heart failure with preserved ejection fraction (HFpEF) is a distinct heart failure syndrome characterized by ejection fraction (EF) >50%, accompanied by various proinflammatory and metabolic comorbidities. Alterations in cell structure and changes at the molecular level (including cardiomyocyte fibrosis, hypertrophy, and inflammation) may cause improper relaxation of the left ventricle, resulting in HFpEF [1, 2]. Studies have shown that epicardial adipose tissue (EAT) is one of the key pathogenic factors in HFpEF [3, 4]. EAT is located directly over the myocardial surface and shares the same microcirculation with myocardium. There is no anatomic boundary between EAT and myocardium; therefore, the secretome from EAT can affect the heart through vasocrine, paracrine, and endocrine signaling pathways [5]. Dysfunctional EAT caused by chronic systemic inflammation or metabolic disorders secretes pro-inflammatory adipokines that may cause myocardial inflammation and myocardial fibrosis, contributing to the pathophysiologic process of HFpEF [6, 7]. Circular RNAs (circRNAs), that are circularized by joining the 3' of the RNA to the 5', are a novel category of non-coding RNA that participate in cardiovascular diseases by regulating the cardiac gene regulator network and inducing cardiomyocyte hypertrophy, proliferation, and cardiac fibrosis [8, 9]. However, there is a paucity of studies that have focused on identifying circRNAs in EAT; in addition, the role of circRNAs in EAT in the pathogenesis of HFpEF has not been previously investigated. The aim

of this study was to characterize the expression profiles of circRNAs in EAT of HFpEF patients by using the RNA sequencing method for the first time. Our findings may help identify novel diagnostic markers and therapeutic targets for HFpEF.

Materials and methods

Study participants and samples

Patients with coronary artery disease (CAD) who underwent coronary artery bypass grafting (CABG) surgery between May and August 2021 were included in our study. According to the cardiac function, patients were divided into a HFpEF group and a non-HF group. Patients with HFpEF were diagnosed according to the HFA-PEFF score, including clinical features of heart failure (HF), the levels of brain natriuretic peptide (BNP) or N-terminal pro-brain natriuretic peptide (NT-proBNP), and echocardiographic criteria [10]. Patients in the HFpEF group exhibited a left ventricular ejection fraction ≥50% with symptoms or signs of HF, a high BNP level, and HFA-PEFF score ≥5. Patients without HF were recruited to the non-HF group.

Information including demographics, medical history, laboratory tests, and echocardiography results were collected from all the enrolled patients after admission. All subjects provided written informed consent, and the study procedures were in compliance with the principles of the Declaration of Helsinki (2013). The study was approved by the Ethics Committee of the Beijing Chaoyang Hospital, Capital Medical University (2021-ke-246, Beijing, China, shown in Supplementary File). EAT samples (1-2 cm^3) were collected immediately before the initiation of cardiopulmonary bypass. The samples were taken from the left-interventricular groove, cut into small pieces approximately 1-2 cm^3 in volume, and washed with PBS. Then the total EAT samples were frozen in liquid nitrogen and stored at -80°C before RNA sequencing.

Library preparation and sequencing of lncRNAs and mRNA

Total RNA from EAT specimens was quantified on a NanoDrop ND-1000 instrument, and the integrity of total RNA samples was checked using agarose gel electrophoresis. For RNA-seq library preparation, total RNA (1-2 μg) from each sample was utilized. The NEBNext® Poly(A) mRNA Magnetic Isolation Module was used to separate mRNA from total RNA, or the RiboZero Magnetic Gold Kit rRNA was used to eliminate rRNA from total RNA. Then, using the KAPA Stranded RNA-Seq Library Prep Kit (Illumina), the enriched mRNA or rRNA-depleted RNA were utilized for library preparation in subsequent procedures, including fragmentation of the RNA molecules, reverse transcription for synthesis of first-strand cDNA, incorporation of dUTP for synthesis of second-strand cDNA, end-repair and A-tailing of the double-stranded cDNA, ligation of Illumina compatible adaptor, and PCR amplification and purification for the final RNA-seq library. Lastly, the absolute quantification qPCR method was used to quantify the completed RNA-seq libraries, which were made using the Agilent 2100 Bioanalyzer. The sequencing was conducted on Illumina HiSeq 6000 as per the manufacturer's instructions.

Verification by qRT-PCR

One of the differentially expressed circRNAs, hsa_circ_0118464, was randomly selected to verify the RNA sequencing outcomes using quantitative real-time polymerase chain reaction (qRT-PCR). The forward primer was 5'-CAGGCTGGTATCTGTTTTTGA-3', and reverse primer was 5'-TGTACTCCTTCTTGTTCTTCTCTG-3'. The housekeeping gene used for qRT-PCR analysis was β-actin. The RNA samples extracted from EAT specimens were reverse transcribed for cDNA synthesis using a PrimeScript™ RT reagent Kit (Takara Bio Incorporated, Japan) following the manufacturer's instructions. The reaction condition was 10 min at 95°C, with 40 PCR cycles (10 seconds at 95°C, 60 seconds at 60°C), and then the fluorescence was detected. The relative expression levels of circRNA were quantified using QuantStudio™ 5 Real-time PCR System (Applied Biosystems) and calculated using the $2^{-\Delta\Delta CT}$ method.

GO and KEGG pathway analysis

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were conducted for functional characterization of the differentially expressed genes identified in our study. Three structured relationships of RNAs were characterized by GO enrichment analysis including biological processes (BP), cellular components (CC), and molecular func-

Table 1. Clinical characteristics of the study population

Characteristic	HFpEF (n=5)	Control (n=5)	*P*-value
Age (years)	67.2±3.96	63.8±6.14	0.329
Females (n (%))	3 (60%)	2 (40%)	0.527
BMI (Kg/m^2)	27.47±1.73	24.8±2.26	0.221
Hypertension (n (%))	3 (60%)	2 (40%)	0.527
Diabetes mellitus (n (%))	3 (60%)	3 (60%)	1.0
Atrial fibrillation (n (%))	2 (40%)	1 (20%)	0.49
Hyperlipidemia (n (%))	4 (80%)	3 (60%)	0.49
Smoking (n (%))	4 (80%)	4 (80%)	1.0
Systolic blood pressure (mmHg)	128.2±11.26	132.4±13.94	0.614
Diastolic blood pressure (mmHg)	71.8±9.2	71.2±7.19	0.911
LVEF (%)	63.76±6.18	63.2±5.29	0.881
LVDD (mm)	56.78±8.59	53.48±6.43	0.511
LAVI (ml/m^2)	42.11±6.66	30.96±2.48	0.008
E/e' (ratio)	16.74±6.19	8.61±2.16	0.024
EAT thickness (mm)	5.7±0.91	4.68±0.23	0.036
NT-proBNP (pg/mL)	2521.2 (1679.4, 4326)	126 (83.3, 177.6)	0.008
Total cholesterol (mmol/L)	3.97±0.78	3.23±0.32	0.089
Triglycerides (mmol/L)	1.0±0.26	1.76±1.35	0.248
LDL (mmol/L)	2.28±0.72	1.64±0.34	0.11
HDL (mmol/L)	0.97±0.31	1.0±0.29	0.894
eGFR (ml/min/1.73 m^2)	111.64±13.90	138.64±16.86	0.025
Hs-CRP (mg/L)	6.5 (2.06, 13.52)	1.33 (1.07, 3.07)	0.115
HbA1C (%)	6.74±1.07	6.24±0.54	0.377

Note: HFpEF: Heart failure with preserved ejection fraction; BMI: body mass index; LVEF: left ventricular ejection fraction; LVDD: left ventricular diastolic diameter; LAVI: left atrial volume index; TRPV: tricuspid regurgitation velocity; EAT: epicardial adipose tissue; NT-proBNP: N-terminal pro-brain natriuretic peptide; LDL: low density lipoprotein; HDL: high density lipoprotein; eGFR: estimated glomerular filtration rate; Hs-CRP: hypersensitive C-reactive protein; HbA1C: Hemoglobin A1C.

tion (MF). KEGG pathway analysis was performed to identify the pathway clusters that the differentially expressed genes were involved in. Fisher Exact test was utilized for classifying the GO category and selecting the significant pathways. The false discovery rates were calculated to adjust the *P* values, and *P* values <0.05 were considered indicative of significance.

Statistical analysis

IBM SPSS Statistics 24.0 was used for data analysis. Continuous variables were expressed as mean ± standard deviation (SD), and categorical variables were expressed as frequencies (percentages). Inter-group differences were assessed using the Student's *t* test, Chi-square test, or Fisher's exact test, based on the distribution of variables. *P*<0.05 was considered of statistical significance. R package was used to calculate the fragments per kilobase of exon per million reads (FPKM) value, differential expression at gene and transcript levels, and RNA-sequencing analysis including hierarchical clustering, GO enrichment analyses, pathway analysis, and the scatter plots and volcano plots were also constructed. Fold change cutoff >1.5, *P* value <0.05 and FPKM ≥0.5 were used to identify differentially expressed genes and transcripts.

Results

Clinical characteristics of the study population

There were no significant differences between HFpEF patients and non-HF patients with respect to demographic characteristics. HFpEF patients had thicker EAT and higher levels of BNP, LAVI, E/e' values, and lower eGFR. A summary of baseline data is presented in **Table 1**.

Expression profiles of circRNAs by RNA sequencing

A total of 1991 EAT circRNAs were detected in the two groups. The lengths of the circRNAs

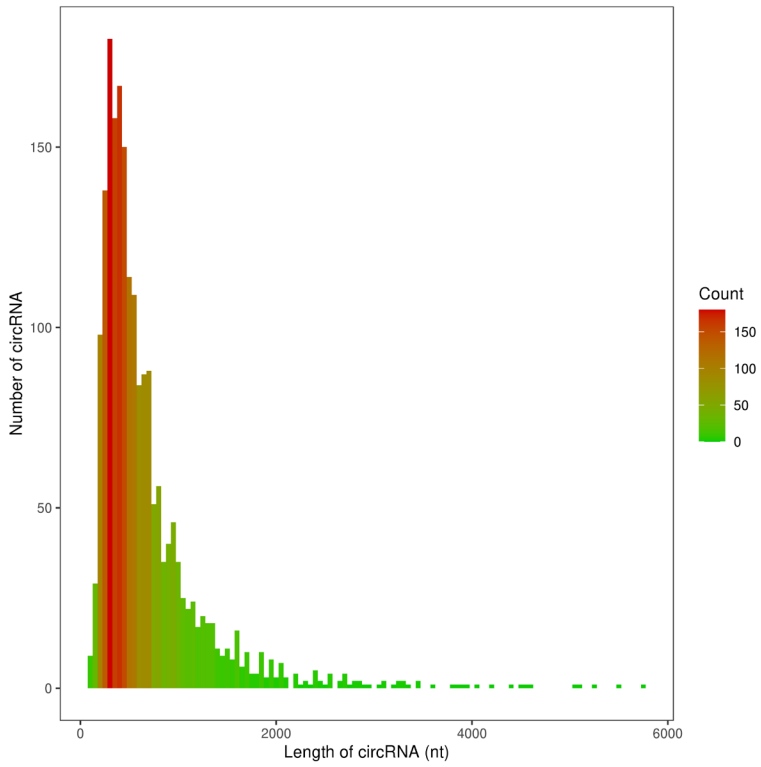

Figure 1. Length distribution of circRNAs in epicardial adipose tissue.

were mainly within 2000 nt (**Figure 1**). We identified 773 circRNAs that were differentially expressed between HFpEF and non-HF groups, including 425 upregulated and 348 downregulated circRNAs. The hierarchical clustering heatmaps and scatter plots of differentially expressed circRNAs are shown in **Figure 2**. In the volcano plots, there were 77 circRNAs upregulated and 54 circRNAs downregulated in the two groups respectively ($P<0.05$, fold change >1.5; **Figure 3**). The top 30 differentially expressed circRNAs are presented in **Table 2**. To verify the results of RNA sequencing, hsa_circ_0118464 was randomly selected and measured by qRT-PCR. The result was consistent with the results of RNA sequencing (**Figure 4**).

Functional analysis

For further functional characterization of the differentially expressed circRNAs, GO enrichment and KEGG analysis were performed (**Figures 5** and **6**). The upregulated circRNAs were mostly involved in regulation of cellular processes, primary metabolic processes, and positive regulation of macromolecule biosynthetic processes, response to protein binding, and activities of transferase, catalytic and phosphatidylinositol kinase. The downregulated circRNAs were linked to regulation of cellular processes, developmental processes, DNA metabolic processes and DNA repair, accompanied by components of cytosol, cytoskeleton and cytoplasm. KEGG pathway analysis showed that the top three pathways of upregulated circRNAs were thyroid hormone signaling pathway (hsa04919), renal cell carcinoma (hsa05211), and platinum drug resistance (hsa01524). For the downregulated circRNAs, signaling pathways were associated with adherens junction (hsa04520), ubiquitin mediated proteolysis (hsa04120), nucleotide excision repair (hsa03420), and morphine addiction (hsa05032).

Discussion

Epicardial adipose tissue shares the same microcirculation with the underlying myocardium, and due to the special anatomy, EAT expansion may play a key role in the development of HFpEF. Various systemic inflammatory or adipogenic metabolic disorders associated with HFpEF have been linked to an increase in epicardial fat volume [6, 11]. Proinflammatory mediators released by accumulated and dysfunctional EAT may lead to microcirculatory dysfunction and fibrosis of myocardium, which may impair the left ventricular distensibility and increase diastolic stiffness and filling pressure [12-14]. Despite growing evidence of the pathogenic role of EAT in HEpEF, the assessment of EAT derangements in HFpEF is challenging. Quantifying EAT volume or testing the circulatory proinflammatory adipocytokines by imaging alone cannot accurately characterize the biologic activity and proinflammatory role of EAT [15]. Thus, a direct focus on EAT samples would provide more precise evidence of its role in the pathogenesis of HFpEF. EAT can secrete various cytokines as well as exosomes carrying non-coding RNAs that may diffuse into the adja-

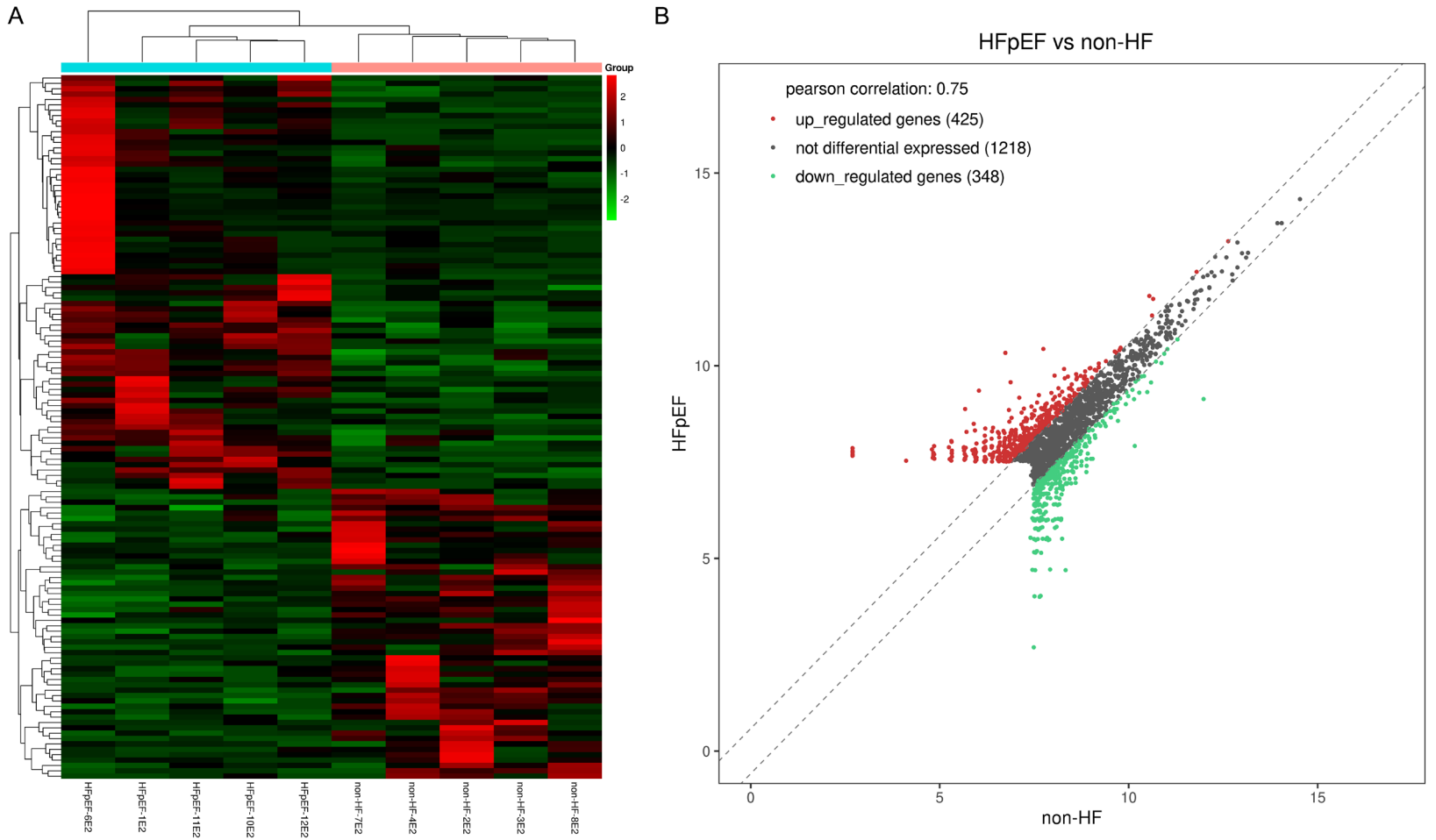

Figure 2. Hierarchical clustering heatmaps and scatter plots of differentially expressed circRNAs between HFpEF and non-HF groups. A: Hierarchical clustering heatmaps; B: Scatter plots. HFpEF: Heart failure with preserved ejection fraction; non-HF: patients without heart failure.

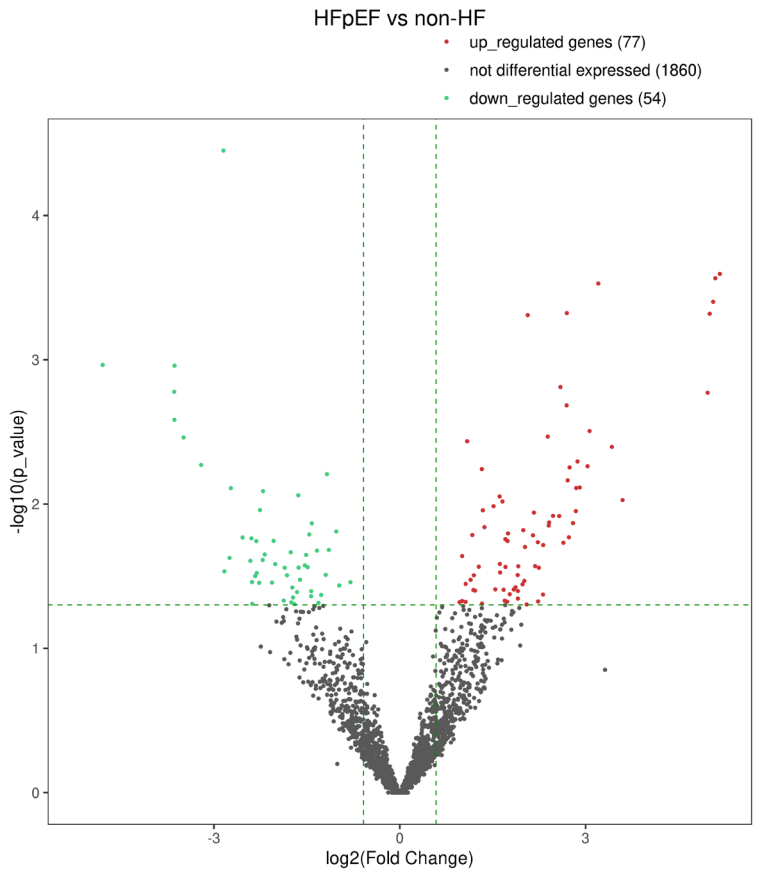

Figure 3. Volcano plots of the differentially expressed circRNAs (P<0.05, fold change >1.5). HFpEF: Heart failure with preserved ejection fraction; non-HF: patients without heart failure.

cent muscle tissues and affect its function. Therefore, our study characterized the expression profiles of circRNAs in EAT of HFpEF patients and explored the role of EAT circRNAs in the pathogenesis of HFpEF.

CircRNAs have been shown to play a role in various cardiovascular diseases, including cardiac hypertrophy, cardiac fibrosis, heart failure, coronary artery disease, and cardiomyopathy [8, 9, 16, 17]. However, there is a paucity of studies that have investigated the circRNAs in EAT of HFpEF patients. In this study, a total of 131 differentially expressed circRNAs were identified between the HFpEF and non-HF groups by RNA-sequencing method. Among them, 77 circRNAs were upregulated and 54 circRNAs were downregulated. Hsa_circ_0118464 in EAT corresponds to HECW2 and is highly upregulated with the highest fold change of 35.939. HECW2 encodes a member of E3 ubiquitin ligases which plays a role in the migration, proliferation, and differentiation of neural crest cells, and also plays a key role in angiogenesis by stabilizing the junctions between endothelial cells [18]. HECW2 contains a C2 domain at its N-terminus and is involved in phospholipid/Ca^{2+} signaling [19]. By gene set enrichment analysis, circ_HECW2 was identified to be involved in cardiac hypertrophy and fibrosis, and knockdown of circ_HECW2 can reduce phenylephrine-induced cardiomyocyte hypertrophy in vitro [8]. It has been suggested that circ_HECW2 can regulate ox-LDL-induced cardiovascular endothelial cell dysfunction through targeting the miR-942-5p/TLR4 axis [20]. In a study, circ_HECW2 was shown to regulate lipopolysaccharide (LPS)-induced endothelial-mesenchymal transition (EndoMT) in human brain microvascular endothelial cells that was caused by proinflammatory mediators through alteration of gene expression [21]. Silencing the circ_HECW2 was shown to enhance cell proliferation and prevent cell apoptosis and EndoMT induced by LPS [22]. Moreover, upregulation of circ_HECW2 in osteoarthritis (OA) patients and a LPS-induced OA cell model was shown to be instrumental in promoting cell apoptosis by regulating miR-93 expression [23]. These findings revealed a possible function of circRNAs in inflammation, which is a key factor in the pathogenesis of HFpEF. We also confirmed upregulation of circRNA-HECW2 in the validation examination. However, further study is required to unravel the biologic role of circRNA_HECW2.

Functional analysis showed that these differentially expressed EAT circRNAs were mainly related to the regulation of cellular and metabolic processes. Hsa_circ_0005583 was shown to originate from the ATM gene locus, which is associated with cell cycle control and DNA repair. A systematic review showed that ATM

Table 2. Top 30 differentially expressed circRNAs

circRNA_ID	Locus	Gene_Name	Length	Regulation	Fold_Change	*p*_value
hsa_circ_0118464	chr2:197084762-197085665:-	HECW2	262	up	35.939	0.0003
hsa_circ_0094798	chr11:107260799-107263621:-	CWF19L2	255	up	34.159	0.0003
hsa_circ_0130086	chr5:96248340-96251473:+	ERAP2	385	up	33.345	0.0004
hsa_circ_0005376	chr8:42780699-42798588:+	HOOK3	257	up	32.073	0.0005
hsa_circ_0001708	chr7:50358643-50367353:+	IKZF1	174	up	31.335	0.0017
hsa_circ_0003553	chr1:24840803-24841057:+	RCAN3	254	up	12.091	0.0094
hsa_circ_0011163	chr1:29010094-29023596:+	GMEB1	628	up	10.736	0.0040
hsa_circ_0000398	chr12:46633461-46637097:-	SLC38A1	330	up	9.217	0.0003
hsa_circ_0121140	chr2:89100615-89104394:+	ANKRD36BP2	3779	v	8.362	0.0031
hsa_circ_0004431	chr3:138400808-138403645:-	PIK3CB	368	up	8.174	0.0055
hsa_circ_0025641	chr12:26217430-26220646:+	RASSF8	1035	up	7.492	0.0077
hsa_circ_0012742	chr1:59147456-59150923:-	MYSM1	860	up	7.305	0.0051
hsa_circ_0115341	chr20:47569241-47580435:+	ARFGEF2	484	up	7.195	0.0077
chr17:67151159-67175149:-	chr17:67151159-67175149:-	ABCA10	1014	up	7.171	0.0112
hsa_circ_0067582	chr3:141231004-141259451:+	RASA2	394	up	6.948	0.0135
hsa_circ_0077077	chr6:76331247-76333676:+	SENP6	155	down	0.036	0.0011
hsa_circ_0003351	chr10:5827814-5842668:-	GDI2	542	down	0.080	0.0017
hsa_circ_0003927	chr3:47960208-47963368:-	MAP4	237	down	0.080	0.0026
hsa_circ_0018180	chr10:34661425-34673182:-	PARD3	817	down	0.080	0.0011
chr17:67181617-67184020:-	chr17:67181617-67184020:-	ABCA10	366	down	0.089	0.0035
hsa_circ_0138526	chr9:2493074-2525717:-	RP11-125B21.2	780	down	0.108	0.0054
hsa_circ_0099634	chr12:97886238-97954825:+	RMST	1314	down	0.139	0.0000
hsa_circ_0000739	chr17:5314022-5320002:-	NUP88	383	down	0.140	0.0293
hsa_circ_0000278	chr11:14880588-14882912:+	PDE3B	366	down	0.149	0.0236
hsa_circ_0001264	chr3:8977554-8983488:-	RAD18	623	down	0.151	0.0078
hsa_circ_0067291	chr3:129182402-129188260:+	IFT122	467	down	0.172	0.0170
hsa_circ_0130906	chr6:144835044-144838084:+	UTRN	597	down	0.188	0.0247
hsa_circ_0120233	chr2:48869540-48898819:+	STON1-GTF2A1L	1082	down	0.190	0.0173
hsa_circ_0136524	chr8:38994175-39068843:+	ADAM32	1095	down	0.191	0.0347
hsa_circ_0095935	chr11:47752925-47772842:-	FNBP4	1371	down	0.192	0.0491

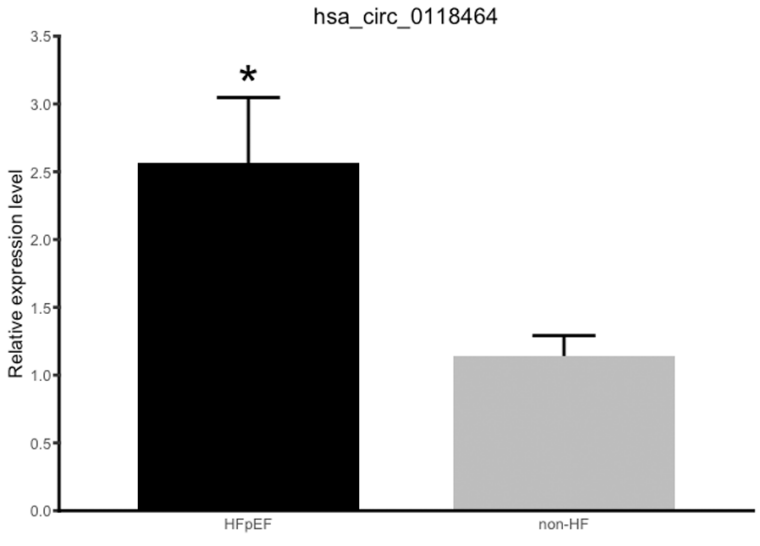

Figure 4. Expression of hsa_circ_0118464 in HFpEF and non-HF groups by qRT-PCR. Data were expressed as mean ± SEM (n=5 for each group). Compared to the non-HF group, *P<0.05. HFpEF: Heart failure with preserved ejection fraction; non-HF: patients without heart failure.

can decrease life expectancy of patients with cancer and ischemic heart disease, and that ATM mutation carriers should be aware of lifestyle factors implicated in the development of cardiovascular diseases and diabetes [24]. Cell cycle and apoptotic regulatory protein 1 (CCAR1) that is possibly modulated by hsa_circ_0002681, plays a critical role in Wnt signaling, adipogenesis, apoptosis, nuclear receptor function, and DNA damage response [25]. Acting as an apoptosis signaling regulator, CCAR1 may also be involved in the pathogenesis of HFpEF; however, this needs to be explored in further studies.

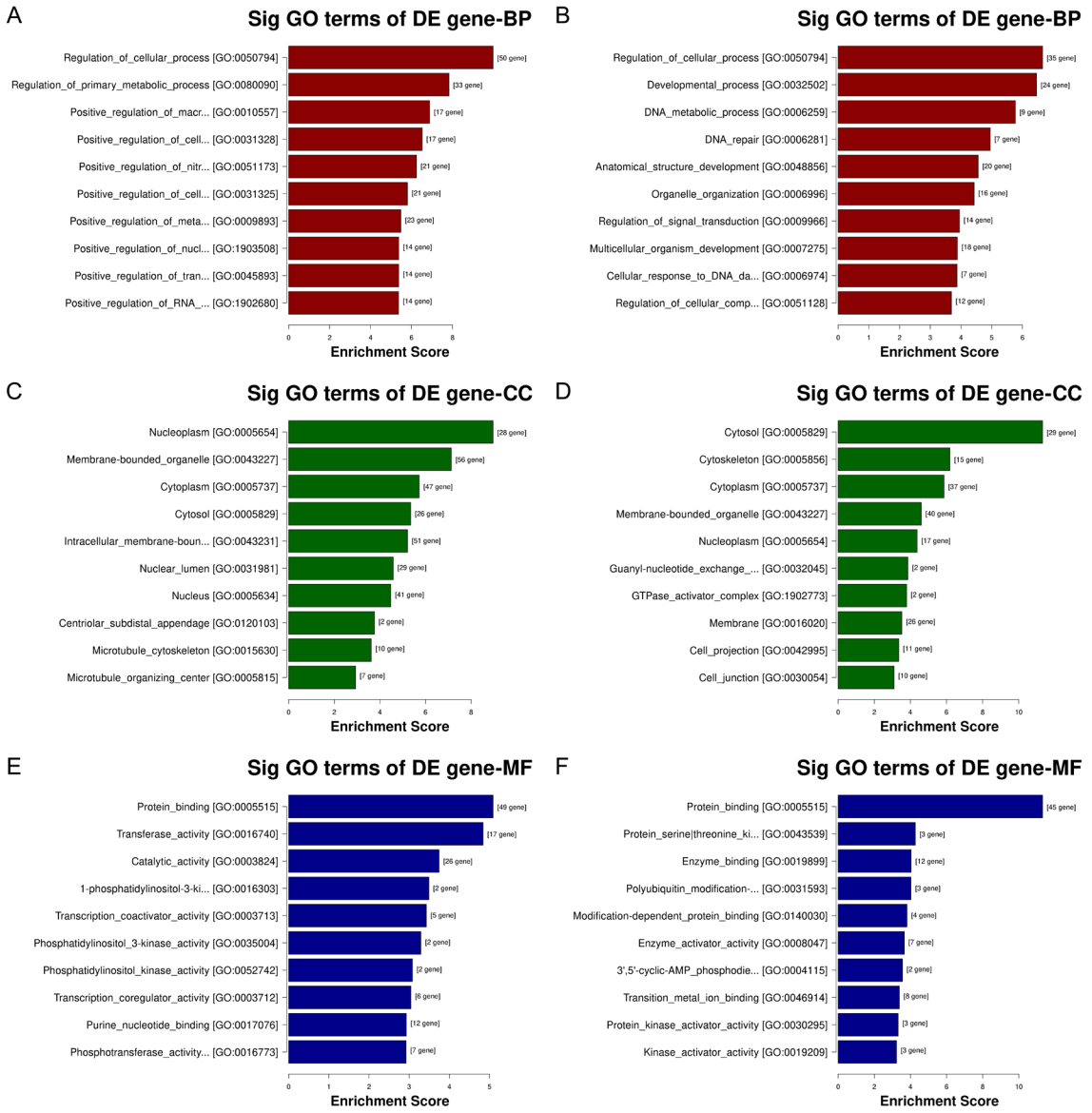

Figure 5. The top ten GO enrichment terms associated with differentially expressed circRNAs. A, C, E: Upregulated circRNAs; B, D, F: Downregulated circRNAs. GO: Gene ontology; BP: Biological Process; CC: Cellular Component; MF: Molecular Function.

Certain limitations of our study should be considered when interpreting our results. First, the limited sample size in this study may have affected the accuracy of RNA-sequencing outcomes. Further studies with larger sample sizes are necessary to confirm the association between candidate EAT circRNAs and HFpEF. Second, we verified only one of the differentially expressed genes in our study. Third, this study was merely observational and theoretically descriptive. Additional experimental and clinical studies are required to validate the biologic function of circRNAs.

In this study, we explored the comprehensive expression profiles of circRNAs in EAT of HFpEF patients by genome-wide screening and identified candidate circRNAs for further investigation. Our work may provide new insights into the molecular and genetic roles of EAT in the pathogenesis of HFpEF and help identify novel diagnostic markers and therapeutic targets for HFpEF.

Disclosure of conflict of interest

None.

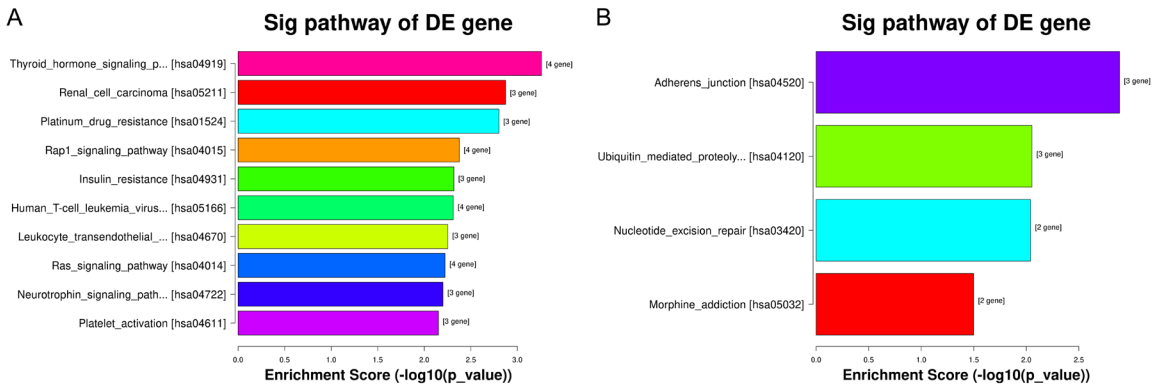

Figure 6. KEGG pathway analysis of differentially expressed circRNAs. A: Upregulated circRNAs; B: Downregulated circRNAs. KEGG: Kyoto Encyclopedia of Genes and Genomes.

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