ASPN is a Potential Promising Biomarker for Common Heart Failure

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Abstract

Background: Heart failure (HF), the leading cause of adult mortality and morbidity worldwide, is the end-stage of various diseases, especially ischemic cardiomyopathy (ICM) and dilated cardiomyopathy (DCM). This study aimed to investigate the common molecular mechanism of ICM and DCM.

Methods: Four gene expression datasets, GSE1869, GSE5406, GSE57338, and GSE79962, were obtained from the Gene Expression Omnibus (GEO) database. The differentially expressed genes (DEGs) of ICM or DCM samples compared with those of nonfailing samples were identified. Gene ontology (GO) annotation, Kyoto encyclopedia of gene and genome (KEGG) pathway analysis, and the protein-protein network (PPI) of the coreregulated DEGs in at least three datasets were performed using the online tools of DAVID, the KOBAS database, and the STRING database, respectively. Hub genes of HF were analyzed for their correlation with left ventricular ejection fraction (LVEF) in dataset GSE19303. The expression levels of notable DEGs were further validated in our tissue microarray (TMA).

Results: Fifty-nine coreregulated ICM and sixty-eight coreregulated DCM relevant DEGs were identified (in at least three datasets). Moreover, 38 common DEGs between ICM and DCM relevant DEGs were obtained that were mainly involved in inflammatory/stress processes, proliferation, and some lipid metabolism pathways. Among the ten hub genes with top degrees, four genes showed a correlation with LVEF, and ASPN had the most significant correlation. Finally, the expression of ASPN protein was validated in our TMA and was significantly increased in ICM and DCM left ventricular samples.

Conclusion: The present study revealed some common molecular mechanisms of HF with different causes. Furthermore, ASPN may be a potential promising biomarker for HF.

Introduction

Heart failure (HF) is the final path of numerous cardiovascular diseases, characterized by impaired ventricular filling and/or ejection function, resulting in insufficient output for the demands of the body, pulmonary and/or systemic congestion[1]. HF is a significant global health concern with a prevalence of more than 26 million patients, representing the leading cause of hospitalization in developed countries[2]. It is projected that the prevalence of HF will increase by 46% until 2030[3], resulting in more than double associated health care expenditures[4]. Despite the significant development in medical therapy for HF, with the marked improvement of prognosis, the mortality remains high[5]. The mortality was reported to be 20.2% within 1 year and 52.6% within 5 years after the HF diagnosis[6]. For patients with end-stage HF, medical therapy has demonstrated little benefit, and mechanical support or heart transplantation may be the final and most efficient option.

HF is a complex clinical disorder that can be initiated by various heart-associated diseases, including ischemic cardiomyopathy (ICM), dilated cardiomyopathy (DCM), valvular heart disease, hypertension, and many other diseases that could impair myocardial function[7, 8]. Among the various reasons, coronary artery disease (CAD) or ICM has emerged as the primary cause, resulting in more than 50% of HF patients,

while DCM is the second most common etiological factor causing approximately 36% of all HF cases[9]. Despite the different causes, HF shows similar symptoms and outcomes based on cardiac dilation and reduced contractility of ventricles[10].

Recently, although many studies have tried to explain the possible underlying mechanisms of HF from various perspectives[1, 5, 10], the molecular mechanisms remain incompletely understood and need further exploration. Comprehensive analysis of the molecular states may provide a better understanding of the underlying mechanism and uncover new therapeutic targets[5]. In recent years, the development of the high-throughput technologies has produced extensive molecular state information on HF and has provided new insight into the pathogenesis of HF[11]. Hundreds or even thousands of samples could be rapidly tested in one batch with a very high accuracy[12]. Presently, numerous sources of gene expression information have been uploaded into the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo>), which is a free public repository[13].

Over the last two decades, tissue microarray (TMA) technology has triggered a revolution in biomarker investigation of paraffin-embedded tissues[14]. The conventional screening method to identify a new biomarker among a large number of samples is inefficient and tedious because of its intrinsic property—one slide with one sample. However, TMAs offer a more cost-effective and efficient resource, containing tens or even hundreds of specimens in one slide[15]. The construction of TMAs involves the repeated transfer of small tissue cores, typically ranging from 0.6 to 2.0 mm in diameter, from paraffin-embedded tissue blocks into a single empty paraffin block[14, 16]. TMAs are an ideal common tool for high-throughput profiling to study tissue morphology, protein or gene expression following different stains, immunohistochemistry or fluorescence in situ hybridization[16, 17]. Because of their inherent characteristics, large numbers of samples can be tested and analyzed in a single running. Therefore, TMAs could reduce the potential interexperiment variability associated with conventional biomarker screening technologies[18].

In the present study, 5 gene expression profiles of human left ventricles with or without failing function were downloaded from the GEO database (<https://www.ncbi.nlm.nih.gov/geo>). These datasets contained nonfailing left ventricular tissues and failing ventricular tissues resulting from ICM and DCM. Differentially expressed genes (DEGs) of ICM or DCM compared with nonfailing samples of each dataset were explored. The hub genes of coreregulated DEGs between ICM and DCM were validated in our TMA comprising left ventricular samples with or without heart failure. This study aimed to identify common molecular mechanisms in general HF that may suggest new therapeutic targets.

Materials And Methods

Data Resources

The gene expression profiles of GSE1869, GSE5406, GSE57338, GSE79962 and GSE19303 with their respective matched probe annotation platform profiles were downloaded from the GEO database. The samples of the former 4 datasets were obtained from explanted hearts or unused donor hearts, while the

samples of GSE19303 were collected from endomyocardial biopsies. The GSE1869 dataset included 6 nonfailing samples, 10 samples of ICM and 14 samples of DCM. The GSE5406 dataset contained 16 nonfailing left ventricular samples and 108 and 86 failing ventricular tissue because of ICM and DCM, respectively. The GSE57338 dataset had 136 normal functional left ventricular samples and 95 and 82 failing samples of ICM and DCM, respectively. The GSE79962 dataset included 11, 11 and 9 samples of nonfailing, ICM and DCM, respectively. The GSE19303 dataset included gene expression information on 81 left ventricular samples with the matched values of the left ventricular ejection fraction (LVEF).

Identification of DEGs

R software (version 3.6.1) was used to process and statistically analyze the expression files. The downloaded matrix files were gene expression annotated with probe names. Therefore, the matched platform file was used to convert the probe name to the gene standard name (gene symbol). The linear models for microarray data (LIMMA) package[12, 13] was used to identify the DEGs between the ICM or DCM compared with normal control specimens of the GSE1869, GSE5406, GSE57338 and GSE79962 datasets. The relative gene expression values with an adjusted P-value < 0.05 and $|\log_2 \text{FC}| > 0.5$ (FC, fold change) were considered as DEGs. Heatmaps of these DEGs were constructed by R software.

To determine the common DEGs of the four datasets, the VENN web server (<http://jvenn.toulouse.inra.fr/app/example.html>) was utilized. The ICM-relative DEGs and DCM-relative DEGs were grouped to explore the overlapped genes, which were considered as highly relevant genes of HF.

GO and KEGG Pathway Enrichment Analysis

Gene ontology (GO) analysis of the coregulated HF genes, including biological process (BP), cellular component (CC) and molecular function (MF), were performed using the DAVID database[19]. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of the highly relevant genes of HF was performed using the KOBAS database (<http://kobas.cbi.pku.edu.cn/>)[20]. A P-value < 0.05 of enriched terms was considered significant.

Protein-protein interaction (PPI) network construction

The PPI network of HF-relevant genes was constructed using the online STRING database tool[21]. An interaction score ≥ 0.4 (medium confidence) was used as the cut-off criterion[22]. Cytoscape software (version 3.6.1) was used to further analyze and visualize the network. The genes with the top 10 connectivity degrees were considered as hub genes of HF.

Human Cardiac Collection

Human left ventricular samples were obtained from patients who had undergone heart transplantation for end-stage HF or from unused donor hearts in Guangdong General Hospital. The tissues were harvested and snap-frozen in liquid nitrogen or fixed in 4% paraformaldehyde for future analysis. The

collection and research use of the heart tissues were approved by the Ethics Committee of Guangdong General Hospital. Informed consent was obtained from all the participants.

TMA construction

For this study, 4 donor samples, 21 DCM samples and 18 ICM samples were included for TMA construction. As previously described[18], the samples fixed in 4% paraformaldehyde were then embedded in paraffin. For the HF groups, one core from each paraffin-embedded sample was removed and inserted into to a blank paraffin block to construct a TMA. Because of the small size of the control group, three cores were collected from each donor sample for TMA construction. Next, the TMA was cut into 4-micrometer-thick cross sections for immunohistochemistry. Each section comprised 51 left ventricular samples to be examined simultaneously.

Immunohistochemistry

For immunohistochemistry, the sections were deparaffinized and rehydrated, followed by heating in a pressure cooker for antigen retrieval. The antigen-retrieved sections were incubated in 0.3% hydrogen peroxide for endogenous peroxidase activity block and then were incubated with primary antibodies (ASPN; 1:250 dilution; Abcam, USA) at 4 °C overnight. Relevant secondary antibodies were used for incubation. All specimens were counterstained with hematoxylin staining solution (Beyotime Institute of Biotechnology, China). Scanning of the TMA slides was performed using the Pannoramic MIDI scanner (3D Histech; Hungary). Quant center, an analysis software supporting the Pannoramic viewer, was used to measure the intensity of ASPN staining (brown color) as described previously[23, 24].

Results

Identification of ICM-related DEGs

ICM-relevant DEGs were identified in the 4 datasets compared with the normal control group. Overall, 225 (including 190 upregulated and 35 downregulated genes), 202 (including 87 upregulated and 115 downregulated genes), 422 (including 239 upregulated and 183 downregulated genes) and 435 (including 253 upregulated and 182 downregulated genes) ICM-relevant DEGs were screened out from the GSE1869, GSE5406, GSE57338 and GSE79962 datasets, respectively. The heatmaps of these DEGs of each dataset are shown in Figure 1A–D.

In the four datasets, 59 coregulated ICM-relevant DEGs were obtained, including 40 co-upregulated genes and 19 co-downregulated genes (coregulated DEGs were defined as DEGs obtained in at least three datasets). Moreover, 7 DEGs, including 2 co-upregulated (ASPN and CRYM) and 5 co-downregulated (FCN3, MYH6, PLA2G2A, RARRES1 and SERPINA3) DEGs, were identified in all four datasets (Figure 1E–G).

Identification of DCM-related DEGs

Similar to ICM, the DEGs of DCM compared with the normal control in each dataset were screened out. Additionally, 2,416 (including 1,645 upregulated and 771 downregulated genes), 235 (including 104 upregulated and 131 downregulated genes), 460 (including 248 upregulated and 212 downregulated genes) and 420 (including 237 upregulated and 183 downregulated genes) DEGs, were identified from the GSE1869, GSE5406, GSE57338 and GSE79962 datasets, respectively (Figure 2A–D).

The DEGs in at least three datasets were defined as coregulated DEGs. Seventy-four coregulated genes were identified. However, only 47 co-upregulated and 21 co-downregulated genes showed the same trends of expression in all the datasets. The other 6 DEGs showed different trends in the four datasets and were excluded from the DCM-related genes. Furthermore, 12 co-up regulated (including ANKMY2, ECM2, LUM, ASPN, ITIH5, CRYM, OMD, ISLR, OGN, MXRA5, LRRC17 and IFI44L) and 5 co-down regulated (MYH6, SERPINA3, FCN3, RARRES1 and ETNPPL) DCM-related DEGs were identified in all the four datasets (Figure 2E–G).

Identification of co-DEGs associated with HF and Their functional enrichment analysis

The common DEGs of ICM and DCM were considered to be highly associated with HF. Additionally, 38 common DEGs, including 26 co-up regulated and 12 co-down regulated genes, were identified (Figure 3A–C). Moreover, 6 coregulated DEGs were identified between ICM- and DCM-related genes in all the four datasets—ASPN, CRYM, MYH6, SERPINA3, FCN3 and RARRES1 (additional Figure 1).

To further determine the role of these common DEGs in HF, GO and KEGG pathway analyses were performed. The variations in MF were primarily involved in binding other factors, such as Toll-like receptor 4 binding, arachidonic acid binding, RAGE receptor binding, and integrin binding (Figure 3D). The results of CC were markedly enriched in the extracellular region, Golgi and lysosomal lumen (Figure 3E). The enriched BP was mainly involved in inflammatory/stress processes and signaling pathways, such as cell adhesion, acute-phase response, positive regulation of inflammatory response, leukocyte migration involved in inflammatory response, activation of cysteine-type endopeptidase activity involved in apoptotic process, positive regulation of cell division, and mediated signaling pathway (Figure 3F). KEGG pathway analysis revealed that HF-related genes are mainly involved in inflammation, proliferation, and lipid metabolism pathways, such as the IL-17 signaling pathway, Ras signaling pathway, PI3K-Akt signaling pathway, adipocytokine signaling pathway, fat digestion and absorption, and alpha-linolenic acid metabolism (Figure 3G).

PPI network analysis

The 38 HF-related genes were imported into the STRING database, and a network of 25 nodes with 37 interactional pairs were constructed (Figure 4A). However, 13 proteins encoded by their genes showed no interaction with other proteins. Ten hub genes with the top connectivity degrees were identified—ASPN, FMOD, OMD, S100A9, OGN, LUM, CD163, S100A8, HBB and ECM2 (Figure 4B).

Correlation between HF-relevant DEGs and LVEF

The GSE19303 dataset showed gene expression of endomyocardial biopsies that contain the LVEF value of each sample. Thus, the 10 HF-related hub genes were analyzed for their correlation with the LVEF in the GSE19303 dataset. Only 4 genes showed a statistical correlation with LVEF: ASPN ($r = -0.43$ with $p < 0.0001$; Figure 5A), ECM2 ($r = -0.27$ with $p = 0.013$; Figure 5C), FMOD ($r = -0.32$ with $p = 0.004$; Figure 5D), and OMD ($r = -0.26$ with $p = 0.018$; Figure 5H). However, the other 6 HF hub genes showed no statistical correlation with LVEF ($p > 0.05$; Figure 5B, E–G, I and J).

Immunohistochemistry validation in TMA

As described above, four HF hub genes showed a correlation with LVEF, and ASPN showed the most significant correlation. Moreover, ASPN among the hub genes was the only DEG identified in all the four datasets (additional Figure 1). Therefore, the expression of ASPN was validated by immunohistochemistry in our TMA comprising 51 cores of left ventricular samples, including 12 cores from four non-HF (NHF) donors, 21 cores from DCM patients, and 18 cores from ICM patients. Masson staining showed aggregated cardiac fibrosis in DCM and ICM compared with that in NHF (Figure 6A). Similarly, the expression levels of ASPN were significantly increased in DCM and ICM compared with that in NHF (Figure 6B and additional Figure 2).

Discussion

HF is the final stage of various heart diseases, especially ICM and DCM, with high mortality and morbidity worldwide[25, 26]. Although numerous studies have suggested the possible mechanisms of HF, they remain largely unknown and need further investigation. In recent years, high-throughput technologies with a large sample size and high reliability have provided numerous valuable information to understand the underlying pathogenesis of disorders, including HF[27].

In the present study, four datasets (GSE1869, GSE5406, GSE57338 and GSE79962) of failing and nonfailing human left ventricular tissues were collected and analyzed. All four datasets contained failing samples resulting from ICM and DCM. Using the LIMMA package of R software, the DEGs of ICM or DCM compared with those of NHF were screened out in each dataset. In total, 59 coregulated ICM-relevant and 68 coregulated DCM-relevant DEGs were identified (in at least three datasets). The overlap of the ICM- and DCM-related DEGs were considered HF-related genes, and 38 common DEGs were identified. These genes may affect the progress of HF through inflammatory/stress processes, proliferation, and some lipid metabolism pathways. Using PPI network analysis, ten hub genes/proteins were identified—ASPN, FMOD, OMD, S100A9, OGN, LUM, CD163, S100A8, HBB and ECM2.

Previous studies have confirmed the association between the inflammatory response and heart failure[28, 29]. Similar to many other homeostatic processes, the role of inflammation in HF depends on the balance of pathological inflammation and physiological inflammation[30]. Cardiac inflammation is essential for repair after myocardial infarction, while the persistence of inflammatory signals could result in maladaptive remodeling and HF[31, 32]. Cardiomyocyte injury and cardiac fibrosis are the two main manifestations of cardiac remodeling[33]. Cardiac fibrosis was reported to be an independent predictor of

overall morality, sudden cardiac death, and HF hospitalization[34]. The excessively increased extracellular matrix could impair myocardial contractility, exaggerate mechanical stiffness, and disrupt electrotonic connectivity[35].

Based on the functional status of the heart (systolic and diastolic function), HF is classified into HF with a reduced ejection fraction (HFrEF) and HF with a preserved ejection fraction (HFpEF)[36]. HFrEF is defined by the impaired LV contraction and ejection function with an EF < 40%, while HFpEF refers to impaired LV relaxation and filling function with an EF > 50% (borderline range from > 40–55%)[1, 36]. Additionally, HFrEF is the most common form. The GSE19303 dataset represents the transcription profiling of left endomyocardial biopsies containing 81 samples with corresponding LVEF. Therefore, the expression levels of the ten HF hub genes were extracted from GSE19303 with LVEF and their correlation with LVEF was determined. However, only four genes showed a statistically significant correlation with LVEF—*ASPN*, *ECM2*, *FMOD* and *OMD*. Among the four genes, *ASPN* had the most significant correlation with LVEF and was the only DEG identified in all four datasets (GSE1869, GSE5406, GSE57338 and GSE79962). Therefore, the expression of *ASPN* was validated in our TMA comprising nonfailing left ventricular samples and failing left ventricular samples caused by ICM and DCM.

TMA is a high-throughput tool for biomarker screening that has been widely applied in cancer studies[37, 38]. Compared with the slow and tedious conventional immunohistochemistry analysis, TMA is a much more cost-effective and efficient because of its high-throughput feature. Moreover, large numbers of samples could be detected and analyzed in a single run using TMA technology, reducing the potential interexperiment variabilities[14, 18]. In the present study, a TMA of 51 left ventricular samples, from 4 nonfailing patients, 21 DCM and 18 ICM patients, was constructed. Additionally, the expression of *ASPN*, a hub gene of HF, was analyzed in TMA. *ASPN* was increased in both DCM and ICM groups.

Tissue heterogeneity is a limitation involved in TMAs that cannot be ignored[39]. In the TMA analysis, the regional cores were assumed to represent the entire heart. However, this assumption may not always be true, especially for ICM. The underlying cause of ICM is severe stenosis of coronary arteries, leading to ischemia or even infarction of the heart. Therefore, the nonischemic regions may exhibit completely different results from those of the ischemic regions. In our TMA study, the expression levels of *ASPN* in some cores of ICM and DCM were relatively lower than those in other samples of ICM and DCM, likely explaining the result of region selection.

Asporin (*ASPN*) is a member of the small leucine-rich proteoglycan family that is expressed in multiple tissues, including osteoarthritic articular cartilage, the aorta, the uterus, the heart, and the liver[40, 41]. Previous studies have suggested the role of *ASPN* in the regulation of the extracellular matrix and collagen mineralization in the bone system[42]. However, the upregulation of *ASPN* in ICM patients and animal models was induced by transverse aortic constriction or coronary artery ligation[27, 43], and few studies have revealed the role of *ASPN* in heart failure, except one study in which *ASPN* increased H9C2 cardiomyocyte apoptosis and fibrosis induced by glycated low-density lipoproteins in vitro[44]. The

specific mechanism of ASPN involved in HF progression needs further exploration. The utilization of gene knockout or transgenic animals may be of great help to better understand the role of ASPN in HF.

Conclusion

In the present study, 38 HF highly relevant genes were identified by analyzing four gene expression datasets. These genes mainly involved the inflammatory process and regulating lipid metabolism involved in the process of HF. Among the hub genes with top degrees, ASPN exhibited the most significant correlation with LVEF. Moreover, using TMA, the expression of ASPN protein was validated, being increased in ICM and DCM left ventricular tissues. Transgenic or gene knockout animals may help better understand the role of ASPN in HF. ASPN may be a potential promising target to prevent HF of various causes.

Abbreviations

HF

heart failure; DEGs: differentially expressed genes; ICM: ischemic cardiomyopathy; DCM: dilated cardiomyopathy; GO: gene ontology; MF: molecular function; CC: cellular component; BP: biological process; KEGG: Kyoto Encyclopedia of Genes and Genomes; LVEF: left ventricular ejection fraction; TMA: tissue microarray; GEO: Gene Expression Omnibus; HFrEF: heart failure with a reduced ejection fraction; HFpEF: heart failure with a preserved ejection fraction.

Declarations

Acknowledgments

None.

Authors' contributions

Concept and design: KZ, MW, XYQ, XWZ, YHW, and JZ. Data analysis: KZ, JRZ, PJW, SXC. Article drafting: KZ. Critical revision of article: YHW and JZ.

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Availability of data and materials

The gene expression profiles of GSE1869, GSE5406, GSE57338, GSE79962 and GSE19303 with their respective matched probe annotation platform profiles were downloaded from the GEO

(<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE1869>, GSE5406, GSE57338, GSE79962 or GSE19303).

Ethics approval and consent to participate

The collection and research use of the heart tissues were approved by the Ethics Committee of Guangdong General Hospital. Informed consent was obtained from all the participants.

Consent for publication

Not Applicable.

Competing interests

None.

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Figures

Figure 1

Identification of ICM-related DEGs. (A-D) Heatmap of the DEGs of ICM compared with control identified in GSE1869, GSE5406, GSE57338, and GSE79962. The red boxes represent upregulated genes, and the green boxes represent downregulated genes. (E-F) Venn diagram showing the coregulated DEGs, co-upregulated DEGs, and co-downregulated DEGs. ICM: ischemic cardiomyopathy; DEGs: differentially expressed genes.

Figure 2

Identification of DCM-related DEGs. (A-D) Heatmap of the DEGs of DCM compared with control identified in GSE1869, GSE5406, GSE57338, and GSE79962. The red boxes represent upregulated genes, and the green boxes represent downregulated genes. (E-F) Venn diagram of the coregulated DEGs, co-upregulated DEGs, and co-downregulated DEGs. DCM: dilated cardiomyopathy; DEGs: differentially expressed genes.

Figure 3

Identification of co-DEGs between ICM and DCM and their functional enrichment. (A-C) Venn diagram showing the coregulated DEGs, co-upregulated DEGs, and co-downregulated DEGs between ICM and DCM. (D-G) Bubble diagram showing GO-MF, GO-CC, GO-BP, and KEGG pathway enrichment analysis of the co-DEGs between ICM and DCM. The dot sizes represent counts of enriched DEGs, and the dot colors represent negative Log10(p values). DEGs: differentially expressed genes; ICM: ischemic cardiomyopathy;

Figure 5

Linear correlation between HF-relevant DEGs and LVEF, including ASPN (A), CD163 (B), ECM2 (C), FMOD (D), HBB (E), LUM (F), OGN (G), OMD (H), S100A8 (I), and S100A9 (J). HF: heart failure; DEGs: differentially expressed genes; LVEF: left ventricular ejection fraction.

Figure 6

Immunohistochemistry validation of ASPN in TMA. (A) Masson staining of the TMA. (B) Immunohistochemistry of the protein expression of ASPN. The TMA comprised 12 nonfailing left ventricular samples, 21 DCM left ventricular samples, and 18 ICM left ventricular samples. TMA: tissue microarray.

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