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Atrial remodeling and metabolic dysfunction in idiopathic isolated fibrotic atrial cardiomyopathy

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Abstract

Background—Idiopathic isolated fibrotic atrial cardiomyopathy (IIF-ACM) is a novel subtype of cardiomyopathy characterized by atrial fibrosis that does not involve the ventricular myocardium and is associated with significant atrial tachyarrhythmia. The mechanisms underlying its pathogenesis are unknown.

Methods—Atrium samples were obtained from 3 patients with IIF-ACM via surgical intervention. Control samples were consisted of 3 atrium biopsies from patients with congenital heart disease and normal sinus rhythm, matched for gender, age and basic clinical characteristics. Comparative histology, immunofluorescence staining, electron microscopy and proteomics analyses were carried out to explore the unique pathogenesis of IIF-ACM.

Results—IIF-ACM atria displayed disordered myofibrils, profound fibrosis and mitochondrial damages compared to the control atria. Proteomics profiling identified metabolic pathways as the most profound changes in IIF-ACM.

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Competing interests

The authors have declared that no competing interests exist.

Ethical approval

The study was approved by the Bioethics Committee of the First Affiliated Hospital of Nanjing Medical University (2014-SR-090). All patients provided written informed consent, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Disclosures

None.

Conclusions—Our study suggested that metabolic changes in the atrial myocardium caused mitochondrial oxidative stress and potential cell damage, which further led to atrial fibrosis and myofibril disorganization, the characteristic phenotype of IIF-ACM.

Keywords

IIF-ACM; Histology; Proteomics; Metabolism; Fibrosis; Oxidative stress

1. Introduction

Atrial myocardium is always affected by multiple cardiac and non-cardiac conditions, such as hypertension [1], obesity [2] and diabetes mellitus [3]. The resulting atrial pathologies, which have a substantial impact on cardiac function, the occurrence of arrhythmias, and stroke risk [4], have been categorized as a new class of cardiomyopathy called atrial cardiomyopathy (ACM) [5]. Isolated atrial lesions can also occur due to age-related amyloid degeneration [6] or mutations in genes such as NPPA [7] and MYL4 [8].

Lately, we reported a group of young patients with unexplained scar-related atrial tachycardia (AT) [9]. Multiple imaging modalities, ventricular voltage mapping, and intracardiac pressure recordings performed during a median 4 years of follow-up ruled out ventricular involvement. In addition, genetic and immunological investigations excluded the involvement of genetic variation or systemic diseases. As the mechanisms of this disease remain unknown, we proposed to name this type of cardiomyopathy as Idiopathic Isolated Fibrotic Atrial Cardiomyopathy (IIF-ACM).

Proteomics is a powerful technology that provides an objective, large-scale analysis of proteins under normal or pathological conditions. It has been applied to the study of complex changes in cardiovascular diseases, such as atrial fibrillation (AF) [10], hypertrophic cardiomyopathy [11] and ischemic cardiomyopathy [3], owing to its ability to examine proteins simultaneously with high throughput [12]. During the last eight years, 3 patients with multiple AT recurrences underwent resections of atrial lesion via minimally invasive surgical interventions in our center to terminate the arrhythmias. These precious atrium samples provided excellent materials for pathological and proteomic studies.

In the present study, we exploited these three atrium specimens from IIF-ACM patients to compare histological, ultrastructural and proteomic characteristics with samples from normal sinus rhythm (NSR) controls. Our results not only provided solid pathological evidence of IIF-ACM, but also revealed proteomic evidence of relationships between atrial remodeling and metabolic dysfunction, shedding light on the inherent mechanism and potential therapeutic strategies for this novel cardiomyopathy.

2. Methods

2.1. Clinical characteristics

A total of three patients (2 males and 1 female) with IIF-ACM underwent resection of atrial lesions to terminate their multiple AT recurrences. The diseased areas of the right atrium were resected via minimally invasive surgical interventions as described previously by our

group [9], which was 3 cm * 1 cm as usual. Controls were three right atrium tissues from patients with congenital heart disease, matched for age and gender. The size of atrial biopsy was routinely 7–8 mm * 4–5 mm. Study procedures were approved by the local Ethics Committee. Before enrollment, patients were screened via questionnaires and pre-operative 12-lead electrocardiography to ensure that they had never experienced AF. Routine 2-D transthoracic echocardiography was performed for all patients. Samples collected during the surgical procedures were snap-frozen in liquid nitrogen and followed by protein analysis and staining.

2.2. Electrophysiological studies

The electro-anatomic mapping was performed as described previously [9]. Briefly, areas of electrical silence or dense scar were defined as those with bipolar voltage 0.1 mV for the atria [13] and 0.5 mV for the ventricles [14], based on previous studies.

2.3. Myocardial biopsies and histology

For the histological analyses, the atrium samples were fixed in 10% buffered formalin and embedded in paraffin. Five micron thick sections were stained with Hematoxylin & Eosin, Masson trichrome and Congo red. Whole fields of samples were scanned using Panoramic MIDI (3D HISTECH). Pictures were taken by CaseViewer software.

2.4. Immunofluorescence staining

Paraffin sections were deparaffined, thoroughly dehydrated and incubated with goat serum for 60 min. These sections were then incubated with the following primary antibodies overnight at 4 °C: mouse anti-cardiac troponin T (1:200, Abcam, ab8295), rabbit anti-Cx43 (1:200, Abcam, ab11370), mouse anti-N-Cadherin (1:200, Abcam, ab98952) and rabbit anti-ANP (1:200, Abcam, ab76743). The secondary antibodies were goat anti-mouse IgG (1:500, Abcam, ab150113) and donkey anti-rabbit IgG (1:500, Abcam, ab150074). Nuclei were visualized with DAPI (Life Technologies, P36931). Images were captured using a Zeiss fluorescence microscope (Axio Imager A2, Zeiss). The immunofluorescence was quantified with ImageJ software (National Institutes of Health, 1.8.0_77) as previously described [15].

2.5. Transmission electron microscopy (TEM)

TEM was carried out as described previously by our group [16]. Briefly, atrium tissues were fixed with 4% glutaraldehyde overnight at 4 °C. Samples were then rinsed with PBS and stained in 2% uranyl acetate. Images were captured using a transmission electron microscope (JEM-1010, Jeol Ltd., Tokyo, Japan).

2.6. Proteomic analyses

Isobaric tags for relative and absolute quantitation (iTRAQ) labeling and quantitative proteomics by mass spectrometry were performed as previously described [17]. Briefly, the tissue samples were preconditioned with Collagenase I and Protease [18]. The extracted atrial proteins were then digested with trypsin. Equal volumes of tryptic peptides were labeled with iTRAQ tags according to the manufacturer's instructions (AB Sciex, Foster City, USA). The samples were then fractionated by off-line strong cation exchange (SCX)

using a Thermo Biobasic SCX column. Each collected fraction was analyzed by liquid chromatography-mass spectrometry (LC-MS, QStar Elite, AB Sciex). The data were analyzed using Protein Pilot software 4.0 (AB SCIEX, Foster City, USA). In the present study, ratios with p -values <0.05 , and fold-changes >1.2 were considered as significant. To further explore the significance of differentially expressed proteins, Ingenuity Pathway Analysis (www.ingenuity.com) was used to search for the relevant molecular functions, cellular processes and pathways of these identified proteins during the pathological changes in the study group. The top canonical pathways identified in this analysis were presented, along with p -values calculated using right-tailed Fisher's exact tests.

2.7. Statistical analyses

All analyses were performed using SPSS Statistics 19.0 (IBM). Unless stated otherwise, all data were expressed as the means $\pm$ SD, as estimated by Student's t -test. A p -value of <0.05 was considered significant for all statistical tests.

3. Results

3.1. Patient population

Clinical and echocardiographic results are presented in Supplement Table. 1. There were no differences as to age, gender, ejection fraction or atrial size between the two groups of patients. In particular, the control patients were in NSR and did not exhibit AF during the recruitment process. The samples were derived from a non-scar-related chamber and were therefore considered to be as close as possible to normal atrium tissues.

3.2. Histological analyses revealed extensive fibrosis in the atria of IIF-ACM patients

Among the IIF-ACM patients, three-dimensional voltage maps revealed large areas of electrical silence in the right atrial free wall (Fig. 1A), whereas other chambers were quite healthy (Supplement Fig. 1). Through thoracotomy, large fibrotic areas were observed, with islands of viable cardiac muscles among the fibrotic tissues (Fig. 1B). Under Hematoxylin-Eosin staining (Fig. 1C), irregularly arranged myocardium and collagen in pale pink was observed in IIF-ACM patients, compared with the NSR controls. Masson's trichrome staining (Fig. 1D, E) of the atrial myocardium samples at the corresponding site showed profound and extensive fibrosis in the IIF-ACM group. In addition, histological appearance under Congo red staining did not differ between the two groups, ruling out the possibility of cardiac amyloidosis in IIF-ACM patients (Fig. 1F). The figures of other two patients and two controls were shown in Supplement Fig. 2.

3.3. Immunofluorescence staining revealed atrial remodeling in IIF-ACM patients

Subsequently, the sample sections were immunostained for a panel of cardiac markers. Cardiac troponin T (cTnT) staining revealed low-density and disordered myofibrils in the IIF-ACM group although both groups showed robustly expressed connexin 43 (Cx43) (Fig. 2A, B). It has been reported that loss of cardiac-specific N-Cadherin leads to slow conduction and arrhythmogenesis [19]. We also found that, compared with NSR controls (Fig. 2C), IIF-ACM showed a dramatically decreased expression of N-Cadherin (844.60 ± 280.58 vs 148.81 ± 57.10 , mean $\pm$ SD, $n = 6$ per group, $***p < 0.001$ estimated by

Student's *t*-test, Fig. 2D). There was no difference in the expression levels of ANP. Taken together, the immunostaining results demonstrated the atrial remodeling of IIF-ACM. The pictures of other two patients and two controls were shown in Supplement Fig. 3.

3.4. Proteomics profiling identified differentially expressed proteins

To provide insight into the pathogenesis of the disease, we used proteomics profiling to compare the protein levels between IIF-ACM atria and the controls. iTRAQ labeling and LC-MS/MS identified a total of 4225 unique proteins. 2D principal component analysis (2D PCA) of all proteins showed an obvious distinction between the IIF-ACM and the control groups (Fig. 3A), indicating a relatively low intragroup variation and significant differences between the groups. 539 proteins (284 decreased, 255 increased) showed differential expression between the IIF-ACM and control groups (Fig. 3B). Ontology analysis revealed significant enrichment for proteins involved in collagen metabolism, action potentials, and skeletal morphogenesis among the differentially expressed proteins (Fig. 3C). These findings were consistent with the observed morphological changes.

3.5. Altered expressions of enzymes in lipid and carbohydrate metabolism in IIF-ACM atria

Identified proteins were further functionally classified using Ingenuity Pathway Analysis to define the main biological processes varied in IIF-ACM and NSR control patients (Supplement Fig. 4). Interestingly, energy metabolism was among the top enriched pathways altered in IIF-ACM atria versus the controls. These metabolic processes included citrate cycle (TCA cycle), 2-oxocarboxylic acid metabolism, glycerophospholipid metabolism, carbohydrates metabolism, phenylalanine metabolism, tyrosine metabolism, and nitrogen metabolism (Fig. 3D).

Myocardium uses both lipids and carbohydrates and maintains a delicate balance between different energy sources under different physiological conditions. Lipids are the predominant substrate under normal physiological conditions [20]. We found that many enzymes involved in lipid metabolism and mitochondrial oxidative metabolism (Fig. 3E–F) were significantly upregulated in IIF-ACM atria compared to the NSR controls. The upregulated proteins were involved in the transportation, synthesis, or catabolism of lipids (Supplement Figs. 5–6). Surprisingly, many proteins involved in carbohydrates metabolism were also upregulated in the IIF-ACM (Fig. 3G). These alterations of key metabolic enzymes suggested a hypermetabolic state in IIF-ACM with increased metabolic fluxes in both lipids and carbohydrates catabolism, which could lead to metabolic inflexibility, traffic jams within the mitochondria, and potentially increase oxidative stress [10].

3.6. Disrupted redox homeostasis and mitochondrial morphology in IIF-ACM atria

Oxidative stress elicits strong adaptive cellular responses [21]. We therefore examined oxidative stress-related proteins in our LC-MS/MS dataset. We found that 18 oxidative stress-related proteins were differentially expressed in the IIF-ACM atria compared to the controls (Fig. 4A). Cytochrome c and peroxiredoxin-5, proteins promoting oxidative stress [22,23], were drastically elevated, accompanied by compensatory changes in superoxide dismutase [24] in the IIF-ACM groups compared to the NSR controls. Antioxidant 1, which

inhibits oxidative stress, was also found to be significantly depleted in IIF-ACM patients [25].

Mitochondria are a major source of oxidative stress in the contracting myocardium. We therefore used TEM to assess mitochondrial morphology as well as the ultrastructure of the myofibril organizations. Consistent with the findings of our immunofluorescence analyses, TEM revealed disorganized myofibrils, increased intracellular empty vacuoles and profound fibrosis in IIF-ACM atrium samples compared to the NSR controls (Fig. 4B, Scale Bar 2 μ m). Compared to normal mitochondrial morphology in the control group, the mitochondria in the IIF-ACM group exhibited remarkable cristolysis, suggesting the potential metabolic disorders and activation of oxidative stress [26] (Fig. 4C, Arrows: typical mitochondria, Scale Bar 500 nm).

4. Discussion

Recently, an expert consensus has emerged identifying and depicting ACM that contributes to atrial arrhythmias including AF, AT or sick sinus syndrome [5]. In the present consensus, most of the listed ACM phenotypes are secondary to the ventricular dysfunctions. We recently reported a series of young patients with unexplained scar-related AT. Based on clear confirmation of atrial fibrosis and strong evidence that excluded the possibility of co-existing ventricular involvement, we defined IIF-ACM as a novel type of cardiomyopathy among these patients. To date, there were no histopathologic or molecular evidence available to elucidate the etiology of IIF-ACM. Here, we reported the pathological and proteomic characteristics of this newly defined entity, with the aim to dissect the potential mechanisms. Our study suggested that metabolic changes in the atrial myocardium cause mitochondrial oxidative stress and potential cell damage, which further led to atrial fibrosis and myofibril disorganization, the characteristic phenotype of IIF-ACM.

In his review articles [27], Dr. Kottkamp has argued that many cases of AF may be attributable to the underlying fibrotic ACM. Atrial fibrosis and AF are closely related. AF itself can cause atrial fibrosis. Even though some cases of AF may belong to the ACM subtype, we excluded patients with a previous AF history. The intracardiac mapping revealed electric silence areas at right atrial free wall in the study group, which may represent an important substrate for the AT sustaining. Indeed, AT was terminated following the resection of the diseased areas of the right atrium (Fig. 1A–B). For the controls, right atrial specimens were obtained via right atrial incision in patients with congenital heart disease undergoing cardiac surgery.

To verify the pathological characteristics of IIF-ACM, multiple histological profiling has been performed. Herein, Hematoxylineosin and Masson staining (Fig. 1C–E) of the atrial myocardium showed profound and extensive fibrosis, while Congo red staining was negative for amyloidosis. Under immunofluorescence staining (Fig. 2), myocardial tissues from IIF-ACM showed a significantly lower expression of N-Cadherin, and disordered myofibrils with cTnT staining. Consistently, TEM investigation exhibited disordered myofibrils, increased intracellular empty vacuoles and profound fibrosis in IIF-ACM (Fig. 4B). In the proteomics analysis, the most significant enrichment for differentially expressed proteins

were involved in collagen metabolism, action potentials and skeletal morphogenesis (Fig. 3C). According to the EHRA/HRS/APHS/SOLAECE expert consensus [5], the newly defined IIF-ACM subtype would be classified as a type III atrial cardiomyopathy, which combines cardiomyocyte pathology and fibrosis. Our findings provided solid pathological evidence for IIF-ACM, which was an important addition to the broad range of atrial cardiomyopathy.

The application of proteomics technology allows novel insights into the pathophysiology of various cardiovascular diseases [10]. The analysis has uncovered plenty of differentially expressed proteins in the present study. Notably, IIF-ACM is associated with changes in energy metabolism processes. The modification of cardiac energy metabolism has been reported to be closely associated with atrial remodeling in AF [28,29]. In this study, many enzymes involved in fatty acids oxidation and carbohydrates metabolism were simultaneously upregulated (Fig. 3E–G), suggesting the increased substrate competition and metabolic inflexibility in the IIF-ACM atria.

The investigation of basic clinical characteristics has ruled out the possibility of metabolic syndromes in our IIF-ACM patients (Table. S1). Given that the energy of myocardial function is obtained primarily from fatty acid through beta-oxidation, those upregulated proteins indicated a hypermetabolic state in IIF-ACM. In line with our findings, Ausma J and colleagues have shown that an increase in energy demand during the early stage of AF was associated with atrial remodeling, including cellular hypertrophy, myolysis and mitochondrial changes [30]. Taken together, these findings suggest that alterations of cardiac energy metabolism may constitute a potential mechanism underlying the disease pathogenesis.

Moreover, a total of 20 differentially expressed proteins related to carbon metabolism were upregulated in the IIF-ACM group (Fig. 3G), which may be compensatory adaptive response to the high energy demand [31]. In fact, those upregulated proteins were closely related to each other, such as pyruvate dehydrogenase, which is responsible for converting pyruvate into acetyl-CoA, representing the irreversible step from glycolysis to the TCA cycle and consequently promoting glucose oxidation. Enhanced glucose utilization could be an adaptive response in cardiac hypertrophy or heart failure [20]. But it could cause oxidative injury in human AF [32]. In our proteomics study, ontology analysis revealed a significant enrichment of oxidative stress-related proteins (Fig. 4A). Interestingly, the ultrastructural scan via TEM exhibited the impaired morphology of mitochondria (Fig. 4C). Mitochondrial dysfunction in heart failure is associated with increased oxidative stress [33]. Collectively, these results suggested that myocardial energetic alterations in IIF-ACM could contribute to oxidative stress and atrial remodeling.

5. Study limitations

The main limitation of this study was the small number of patients. This was partly due to the finite number of IIF-ACM patients who underwent atrial lesion resections to terminate AT recurrences, as most patients received catheter ablations. The limited sample size means that some key proteins that were differentially expressed between the IIF-ACM and NSR

control patients may not have been identified. With the understanding of IIF-ACM as a new concept, further studies are required to confirm these results. Also, more animal and cell experiments are warranted to confirm that metabolic dysfunction and downstream oxidative stress lead to the cardiac injuries in IIF-ACM.

6. Conclusions

To our knowledge, this is the first histological and proteomic study using atrium samples from patients with IIF-ACM. Our results highlighted myofibril impairments and profound fibrosis as key pathological changes in IIF-ACM. iTRAQ proteomics profiling identified marked differences of the protein expressions in the atrium samples from IIF-ACM patients, many of which were relevant to energy metabolism and oxidative stress response. Metabolic dysfunction may be pertinent to the pathogenesis of atrial remodeling. The inherent mechanisms and therapeutic targets warrant further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IIF-ACM	idiopathic isolated fibrotic atrial cardiomyopathy
AF	atrial fibrillation
AT	atrial tachycardia
TEM	transmission electron microscopy
iTRAQ	isobaric tags for relative and absolute quantitation
SCX	strong cation exchange
NSR	normal sinus rhythm
PCA	principal component analysis
KEGG	Kyoto Encyclopedia of Genes and Genomics
RAO	right anterior oblique

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijcard.2018.04.080>.

Fig. 1.

Histological analysis revealed extensive fibrosis in the atria from IIF-ACM patients. (A) Three-dimensional voltage maps in a right anterior oblique (RAO) view revealed large areas of electrical silence in the right atrial free wall. (B) Through a limited right thoracotomy, large fibrotic areas were observed, with islands of viable cardiac muscles amidst the fibrotic tissue. (C) Hematoxylineosin and (D-E) Masson trichrome staining of the atrial myocardium samples at the corresponding sites showed profound and extensive fibrosis. (F) Congo red staining demonstrated no differences between the two groups.

Fig. 2.

Immunofluorescence staining for cardiac markers revealed atrial remodeling in IIF-ACM group. (A–B) Low-density and disordered myofibrils were observed in the IIF-ACM group. Both groups showed robust expression of Cx43 (200 $\times$ Scale bar 200 μ m, 400 $\times$ Scale Bar 100 μ m). (C) IIF-ACM showed a dramatically decreased expression of N-Cadherin compared with control, as shown in the graph (844.60 ± 280.58 vs 148.81 ± 57.10 , mean $\pm$ SD, $n = 6$ per group, $***p < 0.005$ estimated by Student's t -test, D) whereas the expression levels of ANP showed no differences (200 $\times$ Scale Bar 200 μ m). cTnT: cardiac troponin t, Cx43: connexin 43, N-Cad: n-cadherin, ANP: atrial natriuretic peptide.

Fig. 3.

Proteomics profiling identified altered protein expression in IIF-ACM. (A) 2D principal component analysis of all proteins distinguishes IIF-ACM from controls (C1–C3 and ACM1–ACM3 represented three biological replicates from control and IIF-ACM patients, respectively). (B) Heat map depicted the differentially expressed proteins between IIF-ACM and controls. (C) Ontology analysis revealed that the most significantly enriched proteins were involved in collagen metabolism, action potentials and skeletal morphogenesis. (D) The bioinformatic analysis illustrated notable changes in the TCA cycle, oxidative phosphorylation, and protein metabolite processes. (E) List of differentially expressed proteins involved in fatty acid degradation. (F) List of differentially expressed proteins involved in the TCA cycle. (G) List of differentially expressed proteins involved in carbon metabolism.

Fig. 4.

Redox homeostasis and mitochondrial morphology were disrupted in IIF-ACM atria. (A) The expression levels of oxidative stress-related proteins differed between the two groups. (B) TEM studies revealed disordered myofibrils, increased intracellular empty vacuoles and profound fibrosis in IIF-ACM atrium samples (Scale Bar 2 μ m). (C) Mitochondria in control displayed typical normal morphology. While, the mitochondria from IIF-ACM group exhibited remarkable cristolysis (Arrows: typical mitochondria, Scale Bar 500 nm).