International Journal of Cardiology 184 (2015) 190–197

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Identification of microRNA–mRNA dysregulations in paroxysmal atrial fibrillation

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ARTICLE INFO

Article history:
Received 9 October 2014
Received in revised form 8 December 2014
Accepted 26 January 2015
Available online 28 January 2015

Keywords:
microRNA
RNA sequencing
Transcriptomic study
Paroxysmal atrial fibrillation

ABSTRACT

Background: The molecular mechanisms underlying the early development of atrial fibrillation (AF) remain poorly understood. Emerging evidence suggests that abnormal epigenetic modulation via microRNAs (miRNAs) might be involved in the pathogenesis of paroxysmal AF (pAF).
Objective: To identify key molecular changes associated with pAF, we conducted state-of-the-art transcriptomic studies to identify the abnormal miRNA–mRNA interactions potentially driving AF development.
Methods: High-quality total RNA including miRNA was isolated from atrial biopsies of age-matched and sex-matched pAF patients and control patients in sinus rhythm (SR; n = 4 per group) and used for RNA-sequencing and miRNA microarray. Results were analyzed bioinformatically and validated using quantitative real-time (qRT)-PCR and 3′UTR luciferase reporter assays.
Results: 113 genes and 49 miRNAs were differentially expressed (DE) in pAF versus SR patients. Gene ontology analysis revealed that most of the DE genes were involved in the "gonadotropin releasing hormone receptor pathway" and "p53 pathway". Of these DE genes, bioinformatic analyses identified 23 pairs of putative miRNA–mRNA interactions that were altered in pAF (involving 15 miRNAs and 17 mRNAs). Using qRT-PCR and 3′UTR luciferase reporter assays, the interaction between upregulation of miR-199a-5p and downregulation of FKBP5 was confirmed in samples from pAF patients.
Conclusion: Our combined transcriptomic analysis and miRNA microarray study of atrial samples from pAF patients revealed novel pathways and miRNA–mRNA regulations that may be relevant in the development of pAF. Future studies are required to investigate the potential involvement of the gonadotropin releasing hormone receptor and p53 pathways in AF pathogenesis.

1. Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia seen in clinics [1]. The molecular mechanisms underlying AF pathogenesis are complex and not well understood [2]. Because of this, a number of studies in the last decade have utilized microarray technologies to look for transcriptomic changes in both AF patients and animal models of AF [3–9]. Through these studies, a number of key pathways were found to be altered in AF patients such as Ca^{2+}-dependent signaling pathways [3], inflammatory and immune pathways [5], and apoptotic and cell cycle pathways [7]. These prior studies all focused on patients with persistent or permanent AF, otherwise known as chronic AF (cAF), where extensive AF-induced atrial remodeling may have confounded the findings and thereby precluded a clear distinction between AF causes and consequences. By contrast, much less is known about the early changes that may occur in the atria of patients with paroxysmal AF (pAF), which is an earlier stage of AF development where atrial remodeling is still mostly limited [10].

MicroRNAs (miRNAs) are a class of small non-coding RNAs (ncRNAs) (containing 21–24 nucleotides) that function in RNA silencing and post-transcriptional regulation of gene expression [11]. Since miRNAs may

Abbreviations: AF, Atrial fibrillation; cAF, Chronic atrial fibrillation; DE, Differentially expressed; miRNA, microRNA; pAF, Paroxysmal atrial fibrillation; qRT-PCR, Quantitative real-time polymerase chain reaction; RNA-Seq, RNA sequencing; SR, Sinus rhythm.

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http://dx.doi.org/10.1016/j.ijcard.2015.01.075
0167-5273/

potentially drive mRNA changes observed in AF patients, some studies performed microarray experiments using samples from AF patients [12,13] while others have validated key roles for miRNAs such as miR-1, miR-328, and miR-26 in AF pathogenesis [14–16]. However, since these studies were all performed in patients with cAF, they were limited in their contribution to our understanding of the factors involved in early AF development.

With this in mind, and in light of the recent advance in RNA-sequencing (RNA-Seq) technology, we were motivated to conduct an unbiased transcriptomic study using atrial samples from pAF patients. The objective of this study is to identify pathways and miRNA–mRNA interactions that may play a role in early AF pathogenesis. Using both RNA-Seq and miRNA microarray, we uncovered 113 genes and 49 miRNAs that are differentially expressed in patients with pAF compared to those in sinus rhythm (SR). Additional bioinformatic analyses identified 23 pairs of putative miRNA–mRNA interactions (involving 15 miRNAs and 17 mRNAs) that are dysregulated in pAF patients. Several candidates were validated using quantitative real-time (qRT)-PCR, and one miRNA–mRNA pair (miR-199a-5p/FKBP5) was further validated using 3′UTR luciferase assay. Together, our study revealed novel signaling pathways and miRNA–mRNA interactions that are associated with the early development of AF.

2. Materials and methods

Detailed information is provided in the Online Supplement.

2.1. Human atrial samples

After written informed consent was obtained, the tip of the right atrial appendage was removed as part of the general surgical procedure in patients undergoing open-heart surgery for bypass grafting, valve replacement or a combination of both. All biopsies were taken at the same site, which is at the tip of the right atrial appendage. After collection, the samples were flash frozen in liquid nitrogen, under protocols approved by the ethics committee of the Faculty of Medicine, University Duisburg-Essen (12-5268-BO). Characteristics of the patients are listed in Tables 1 and 2.

2.2. RNA sequencing and miRNA microarray

High quality total RNA including miRNAs was isolated from frozen human atrial samples using the miRNeasy Mini Kit from Qiagen (Venlo, The Netherlands). A portion of the RNA was converted to barcoded cDNA libraries for RNA-Seq using Ion Total RNA-Seq Kit v2.0 and sequenced on the Ion Proton™ System (Life Technologies, CA). Data analysis and visualization were accomplished using SNP & Variation Suite (Version 8; Golden Helix, Bozeman, MT) while gene ontology (GO) analysis was performed using PANTHER 9.0. [17] Another portion of the RNA was used for miRNA microarray, performed by LC Sciences (Houston, TX) on their in-house μParaflo® technology platform that included all human mature miRNAs available in the latest version of the miRBase database (Release 20).

Table 1
Characteristics of patients for the transcriptomic studies.

	SR (RNA-Seq)[a]	SR (miR microarray)	pAF
Patients, n	4	4	4
Gender, m/f	2/2	3/1	3/1
Age, y	65.0 ± 4.1	70.5 ± 1.9	71.3 ± 2.9
Body mass index, kg/m^2	27.4 ± 1.5	26.9 ± 1.5	25.2 ± 1.9
CAD, n	3	3	3
MVD/AVD, n	0	0	0
CAD + MVD/AVD, n	1	1	1
Hypertension, n	4	4	4
Diabetes, n	2	1	1
Hyperlipidemia, n	4	4	3
LVEF, %	49.7 ± 12.9	51.5 ± 9.8	51.3 ± 8.1
Digitalis, n	1	1	1
ACE inhibitors, n	3	2	4
AT1 blockers, n	0	0	0
β-Blockers, n	4	3	3
Dihydropyridines, n	1	1	3
Diuretics, n	2	2	1
Nitrates, n	0	0	1
Lipid-lowering drugs, n	4	3	3

[a] With the exception of one SR sample, all the other SR and pAF samples were used for both the RNA-Seq and miR microarray experiments. Values are presented as mean ± SEM or number of patients. SR, patients in sinus rhythm; pAF, paroxysmal atrial fibrillation patients; CAD, coronary artery disease, MVD/AVD, mitral/aortic valve disease; LVEF, left ventricular ejection fraction; ACE, angiotensin-converting enzyme; and AT, angiotensin receptor. No statistical differences were found between SR (RNA-Seq) vs. pAF or SR (miR microarray) vs. pAF using two-tailed Student's *t*-test for continuous variables and Fisher's exact test for categorical variables (all P > 0.1).

Table 2
Characteristics of patients for the qRT-PCR experiments.

	SR[a]	pAF
Patients, n	8	10
Gender, m/f	6/2	5/5
Age, y	64.5 ± 3.0	67.5 ± 3.8
Body mass index, kg/m^2	27.2 ± 1.0	30.5 ± 2.9
CAD, n	6	6
MVD/AVD, n	1	2
CAD + MVD/AVD, n	1	1
Hypertension, n	7	10
Diabetes, n	3	3
Hyperlipidemia, n	6	5
LVEF, %	55.0 ± 6.0	44.6 ± 6.2
Digitalis, n	1	2
ACE inhibitors, n	6	6
AT1 blockers, n	0	1
β-Blockers, n	6	6
Dihydropyridines, n	1	5
Diuretics, n	3	4
Nitrates, n	0	2
Lipid-lowering drugs, n	5	6

[a] The qRT-PCR experiments included additional samples not used for the RNA-Seq and miR microarray experiments. Values are presented as mean ± SEM or number of patients. SR, patients in sinus rhythm; pAF, paroxysmal atrial fibrillation patients; CAD, coronary artery disease, MVD/AVD, mitral/aortic valve disease; LVEF, left ventricular ejection fraction; ACE, angiotensin-converting enzyme; and AT, angiotensin receptor. No statistical differences were found between SR and pAF groups using two-tailed Student's *t*-test for continuous variables and Fisher's exact test for categorical variables (all P > 0.1).

2.3. Quantitative real-time PCR

To measure levels of mRNAs, reverse transcription was performed using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). To measure levels of miRNAs, reverse transcription and quantitative real-time (qRT)-PCR were carried out using a modified protocol as described [18–20]. qRT-PCR reactions were performed in triplicate in 96-well plates using PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences, Gaithersburg, MD) in Mastercycler ep realplex (Eppendorf, Hamburg, Germany). Relative expression levels were calculated using the CT (cycle number) method after normalization to *RPL7*. All primers used for mRNA and miRNA detection are listed in Supplemental Tables S1 and S2, respectively [21].

2.4. Mutagenesis and luciferase assay

The *Homo sapiens* FKBP5 3′UTR luciferase construct was purchased from SwitchGear Genomics (S811120, Active Motif). The putative interaction site between hsa-miR-199a-5p and the FKBP5 3′UTR was searched using both TargetScanHuman 6.2 [22] and microRNA.org [23]. Mutagenesis was carried out using QuikChangeII (Agilent Technologies, Santa Clara, CA) to disrupt this putative interaction using forward primer 5′-AATAAAAACTAATTTAAAATATAACTGCGAATTCTTTTTAGGATTTAAAAAGGTGAGATGTTCCAGGTTTAAGCAAATGG-3′ and reverse primer 5′-CCATTTGCTTAAACCTGGAACATCTCACCTTTTTAAATCCTAAAAAGAATTCGCAGTTATATTTTAAATTAGTTTTTATT-3′, according to the manufacturer's instructions.

3′UTR luciferase reporter assays were performed as described previously, with modifications [20]. Hsa-miR-199a-5p and a non-targeting scramble miRNA mimic were purchased from Life Technologies (Carlsbad, CA) and used at a final concentration of 10 nM. The luciferase constructs and miRNA mimics were co-transfected in pairs into HEK293 cells according to the protocol provided by SwitchGear Genomics (Active Motif) using LipoD293 (SignaGen Laboratories, Rockville, MD). Cells were harvested after 24 h and assayed using the LightSwitch Luciferase Assay Reagents (Active Motif) according to the manufacturer's instructions. Luciferase signals were normalized to the protein concentration of the respective lysates and to the mimics' effect on the empty 3′UTR vector (S890005, Active Motif).

2.5. Statistical analysis

Two-tailed student's *t*-test and Fisher's exact test were used where appropriate. Data are presented as mean ± SEM and a P-value less than 0.05 was considered statistically significant, except for the miRNA microarray data analyzed by LC Sciences (Houston, TX) using a validated method.