Int J Clin Exp Pathol 2017;10(4):4221-4231
www.ijcep.com /ISSN:1936-2625/IJCEP0046822

Original Article

MiR-499 is a diagnostic biomarker of paroxysmal atrial fibrillation involved in the development of atrial fibrillation

Runwei Ma[1], Jiyang Wang[1], Xingjian Wu[2], Zongning Chen[3], Xiaoyu Zhang[1], Xu Yang[1], Yi Song[1]

[1]Department of Cardiovascular Surgery, The Second People's Hospital of Yunnan Province, Kunming, Yunnan Province, China; [2]Department of Cardio-thoracic Surgery, People's Hospital of Wen Shan, Yunnan Province, China; [3]Department of Cardiology, People's Hospital of Li Jiang, Yunnan Province, China

Received December 17, 2016; Accepted January 20, 2017; Epub April 1, 2017; Published April 15, 2017

Abstract: Background: microRNAs (miRNAs) are associated with cardiovascular disease, regulate gene expression, and are detectable in blood samples. Objective: We sought to determine the diagnostic value of miRNAs in paroxysmal atrial fibrillation (AF) and the mechanisms underlying miRNA involvement in the pathophysiological of AF. Methods: miRNA profiles in plasma samples from AF patients and healthy individuals were obtained using a microarray assay and validated by qPCR. Subsequently, the diagnostic performance of selected miRNAs was analyzed using receiver operating characteristic (ROC) curves. *In vitro*, western blot, and luciferase reporter assays were conducted to determine downstream targets of miR-499 in HL-1 cells. The whole-cell patch-clamp technique was then performed to observe the function of miR-499 on Ca^{2+} channels. Results: miRNA microarray data revealed 10 aberrantly expressed miRNAs between groups and qPCR validated three upregulated miRNAs, miR-21, miR-208, and miR-499. ROC analysis showed miR-499 had a higher diagnostic performance than the other two, with an area under the curve value of 0.83 (95% CI, 0.74-0.90). Furthermore, miR-499 directly regulated the expression of the L-type Ca^{2+} channel Cav1.2 (encoded by the *CACNA1C* gene). The results from the whole-cell patch-clamp technique showed a reduction in L-type Ca^{2+} currentin HL-1 cells. Conclusions: miR-499 could serve as a diagnostic biomarker for paroxysmal AF and targets the *CACNA1C* gene regulating Cav1.2 expression and function, which is involved in the development of AF.

Keywords: Diagnosis, Cav1.2, miRNA, paroxysmal atrial fibrillation

Introduction

Atrial fibrillation (AF) is a global healthcare problem affecting 2.5% of the current population worldwide and its incidence increases with age [1]. Currently, the prevalence of AF is 5.2 million in the US and is predicted to rise to 12.1 million by 2030 [2]. Despite progress in the management of AF patients, this arrhythmia remains one of the major causes of stroke, heart failure, sudden death, and cardiovascular morbidity in the world [3]. Approximately one in five ischemic strokes is attributable to cardioembolic events from AF. Unfortunately, strokes due to AF are associated with increased disability and mortality compared to strokes from other etiologies [4, 5]. Because AF, especially paroxysmal AF, may be undetected, there is a critical need to identify those at greatest risk for the arrhythmia.

miRNAs are a class of single-stranded, noncoding endogenous RNAs that inhibit gene expression at the post-transcriptional level by impeding mRNA translation or degrading target mRNAs [6]. Accumulating evidence suggests that miRNAs play important roles in the growth, development, and stress responses of the heart [7]. miRNAs are also associated with pathological processes in the cardiovascular system [8-10]. For example, miR-208 was significantly upregulated in human and ovine AF atrial tissue contributing to impaired Ca^{2+} handling during atrial remodeling [11]. miR-1 levels were greatly reduced in left atrial tissue samples from AF patients and might be relevant to AF-associated inwardly rectifying potassium channel Kir2.1 remodeling, which is important for AF maintenance [8]. In addition, the miR-30a target, snail 1 protein, may be related to AF-induced myocardial fibrosis [12]. Studies

Table 1. Characteristics of study subjects

Characteristic	Healthy	Paroxysmal AF	Persistent AF	Permanent AF	*P* value
Number	76	64	95	100	
Male	41 (54%)	31 (48%)	52 (54%)	43 (43%)	0.873
Mean age	64±6.2	67±9.0	66±8.7	70±7.4	0.105
BMI kg/m^2	22.5±1.1	21.2±2.3	21.8±1.9	22.0±2.8	0.584
Diabetes	11 (14%)	20 (31%)	28(30%)	34 (34%)	0.004*
Hypertension	13 (17%)	39 (61%)	41 (43%)	62 (62%)	0.019*
TC (mmol/L)	4.6±0.8	4.9±0.5	5.2±0.4	5.1±0.5	0.137
TSH mIU/mL	1.8±0.2	1.9±0.3	1.7±0.3	1.8±0.4	0.213
History of AMI	-	24 (38%)	53 (56%)	59 (59%)	0.002*

BMI=body mass index; TC=Total cholesterol; TSH=Thyroid stimulating hormone; AMI=acute myocardial infarction; *P < 0.05.

show that miRNAs are measurable in blood and body fluids [13], which makes them ideal potential diagnostic and prognostic biomarkers for diseases such as AF [14]. However, thus far, published studies have provided a limited understanding of the factors involved in early AF development.

The aim of the present study was to determine the feasibility of detecting and quantifying the expression level of miRNAs in plasma samples from patients at different stages of AF, to find practical diagnostic biomarkers for paroxysmal AF in clinical settings, and to ascertain the molecular mechanism of miRNAs involved in the development of AF. We identified dysregulated miRNAs in plasma using an miRNA microarray that were validated by qPCR. Receiver operating characteristic (ROC) curveswere used to determine the diagnostic values of the selected miRNAs: miR-21, miR-499, and miR-208. In addition, the effects of miR-499 on Ca^{2+} channel expression were testedto determine how the candidate miRNA integrates into AF pathophysiology. We determined the diagnostic value of miRNAs for paroxysmal AF and identified a crucial association of miR-499 upregulation with AF development.

Materials and methods

Patients and plasma samples

Between November 2010 and September 2014, 312 consecutive patients diagnosed with atrial fibrillation were considered for inclusionin The Second People's Hospital of Yunnan Province. The exclusion criteriawere: a) history or symptoms of heart failure; b) cardiac valvular disease, left atrial or left atrial appendage thrombosis; c) major surgical procedure or trauma within 60 days; or d) malignancy, immune system diseases, serious liver or renal dysfunctions, cerebrovascular accident, or nervous system diseases. Thus, 259 patients presenting with paroxysmal AF (64 patients), persistent AF (95 patients), and permanent AF (100 patients) were included. Local and regional research ethics committees approved the study. The medical history of each AF patient was recorded. The clinical characteristics of study subjects are shown in **Table 1**. Plasma samples from 259 patients with AF and 76 healthy volunteers were centrifuged at 3000× g for 10 min within 1 h of acquisition and stored at -80°C. Written informed consent was obtained from each patient.

miRNA microarrays and quantitative real-time PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and purified with a PureLink® RNA Mini Kit (Invitrogen). For microarrays, three samples were randomly chosen from the four groups and mRNA was amplified, labelled, and hybridized according to the instructions provided by CapitalBio Corporation. The data was read by a confocal LuxScan scanner (CapitalBio Corp, Beijing, China) and analyzed by SpotData Pro software (CapitalBio Corp). "Fold Change" and "Regulation" columns were added in Excel for estimation of gene expression and normalization. For qRT-PCR, first strand cDNA was synthesized according to the manufacturer's instructions using PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Shiga, Japan). Reverse transcription was performed at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Relative expression levels of differentially expressed miRNAs obtained from the microarray analysis were obtained using SYBR Premix Ex Taq™ (Takara) on a ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). All reactions were performed with at least three replicates. Relative quantification of miRNA and mRNA expression levels were calculated using the

Table 2. The TaqMan stem-loop primers for reverse transcription PCR and the forward and reverse primers for real-time PCR

Accession	ID		Sequence
MIMAT0000416	Has-miR-1	Sequence	UGGAAUGUAAAGAAGUAUGUAU
		Stem loop primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACATACAT
		PCR-F	TGGAATGTAGAGAAGT
MIMAT0000076	Has-mir-21	Sequence	UAGCUUAUCAGACUGAUGUUGA
		Stem loop primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCAACA
		PCR-F	TAGCTCGTCAGACTGA
MIMAT0000241	Has-mir-208	Sequence	AUAAGACGAGCAAAAAGCUUGU
		Stem loop primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACTTGC
		PCR-F	ATACGACGAGCAAGAA
MIMAT0002870	Has-mir-499	Sequence	UUAAGACUUGCAGUGAUGUUU
		Stem loop primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAAACAT
		PCR-F	GTAAGACTTGCAGTG
MIMAT0000752	Has-mir-328	Sequence	CUGGCCCUCUCUGCCCUUCCGU
		Stem loop primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACGGAA
		PCR-F	CTGGACCTCTCTGCAC
MIMAT0026478	Has-mir-133	Sequence	AGCUGGUAAAAUGGAACCAAAU
		Stem loop primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACATTTGG
		PCR-F	AGCTGGTAAAGTGGAA
M14486	U6	PCR-F	CTCGCTTCGGCAGCACA
		PCR-R	AACGCTTCACGAATTTGCGT
	β-actin	PCR-F	AGTTGCGTTACACCCTTTCTTG
		PCR-R	CACCTTCACCGTTCCAGTTTT
	CACNA1C	PCR-F	TGGGTGTACAGGTGGGT
		PCR-R	GTGGTTTGTTCTTGCTTTCAAAC
	CACNB2	PCR-F	AAGAAGACAGAGCACACTCC
		PCR-R	TGCGTGCTTACTGGGATT

$2^{-\Delta\Delta Ct}$ method normalized with respect to U6 and β-actin, respectively. **Table 2** shows the primers used for qPCR.

miRNA target prediction

Computational prediction of putative targets for miR-499 was performed by searching miRNA databases including TargetScan, miRanda, miRWalk, and miRDB.

Cell culture and transfection

Human atrial myocytes HL-1 (GF015, Shanghai Gefan Biotechnology. Co., LTD, China) were cultured in DMEM (Invitrogen) supplemented with 50 unit/ml penicillin/streptomycin, 10% calf serum, and 10% fetal calf serum (Gibco, CA, USA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

miR-499 mimic and inhibitor were synthesized by RiboBio Biological Technology Co., Ltd (Guangzhou, China). The negative control was a scrambled 22-nucleotide miRNA. Transfections with miR-499 mimic, inhibitor, or their negative controls and luciferase reporter plasmid were carried out using Lipofectamine® 2000 (11668-029, Invitrogen) according to the manufacturer's instructions. Transfection efficiency was determined through fluorescence detection.

Western blot

HL-1 cells with miR-499 mimics or inhibitors were lysed with RIPA buffer (Beyotime, China) for 30 min on ice. The lysates were cleared by centrifugation at 10,000× g for 5 min and the BCA Protein Assay kit (Beyotime, China) was used for protein quantification. Equal amounts of protein were used for SDS-PAGE, transferred onto PVDF membranes, and blocked with 5% non-fat milk. The membranes were incubated with first antibodies included anti-Cav1.2 (1:1000, Abcam), Cavβ2 (1:500, Abcam), and β-actin (1:2000, Abcam) overnight at 4°C fol-

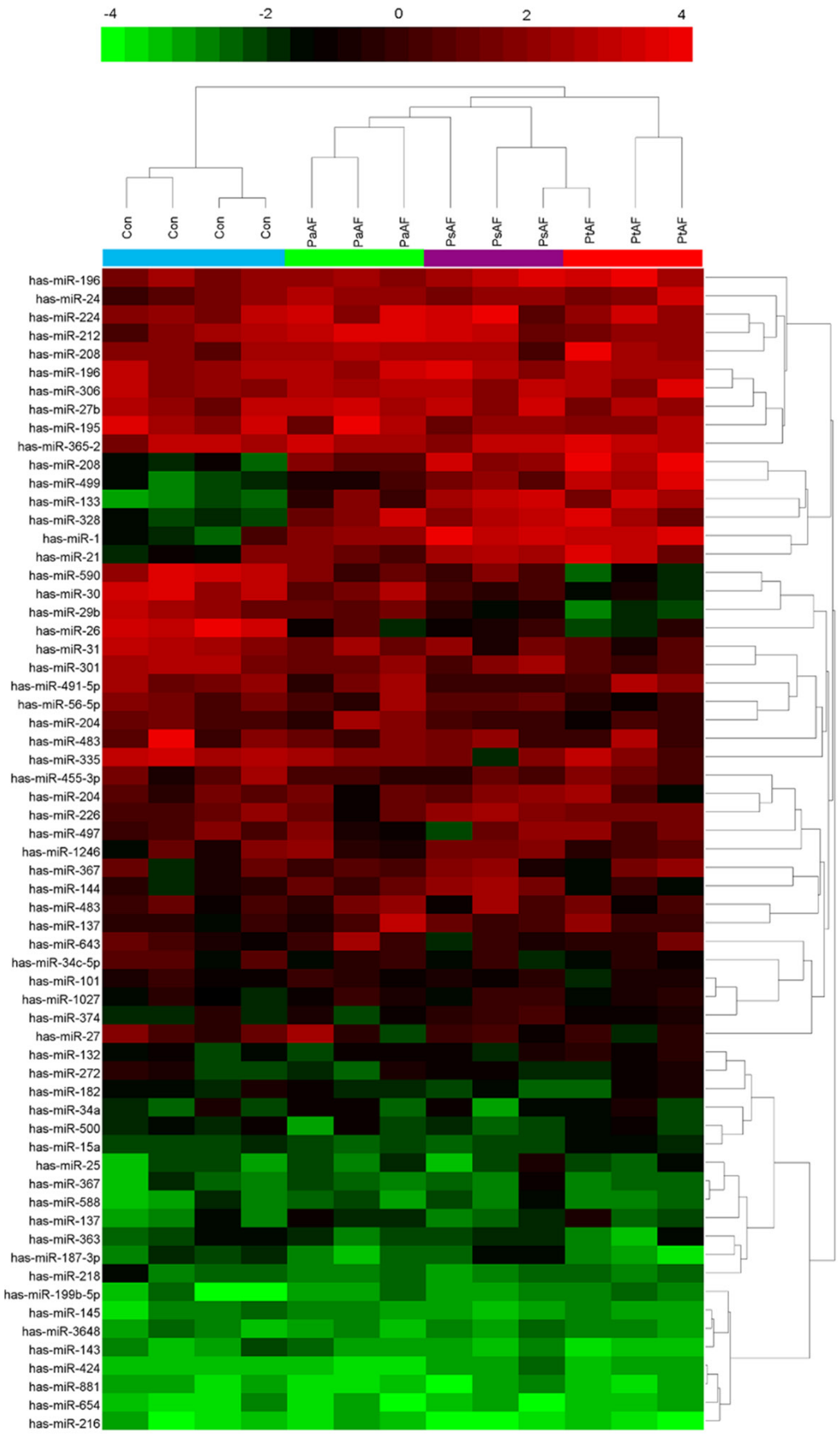
-4
-2
0
2
4
Con
Con
Con
Con
PaAF
PaAF
PaAF
PsAF
PsAF
PsAF
PtAF
PtAF
PtAF
has-miR-196
has-miR-24
has-miR-224
has-miR-212
has-miR-208
has-miR-196
has-miR-306
has-miR-27b
has-miR-195
has-miR-365-2
has-miR-208
has-miR-499
has-miR-133
has-miR-328
has-miR-1
has-miR-21
has-miR-590
has-miR-30
has-miR-29b
has-miR-26
has-miR-31
has-miR-301
has-miR-491-5p
has-miR-56-5p
has-miR-204
has-miR-483
has-miR-335
has-miR-455-3p
has-miR-204
has-miR-226
has-miR-497
has-miR-1246
has-miR-367
has-miR-144
has-miR-483
has-miR-137
has-miR-643
has-miR-34c-5p
has-miR-101
has-miR-1027
has-miR-374
has-miR-27
has-miR-132
has-miR-272
has-miR-182
has-miR-34a
has-miR-500
has-miR-15a
has-miR-25
has-miR-367
has-miR-588
has-miR-137
has-miR-363
has-miR-187-3p
has-miR-218
has-miR-199b-5p
has-miR-145
has-miR-3648
has-miR-143
has-miR-424
has-miR-881
has-miR-654
has-miR-216

Figure 1. miRNA expression profiles of plasma samples from patients with atrial fibrillation. Aheat map showing all miRNA expression differences between paroxysmal AF (PaAF), persistent AF (PsAF), permanent AF (PtAF), and healthy volunteer (Con) groups. The green in the legend represents downregulation and the red represents upregulation. For all array analyses, a *p*-value < 0.05 and a±2-fold change cut-off was applied.

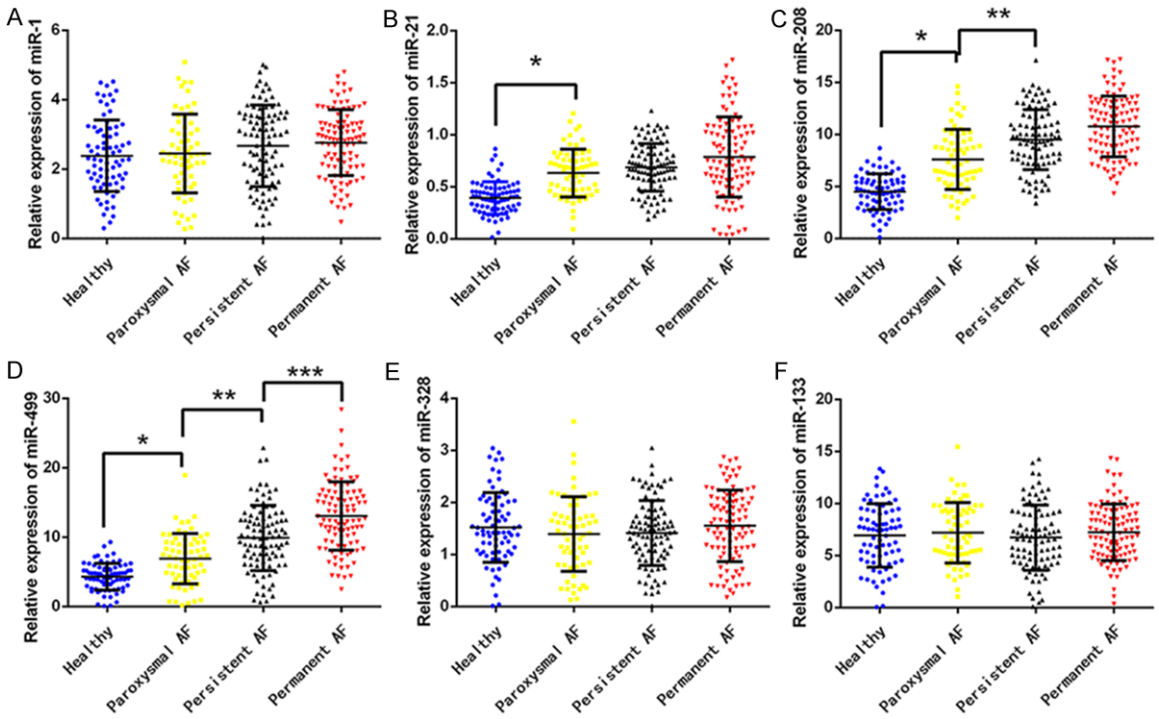

Figure 2. The expression levels of selected miRNAs detected by qPCR. *P < 0.05 vs Healthy group; **P < 0.05 vs paroxysmal AF; ***P < 0.05 vs persistent AF.

lowed by incubation with secondary antibodies (Merck, 1:5000) for 1.5 h at room temperature. After extensive washes with TBST, the proteins were detected by chemiluminescence (Chemi-Doc XRS, Bio-Rad).

Luciferase reporter assay

The luciferase assay was used to assess whether miR-499 targets the 3'-UTR of *CACNA1C or CACNB2*. HL-1 cells were transfected with the pMir-Target luciferase expression reporter vector carrying the 3'UTR of *CACNA1C*, *CACNB2,* or an empty control vector and the promoter-luciferase fusion plasmid. In brief, cells were cultured in 48-well plates and the Dual-Luciferase Reporter Assay System (Promega, E19-80, Wisconsin, USA) was performed according to the manufacturer's instructions.

Calcium current recordings

Voltage-gated Ca^{2+} channel function was evaluated by recording inward Ca^{2+} currents (ICa, L) in HL-1 cells using the whole-cell patch-clamp technique (AXON-200B, Molecular Devices, Inc., Sunnyvale, CA USA). HL-1 cells were stimulated on a ramp protocol with a velocity of 1.5 mV/sec and intracellular Ca^{2+} signals were analyzed by confocal microscopy in HL-1 cells loaded with pClamp10. The membrane capacitance (Cm) was calculated according to the equation Cm=I/(dV/dt) and the current value was the ratio of the current amplitude to the cell capacitance (pA/pF).

Statistical analysis

Data are mean ± standard deviationfrom three independent experiments. An unpaired t-test was used for two-group comparisons and a one-way ANOVA was used followed by a Fisher's Least Significant Difference (LSD) t-test for more than two groups. ROC curves were constructed, and the area under the curve (AUC) was used to assess the diagnostic values of miRNAs. P < 0.05 was considered statistically

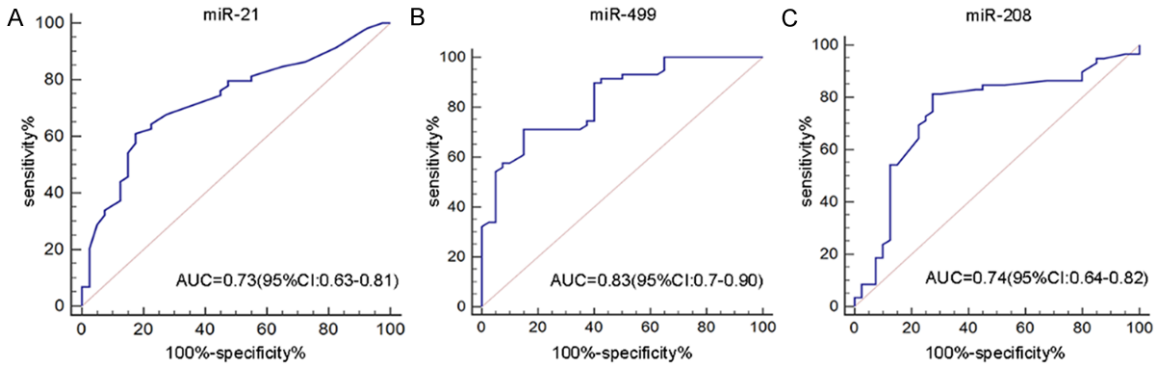

Figure 3. ROC analysis of miRNAs for diagnosing paroxysmal AF. A: Diagnostic performance of miR-21 in groups of paroxysmal AF with healthy individuals and its ROC curve: AUC 0.73 (95% CI, 0.63-0.81), Cut-off value is 2.53, sensitivity 61.02%, and specificity 80.50%. B: ROC analysis of miR-499; AUC 0.83 (95% CI, 0.74-0.90); Cut-off value is 6.35, sensitivity 81.36%, and specificity 72.51%. C: ROC analysis of miR-208; AUC 0.74 (95% CI, 0.64-0.82); Cut-off value is 4.74, sensitivity 75.22%, and specificity 85.00%.

Table 3. The receiver operating characteristic (ROC) analysis of miRNA in patients with paroxysmal AF

miRNAs	AUC	95% CI	*P* value	Youden	Cut-off	Sensitivity	Specificity
miR-21	0.73	0.63-0.81	<0.001	0.44	2.53	61.02%	80.50%
miR-208	0.74	0.64-0.82	<0.001	0.57	6.35	81.36%	72.51%
miR-499	0.83	0.74-0.90	<0.001	0.56	4.74	75.22%	85.00%

Note. AUC, area under the receiver operating characteristic curve; CI, confidence interval; *P*-value, compared with AUC of 0.5.

significant. GraphPad Prism 5.0 (La Jolla, California, USA) was used for graphing. Data were analyzed using SPSS software version 20.0 (SPSS Inc., Chicago, IL).

Results

The changes in miRNAs in atrial fibrillation patients

miRNA signatures in AF patient plasma were obtained by comparing microRNA microarrays between the paroxysmal AF group, persistent AF group, permanent AF group, and healthy volunteers group. As illustrated in **Figure 1**, 10 miRNAs exhibited significantly altered expression levels ($P < 0.05$ and fold change ≥ 2). Of these, six miRNAs were significantly upregulated and four were suppressed. qPCR was performed to confirm this data. We hypothesized that miRNAs may contribute to atrial electrical remodeling and involvement in arrhythmogenesis. Therefore, we investigated the six upregulated miRNAs: miR-1, miR-21, miR-208, miR-499, miR-328, and miR-133. However, miR-1, miR-328, and miR-133 did not show upregulation in any subgroup of AF patients compared with healthy patients (**Figure 2A**, **2E**, **2F**). The expression levels of miR-21, miR-208, and miR-499 were consistent with the microarray findings (**Figure 2B-D**, $P < 0.05$ for all). Interestingly, only the miR-499 expression level gradually increased between AF groups suggesting that it may participate in the development of AF and could serve as a biomarker for early stage AF.

Diagnostic performance of miRNAs in patients with paroxysmal AF

Based on the above hypothesis, we constructed receiver operating characteristic curves to assess the diagnostic value of selected miRNAs in plasma from paroxysmal AF patients. The AUC value for miR-21 and miR-208 was 0.73 (95% CI, 0.63-0.81) and 0.74 (95% CI, 0.64-0.82), respectively ($P < 0.001$; **Figure 3A**, **3C**). miR-499 had a greater power to diagnose paroxysmal AF with an AUC value of 0.83 (95% CI, 0.74-0.90), a cut-off value of 4.74, and sensitivity and specificity of 75.22% and 85.00%, respectively (**Figure 3B**). Detailed information about the diagnostic performance of miRNAs is shown in **Table 3**.

miR-499 alters inward L-type calcium channel expression and function in human myocardial cells

Because the expression of miR-499 differed between AF subtypes, it was valuable to explore

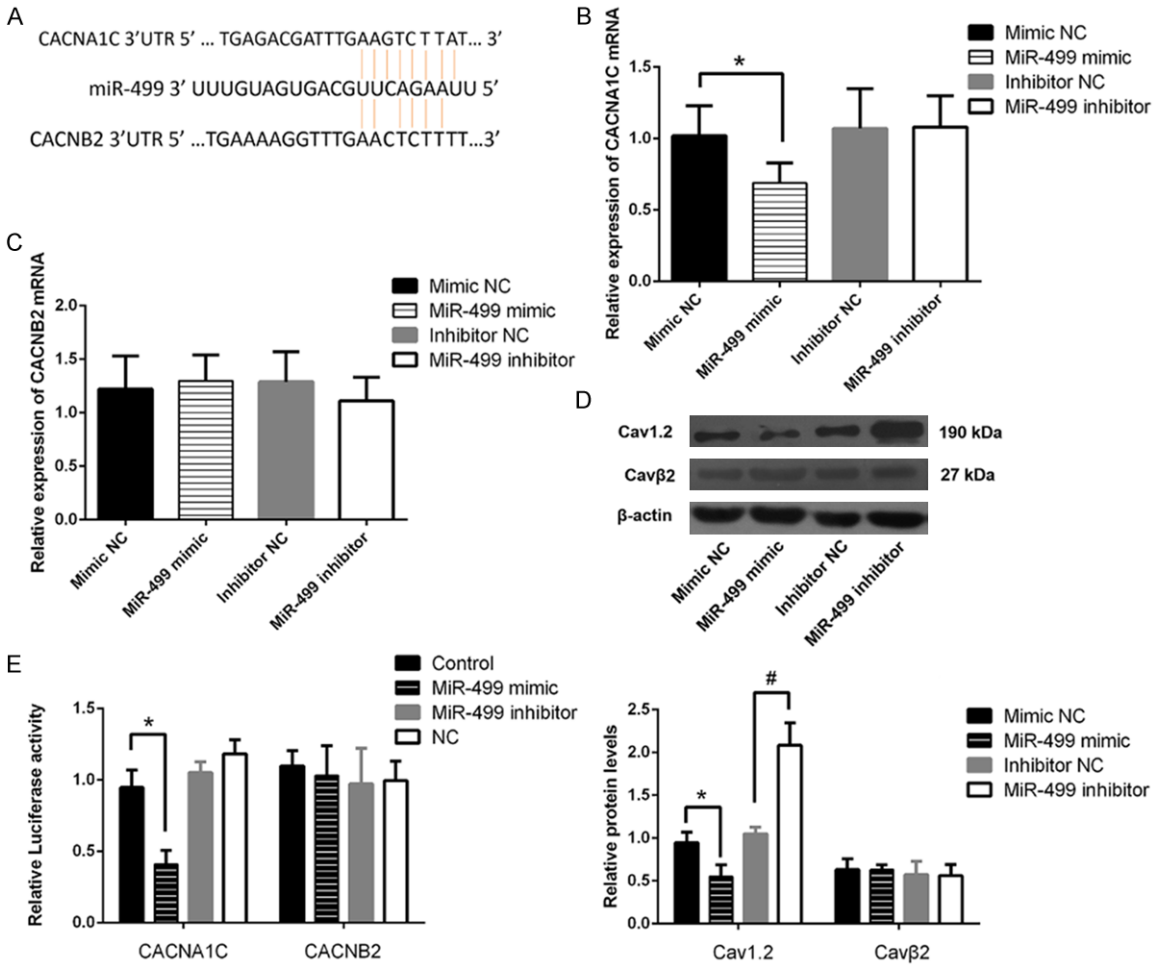

Figure 4. miR-499 directly targets CACNA1C in HL-1 cells. A: Putative binding sites between miR-499 and CACNA1C. B, C: The relative expressions of CACNA1C and CACNB2 mRNA using qPCR. D: Western blot showing the altered expression of Cav1.2 and Cavβ2 proteins (encoded by CACNA1C and CACNB2 mRNA, respectively) in the different groups. E: Luciferase assays in HL-1 cells (transfected with miR-499 mimic, miR-499 inhibitor, or corresponding negative controls) for posttranscriptional excitation of CACNA1C and CACNB2.

whether miR-499 was involved in the pathogenesis of AF.We assumed that CACNA1C and CACNB2 (L-type Ca^{2+} channel subunits α1c and β2 coding genes) were the putative target genes of miR-499. The potential complementary sequence between miR-499 and the candidates is illustrated in **Figure 4A**. CACNA1C and CACNB2 mRNA amounts were evaluated by qPCR in HL-1 cells transfected with miR-499 mimic or inhibitor or matched negative control (NC). Interestingly, lower CACNA1C mRNA amounts were observed in the miR-499 mimic group compared with the other three groups (**Figure 4B**, $P < 0.01$). However, CACNB2 mRNA levels did not change between groups (**Figure 4C**). Subsequently, Cav1.2 and Caβ2 protein (products of CACNA1C and CACNB2 mRNA, respectively) levels were evaluated by western blot in HL-1 cells. The results showed that Cav1.2 protein levels decreased in the miR-499 overexpression group and increased when miR-499 was inhibited (**Figure 4D**, $P < 0.01$). In accordance with the qPCR results, there was no statistically significant change in Caβ2 protein expression (**Figure 4D**). We further used a standard 3'UTR luciferase reporter assay to validate the interaction between miR-499 and CACNA1C. miR-499 mimic induced a reduction in luciferase activity in cells co-transfected with CACNA1C 3'UTR regions, whereas transfection with the negative control miRNA (NC) or with the CACNB2 3'UTR region showed no effect (**Figure 4E**). These findings suggest that CACNA1C, but not CACNB2, is subject to regula-

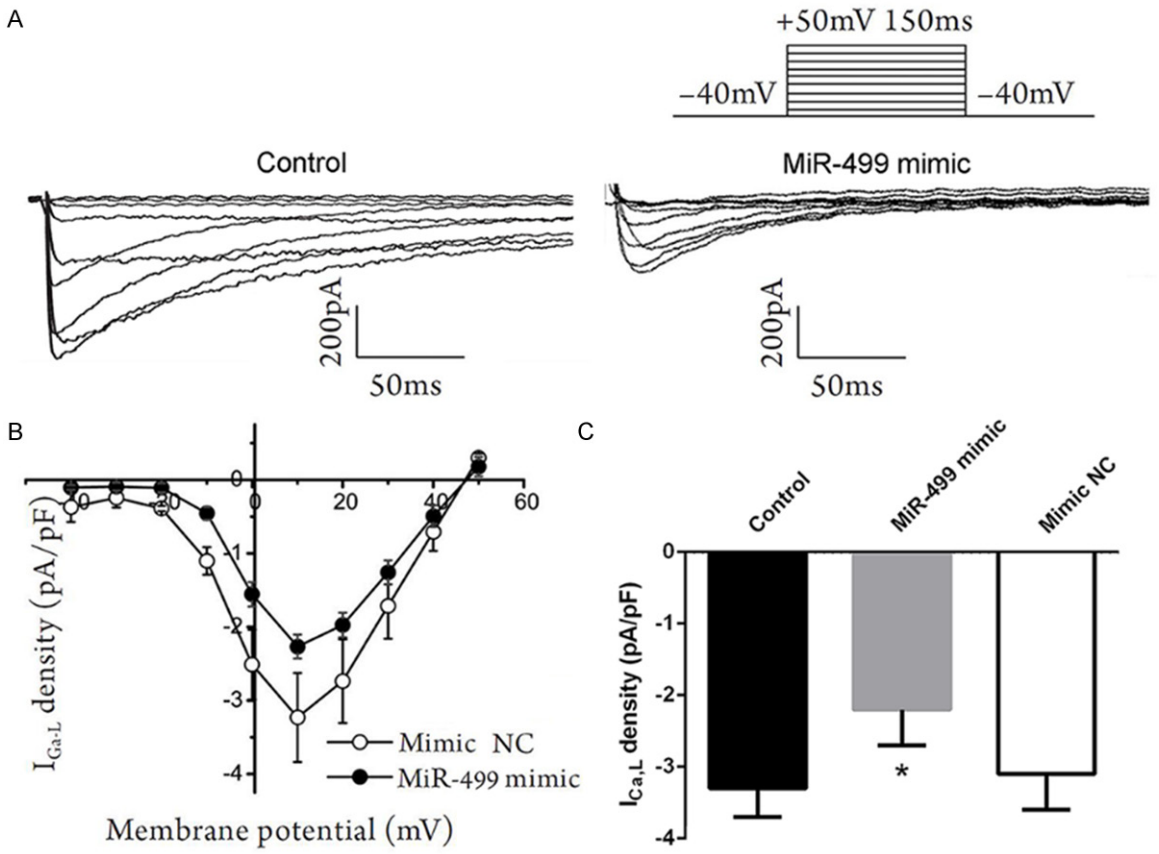

Figure 5. miR-499 regulated inward L-type Ca^{2+} current. $I_{ca,L}$ was recorded with the voltage-clamp mode with the whole-cell patch-clamp configuration using the voltage protocols shown in the inset. A: Representative $I_{ca,L}$ traces recorded in sham (control) and in miR-499 transfected HL-1 cells. B: *I-V* relationships showed that miR-499 reduced L-type Ca^{2+} current by 30.01%. C: Density of $I_{ca,L}$ recorded at +10 mV in HL-1 cells transfected with miR-499 mimics or mimics negative control. n = 15 cells in each group. *P < 0.01 vs mimics NC.

tion by miR-499 through binding to its 3'-UTR. To evaluate the correlation between $I_{Ca,L}$ density and miR-499 levels, we recorded $I_{Ca,L}$ in HL-1 cells transfected with miR-499 mimic or mimic NC using the whole-cell patch-clamp technique. Current density reached the maximum (-3.23± 0.64 pA/pF) at +10 mV in the control group, whereas transfection of miR-499 significantly reduced $I_{Ca,L}$ density (-2.26±0.36 pA/pF) without modifying the activation/inactivation time course and mimic NC did not modify the $I_{Ca,L}$ density (**Figure 5**). Collectively, these results reveal the underlying mechanism of miR-499 involvement in AF pathogenesis.

Discussion

Atrial fibrillation (AF) is independently associated with a two-fold increased risk of all-causes of mortality in women and a 1.5-fold increase in men [15, 16]. AF-related hospital admissions (the single most important determinant of cost) are twice as high as those in the general population [17]. Early detection of AF could dramatically reduce the risk by facilitating timely treatment with oral anticoagulants [18]. Likewise, the chance for curative treatment of AF also increases through early identification. The potential for microRNA to act as non-invasive molecular biomarkers of systemic disease is of great interest owing to their high level of stability in many different biofluids [19]. However, only a small set of miRNAs have been validated as altered in AF, and few studies provide the regulatory mechanism of atrial fibrosis in non-valvular paroxysmal AF [20].

In this work, we generated miRNA profiles of AF patients using microarray assays and validated the upregulated miRNAs through qPCR. Our

results show that miR-21, miR-208, and miR-499 were at higher expression levels in plasma from paroxysmal AF patients than healthy volunteers. A number of studies have reported that miR-499 is a cardiac-abundant miRNA under physiological conditions [21, 22]. miR-499 can inhibit cardiomyocyte apoptosis through its suppression of calcineurin-mediated dephosphorylation of dynamin-related protein-1 and p53 transcriptionally downregulates miR-499 expression [23]. miRNA-499 is a newly discovered member of miRNAs encoded by the myosin gene family and is located in an intron of the *Myh7B* (skeletal muscle and cardiac myosin) gene [24]. A recent study compared and analyzed the miR-499 expression profiles between non-ST-segment elevation myocardial infarction (NSTEMI) and congestive heart failure patients and found miR-499 was significantly increased in NSTEMI patients, suggesting circulating miR-499 is a sensitive biomarker of acute NSTEMI [25]. In agreement with this, Zhang et al. reported that miR-499 was significantly elevated in acute myocardial infarction (AMI) patients. Importantly, miR-499 was detectable in the plasma as early as 1 h after the onset of chest pain in AMI patients andwas shown to substantially increase the diagnostic accuracy of creatine kinase-MB and troponin in the diagnosis of AMI [26]. Furthermore, researchers also identified significantly upregulated atrial miR-499 in patients with permanent AF and found that miR-499 targets the small conductance calcium-activated potassium channel 3, contributing to the electrical remodeling in AF [27]. In the present study, miR-499 was significantly upregulated in paroxysmal AF plasma samples compared with negative controls and showed excellent diagnostic performance of paroxysmal AF with the AUC value of 0.83 (95% CI: 0.74-0.90).

Furthermore, we identified CACNA1C as the downstream target of miR-499, which may explain how miR-499 is involved in AF development. Previous studies suggest thatmultiple miRNAs contribute to controlling arrhythmogenicity of the heart and different miRNAs are involved in different types of arrhythmias under different pathological conditions of the heart [28, 29]. Cardiomyocytes possess distinct calcium channels that are dynamically regulated during development [30, 31]. CACNA1C is not expressed in cardiomyocytes initially, but is one of the hallmarks for cardiac electrophysiological in later developmental stages [32]. Mutations in *CACNA1C* may cause life-threatening arrhythmias in humans [33, 34]. Reduction of the density of the L-type Ca^{2+} current ($I_{Ca,\ L}$) is a hallmark of the electrical remodeling in AF [35]. Recently, studies report that miRNA-223 could regulate the expression of *CACNA1C* gene and its encoded cardiac L-type calcium channel α1c subunit (Cav1.2) in rat cardiomyocytes [36]. Here, computational analysis suggested that miR-499 regulates the *CACNA1C* gene, which we validated using a series of biological experiments. In addition, overexpression of miR-499 could reduce $I_{Ca,\ L}$ density. Our findings suggest that miRNA-499 targets the *CACNA1C* gene and regulates its encoded L-type Ca^{2+} channel Cav1.2, thus contributing to thearrhythmia.The calcium channel β2 (*CACNB2*), another voltage-gated calcium channel, is required during embryogenesis for normal ventricular cardiomyocyte proliferation, cardiac looping, chamber morphogenesis, and cardiac contractility [37], and was assumed to be a target of miR-499. However, our experiments did not identify *CACNB2* as a target of miR-499.

In addition, the expression level of plasma miR-21 and miR-208 were increased in AF patients, which was in line with other studies [11, 38]. These two miRNAs were able to properly diagnose paroxysmal AF patients according to our data.

However, this study has limitations. On the one hand, the miRNA profile was acquired from blood samples but not atrial samples as lot of patients didn't receive cardiac surgery and the diagnostic value of selected miRNAs was not validated in another study group. On the other hand, we only highlight the status of miR-499 and may miss the importance of other miRNAsthat are pertinent to the development of AF. Within these limitations, more studies need to be conducted to clarify the regulation mechanisms of miRNA in AF.

Conclusions

In summary, our data present the first demonstration that plasma miRNAs could serve as diagnostic biomarkers in paroxysmal AF. This study also establishes that miR-499 directly targets the *CACNA1C* gene and regulates the expression and function of Cav1.2. These find-

ings suggest that miR-499 could be a potential therapeutic target in paroxysmal AF.

Acknowledgements

This study was supported by Joint special applied basic research Projcet from Yunnan Province Technology Hall and Kunming Medical University (NO. 2014FZ044, 2014FZ045).

Disclosure of conflict of interest

None.

Address correspondence to: Yi Song, Department of Cardiovascular Surgery, The Second People's Hospital of Yunnan Province, Kunming City, Yunnan Province, China. E-mail: marunweiynkm@163.com

References

[1] Go AS, Hylek EM, Phillips KA, Chang Y, Henault LE, Selby JV and Singer DE. Prevalence of diagnosed atrial fibrillation in adults: national implications for rhythm management and stroke prevention: the AnTicoagulation and Risk Factors in Atrial Fibrillation (ATRIA) Study. JAMA 2001; 285: 2370-2375.

[2] Colilla S, Crow A, Petkun W, Singer DE, Simon T and Liu X. Estimates of current and future incidence and prevalence of atrial fibrillation in the U.S. adult population. Am J Cardiol 2013; 112: 1142-1147.

[3] Ravens U and Odening KE. Atrial fibrillation: Therapeutic potential of atrial K+ channel blockers. Pharmacol Ther 2016; [Epub ahead of print].

[4] Landenhed M, Engstrom G, Gottsater A, Caulfield MP, Hedblad B, Newton-Cheh C, Melander O and Smith JG. Risk profiles for aortic dissection and ruptured or surgically treated aneurysms: a prospective cohort study. J Am Heart Assoc 2015; 4: e001513.

[5] Howie BN, Donnelly P and Marchini J. A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. PLoS Genet 2009; 5: e1000529.

[6] Shukla GC, Singh J and Barik S. MicroRNAs: processing, maturation, target recognition and regulatory functions. Mol Cell Pharmacol 2011; 3: 83-92.

[7] van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J and Olson EN. Control of stress-dependent cardiac growth and gene expression by a microRNA. Science 2007; 316: 575-579.

[8] Girmatsion Z, Biliczki P, Bonauer A, Wimmer-Greinecker G, Scherer M, Moritz A, Bukowska A, Goette A, Nattel S, Hohnloser SH and Ehrlich JR. Changes in microRNA-1 expression and IK1 up-regulation in human atrial fibrillation. Heart Rhythm 2009; 6: 1802-1809.

[9] Kontaraki JE, Marketou ME, Zacharis EA, Parthenakis FI and Vardas PE. MicroRNA-9 and microRNA-126 expression levels in patients with essential hypertension: potential markers of target-organ damage. J Am Soc Hypertens 2014; 8: 368-375.

[10] Roldan V, Arroyo AB, Salloum-Asfar S, Manzano-Fernandez S, Garcia-Barbera N, Marin F, Vicente V, Gonzalez-Conejero R and Martinez C. Prognostic role of MIR146A polymorphisms for cardiovascular events in atrial fibrillation. Thromb Haemost 2014; 112: 781-788.

[11] Canon S, Caballero R, Herraiz-Martinez A, Perez-Hernandez M, Lopez B, Atienza F, Jalife J, Hove-Madsen L, Delpon E and Bernad A. miR-208b upregulation interferes with calcium handling in HL-1 atrial myocytes: implications in human chronic atrial fibrillation. J Mol Cell Cardiol 2016; 99: 162-173.

[12] Yuan CT, Li XX, Cheng QJ, Wang YH, Wang JH and Liu CL. MiR-30a regulates the atrial fibrillation-induced myocardial fibrosis by targeting snail 1. Int J Clin Exp Pathol 2015; 8: 15527-15536.

[13] Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB and Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008; 105: 10513-10518.

[14] Wei XJ, Han M, Yang FY, Wei GC, Liang ZG, Yao H, Ji CW, Xie RS, Gong CL and Tian Y. Biological significance of miR-126 expression in atrial fibrillation and heart failure. Braz J Med Biol Res 2015; 48: 983-989.

[15] Stewart S, Hart CL, Hole DJ and McMurray JJ. A population-based study of the long-term risks associated with atrial fibrillation: 20-year follow-up of the Renfrew/Paisley study. Am J Med 2002; 113: 359-364.

[16] Andersson T, Magnuson A, Bryngelsson IL, Frobert O, Henriksson KM, Edvardsson N and Poci D. All-cause mortality in 272,186 patients hospitalized with incident atrial fibrillation 1995-2008: a Swedish nationwide long-term case-control study. Eur Heart J 2013; 34: 1061-1067.

[17] Wolowacz SE, Samuel M, Brennan VK, Jasso-Mosqueda JG and Van Gelder IC. The cost of illness of atrial fibrillation: a systematic review of the recent literature. Europace 2011; 13: 1375-1385.

[18] Gladstone DJ, Spring M, Dorian P, Panzov V, Thorpe KE, Hall J, Vaid H, O'Donnell M, Laupa-

cis A, Cote R, Sharma M, Blakely JA, Shuaib A, Hachinski V, Coutts SB, Sahlas DJ, Teal P, Yip S, Spence JD, Buck B, Verreault S, Casaubon LK, Penn A, Selchen D, Jin A, Howse D, Mehdiratta M, Boyle K, Aviv R, Kapral MK and Mamdani M. Atrial fibrillation in patients with cryptogenic stroke. N Engl J Med 2014; 370: 2467-2477.

[19] Xu J, Zhao J, Evan G, Xiao C, Cheng Y and Xiao J. Circulating microRNAs: novel biomarkers for cardiovascular diseases. J Mol Med (Berl) 2012; 90: 865-875.

[20] Rosenheck S. A new insight into the molecular mechanism of atrial fibrillation: the role of microRNAs. Cardiology 2015; 131: 55-57.

[21] van Rooij E, Quiat D, Johnson BA, Sutherland LB, Qi X, Richardson JA, Kelm RJ Jr and Olson EN. A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. Dev Cell 2009; 17: 662-673.

[22] Kim HJ, Cui XS, Kim EJ, Kim WJ and Kim NH. New porcine microRNA genes found by homology search. Genome 2006; 49: 1283-1286.

[23] Wang JX, Jiao JQ, Li Q, Long B, Wang K, Liu JP, Li YR and Li PF. miR-499 regulates mitochondrial dynamics by targeting calcineurin and dynamin-related protein-1. Nat Med 2011; 17: 71-78.

[24] Zhu J, Yao K, Wang Q, Guo J, Shi H, Ma L, Liu H, Gao W, Zou Y and Ge J. Ischemic postconditioning-regulated miR-499 protects the rat heart against ischemia/reperfusion injury by inhibiting apoptosis through PDCD4. Cell Physiol Biochem 2016; 39: 2364-2380.

[25] Olivieri F, Antonicelli R, Lorenzi M, D'Alessandra Y, Lazzarini R, Santini G, Spazzafumo L, Lisa R, La Sala L, Galeazzi R, Recchioni R, Testa R, Pompilio G, Capogrossi MC and Procopio AD. Diagnostic potential of circulating miR-499-5p in elderly patients with acute non ST-elevation myocardial infarction. Int J Cardiol 2013; 167: 531-536.

[26] Zhang L, Chen X, Su T, Li H, Huang Q, Wu D, Yang C and Han Z. Circulating miR-499 are novel and sensitive biomarker of acute myocardial infarction. J Thorac Dis 2015; 7: 303-308.

[27] Ling TY, Wang XL, Chai Q, Lau TW, Koestler CM, Park SJ, Daly RC, Greason KL, Jen J, Wu LQ, Shen WF, Shen WK, Cha YM and Lee HC. Regulation of the SK3 channel by microRNA-499--potential role in atrial fibrillation. Heart Rhythm 2013; 10: 1001-1009.

[28] Yang B, Lin H, Xiao J, Lu Y, Luo X, Li B, Zhang Y, Xu C, Bai Y, Wang H, Chen G and Wang Z. The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nat Med 2007; 13: 486-491.

[29] Lu Y, Zhang Y, Wang N, Pan Z, Gao X, Zhang F, Zhang Y, Shan H, Luo X, Bai Y, Sun L, Song W, Xu C, Wang Z and Yang B. MicroRNA-328 contributes to adverse electrical remodeling in atrial fibrillation. Circulation 2010; 122: 2378-2387.

[30] Qu Y and Boutjdir M. Gene expression of SERCA2a and L- and T-type Ca channels during human heart development. Pediatr Res 2001; 50: 569-574.

[31] Cribbs LL, Martin BL, Schroder EA, Keller BB, Delisle BP and Satin J. Identification of the t-type calcium channel (Ca(v)3.1d) in developing mouse heart. Circ Res 2001; 88: 403-407.

[32] Takemura H, Yasui K, Opthof T, Niwa N, Horiba M, Shimizu A, Lee JK, Honjo H, Kamiya K, Ueda Y and Kodama I. Subtype switching of L-Type Ca 2+ channel from Cav1.3 to Cav1.2 in embryonic murine ventricle. Circ J 2005; 69: 1405-1411.

[33] Splawski I, Timothy KW, Sharpe LM, Decher N, Kumar P, Bloise R, Napolitano C, Schwartz PJ, Joseph RM, Condouris K, Tager-Flusberg H, Priori SG, Sanguinetti MC and Keating MT. Ca(V)1.2 calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. Cell 2004; 119: 19-31.

[34] Li W, Zheng NZ, Yuan Q, Xu K, Yang F, Gu L, Zheng GY, Luo GJ, Fan C, Ji GJ, Zhang B, Cao H and Tian XL. NFAT5-mediated CACNA1C expression is critical for cardiac electrophysiological development and maturation. J Mol Med (Berl) 2016; 94: 993-1002.

[35] Wakili R, Voigt N, Kaab S, Dobrev D and Nattel S. Recent advances in the molecular pathophysiology of atrial fibrillation. J Clin Invest 2011; 121: 2955-2968.

[36] He F, Xu X, Yuan S, Tan L, Gao L, Ma S, Zhang S, Ma Z, Jiang W, Liu F, Chen B, Zhang B, Pang J, Huang X and Weng J. Oxidized low-density lipoprotein (ox-LDL) cholesterol induces the expression of miRNA-223 and L-type calcium channel protein in atrial fibrillation. Sci Rep 2016; 6: 30368.

[37] Chernyavskaya Y, Ebert AM, Milligan E and Garrity DM. Voltage-gated calcium channel CACNB2 (beta2.1) protein is required in the heart for control of cell proliferation and heart tube integrity. Dev Dyn 2012; 241: 648-662.

[38] Barana A, Matamoros M, Dolz-Gaiton P, Perez-Hernandez M, Amoros I, Nunez M, Sacristan S, Pedraz A, Pinto A, Fernandez-Aviles F, Tamargo J, Delpon E and Caballero R. Chronic atrial fibrillation increases microRNA-21 in human atrial myocytes decreasing L-type calcium current. Circ Arrhythm Electrophysiol 2014; 7: 861-868.