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Plasma Circular RNAs, Hsa_circRNA_025016, Predict Postoperative Atrial Fibrillation After Isolated Off-Pump Coronary Artery Bypass Grafting

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Background—Circular RNAs (circRNAs) are pervasively expressed in highly divergent eukaryotes and are stable in body fluids. However, the link between circRNAs and onset of atrial fibrillation (AF) has not previously been investigated. We aimed to identify plasma circRNAs that are able predict AF after cardiac surgery.

Methods and Results—Plasma circRNA expression profiles were investigated in a total of 769 patients with or without AF after isolated off-pump coronary artery bypass grafting. First, a circRNA microarray was used to screen 13 617 human circRNAs in plasma samples from patients in the discovery cohort (n=30). A quantitative polymerase chain reaction assay was then applied to evaluate the expression of 9 selected circRNAs in the training cohort (n=365). This approach revealed that hsa_circRNA_025016 was upregulated in patients with new-onset AF with a high diagnostic accuracy as assessed by the area under the receiver operating characteristic curve (=0.802). Additionally, a satisfactory diagnostic performance of hsa_circRNA_025016 was found in the validation cohort (n=284). Furthermore, Kyoto Encyclopedia of Genes and Genomes biological pathway analysis indicated that hsa_circ_025016 participated in melanogenesis, insulin secretion, and the thyroid hormone signaling pathway. A positive correlation between hsa_circ_025016 and fasting blood glucose was also identified in both cohorts.

Conclusions—Hsa_circ_025016 is a potential biomarker for predicting new-onset AF after isolated off-pump coronary artery bypass grafting. (*J Am Heart Assoc*. 2018;7:e006642. DOI: 10.1161/JAHA.117.006642.)

Key Words: atrial fibrillation • coronary artery bypass graft surgery • circular RNAs

P ervasive expression of circular RNAs (circRNAs) in highly divergent eukaryotes was discovered in 2012.[1] Recently, thousands of human circRNAs have been identified using molecular biology strategies coupled with new bioinformatic approaches.[2] CircRNAs are expressed in tissue-specific and developmental stage-dependent manners.[3] The dysregulations of circRNAs contribute to many diseases because they act as microRNA target decoys, RNA binding protein sponges, and transcriptional regulators.[4] Additionally, circRNAs are highly stable because of a resistance to debranching enzymes and RNA exonucleases. Therefore, circRNAs possess distinct advantages and may be useful as new biomarkers for diagnosis, prognosis, and therapeutic response prediction.[2]

Postoperative atrial fibrillation (PoAF) is one of the most common complications following coronary artery bypass grafting (CABG).[5] The reported incidences of atrial fibrillation (AF) after CABG range from 15% to 50%. PoAF is associated with significantly increased morbidity, mortality, and total treatment costs.[6] Although potential biomarkers of PoAF, such as mtDNA copy numbers and microRNAs,[7,8] have been actively researched, the diagnostic use of plasma circRNAs for PoAF has never been investigated. Our study screened the plasma circRNAs expression profiles (13 617 circRNAs) of patients with and without PoAF. The selected circRNAs that were found to be associated with PoAF were then tested in 2 additional independent populations of patients who underwent CABG.

Methods

Because of privacy, the data, analytic methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure.

Clinical Perspective

What Is New?

- For the first time, this study revealed the high expression of circular RNA (hsa_circRNA_025016) in plasma before surgery predicted new-onset atrial fibrillation after isolated off-pump coronary artery bypass grafting.

What Are the Clinical Implications?

- The present study demonstrates that this plasma hsa_-circ_025016 holds potential as a biomarker for the prediction of postoperative atrial fibrillation and could enable us to more accurately target patients who are at risk and thereby improve the effects of prophylactic treatments.

Study Design and Patients

Seven hundred sixty-nine blood samples were collected from the Department of Cardiovascular Surgery of the General Hospital of Shenyang Military Area Command from March 1, 2015 to June 31, 2016. All patients underwent off-pump CABG. The inclusion criteria, surgical procedure, and monitoring of AF onset were performed as described previously.[8] Patients' blood samples were collected within 1 week before surgery. Blood samples from 15 healthy donors were collected from the Health Examination Center of the General Hospital of Shenyang Military Area Command. All samples in this study were collected after informed consent was obtained from the participants, and all procedures were conducted according to an established protocol that was approved by the Ethics Committee of the General Hospital of Shenyang Military Area Command. The samples were allocated to 3 phases in chronological order (Figure 1A).

Discovery Phase

Potential associations between detectable circRNAs in EDTA-plasma and the onset of PoAF were analyzed in a retrospective case-controlled clinical trial (ClinicalTrials.gov, NCT02807532). Overall, 120 patients who underwent off-pump CABG were recruited to this study, and the incidence of PoAF was 16.7% (20/120). In this cohort, 15 patients who developed PoAF were selected as case subjects, and 15 non-PoAF patients with matched characteristics (ie, age, sex, smoking, left atrial diameter, left ventricle end-diastolic volume, and the use of statins before surgery) were selected as control subjects. A circRNA microarray was used to screen 13 617 human circRNAs in these 15 pairs of case–control

samples. The microarray information was submitted to the Gene Expression Omnibus under the accession number GSE97455.

Training Phase

The 9 circRNAs discovered via the microarray analyses were first tested by quantitative real-time polymerase chain reaction in an independent cohort of plasma samples from 365 consecutive participants who were recruited from June 2015 to December 2015. Of these circRNAs, 6 were consistently amplified in all individual samples. Two circRNAs were differentially expressed between the PoAF and non-PoAF groups in this cohort. One of the 2 circRNAs exhibited the greatest association with PoAF.
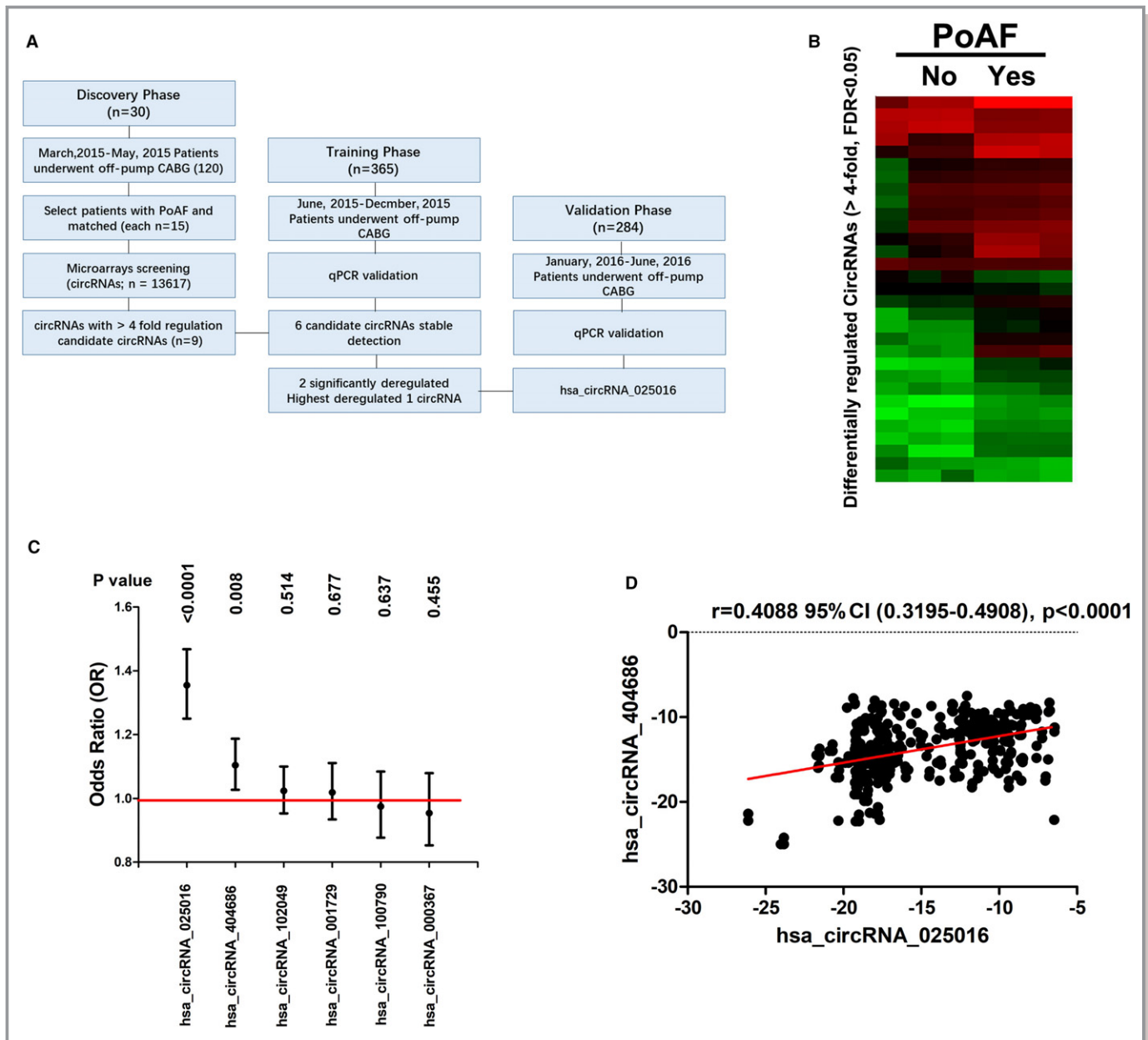
Validation Phase

The optimal mean cutoff generated from the training phase was applied to an independent cohort of 284 consecutive participants who were included from January 2016 to June 2016 to evaluate the diagnostic performance of the circRNA (hsa_circRNA_025016).

Plasma Preparation and RNA Isolation

For the plasma preparation, peripheral blood (3–4 mL) was drawn into EDTA tubes. Within 30 minutes, each tube was subjected to centrifugation at 1000 g for 15 minutes. Next, 1 mL plasma was transferred to a 1.5-mL Eppendorf tube and centrifuged at 11 000 g for 10 minutes to pellet any remaining cellular debris. Each EDTA tube can isolate nearly 2 mL plasma. Subsequently, the supernatants were transferred to fresh tubes and stored at −80°C. For the plasma samples, the total RNA was extracted using TRIzol LS according to the instructions from the manufacturer (Thermo Scientific, Waltham, MA). The concentrations were quantified with a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Waltham, MA). The integrity of the RNA was assessed by electrophoresis on a denaturing agarose gel.

Microarray Analysis

The sample preparation and microarray hybridization were performed based on the Arraystar's standard protocols. The microarray contained probes for 13 617 human circRNAs. Briefly, the total RNAs were digested with Rnase R (Epicentre, Madison, WI) to remove linear RNAs and enrich the circRNAs. Then, the enriched circRNAs were amplified and transcribed

Figure 1. Representation of the workflow of the screening and validation of the circular RNAs (circRNAs) in various populations with and without postoperative atrial fibrillation (PoAF). A, A total of 769 samples were collected from patients who underwent isolated off-pump coronary artery bypass grafting. This approach identified that hsa_circRNA_025016 predicted PoAF. B, Hierarchical clustering based on the levels of circulating circRNAs in patients with and without PoAF. C, The relationships between circulating circRNAs and PoAF. The data are presented as the odds ratios and 95% confidence intervals (CI) per 1 SD. D, Showing the relationship between hsa_circRNA_404686 and hsa_circRNA_025016. CABG indicates coronary artery bypass; CI, confidence interval; FDR, false discovery rate; qPCR, quantitative polymerase chain reaction.

into fluorescent cRNA utilizing a random priming method (Arraystar Super RNA Labeling Kit; Arraystar, Rockville, MD). The labeled cRNAs were hybridized onto the Arraystar Human circRNA Array (8×15 K). After washing the slides, the arrays were scanned with an Agilent Scanner G2505C (Arraystar, Rockville, MD).

Quantitative Real-Time Polymerase Chain Reaction

To test the candidate circRNAs that were acquired on the microarrays, circRNA detection was next validated by real-time polymerase chain reaction. For this purpose, the isolated RNAs

Table 1. Sequence of PCR Primers Used in This Study

18s	F:5′ CGGCTACCACATCCAAGGAA3′	R :5′ GCTGGAATTACCGCGGCT3′
hsa_circRNA_404686	F:5′CTCAGAACAAGAGCGTCCATT3′	R:5′AAGGCTGATGAAGAGGGAGG3′
hsa_circRNA_000367	F:5′CATCGAAGACTGGCGTGAAAC3′	R:5′CAGTTACAGAATCGTATGGAATGG3′
hsa_circRNA_001729	F:5′AGACCATTCCATTCTGGCTACA3′	R:5′TACCCCCTGGCTGCTGTGT3′
hsa_circRNA_100790	F:5′AAGTATTTCAGGCTGGGACAAG3′	R:5′TGTACCCCTCACTTGTGGATG3′
hsa_circRNA_030162	F:5′TGGGACTGATGTCATCTTGAGC3′	R:5′ATTTCCACCAATGAGCGAGTC3′
hsa_circRNA_100789	F:5′TGTAAATGCAGCTCCATTCCAA3′	R:5′ACCTGTGTCGCTTCTGGTTGTA3′
hsa_circRNA_025016	F:5′GCCTTAGCGATCTATATTCCCT3′	R:5′GCATTGGCATTCATGTTGG3′
hsa_circRNA_104270	F:5′CGACAGCGGGTCTACTCACTCT3′	R:5′GGTACTTCCCACAAATCCTTGC3′
hsa_circRNA_102049	F:5′GCATCTACCCTGCTGAACCT3′	R:5′CATTTCTTCTTGAGCAGTCCAG3′

PCR indicates polymerase chain reaction.

Table 2. Demographic and Clinical Characteristics by Presence of PoAF in Discovery Phase

Characteristics	PoAF Group n=20	No PoAF Group n=100	*P* Value
Sex (male/female)	15/5	84/16	0.519
Age, y	61.50±6.94	62.01±7.23	0.772
BMI, kg/m^2	24.10±2.23	24.97±2.78	0.189
Smoking, %	16 (80.0)	62 (62.0)	0.123
Heart rate, beats/min	73.75±8.76	71.37±10.34	0.291
NYHA III–IV, %	4 (20.0)	17 (17.0)	0.747
Hypertension, %	14 (70.0)	60 (60.0)	0.401
DM, %	8 (40.0)	41 (41.0)	0.934
Pre–myocardial infarction, %	9 (45.0)	54 (54.0)	0.462
Blood glucose, mmol/L	6.10±2.79	6.67±5.32	0.490
Cholesterol, mmol/L	4.33±0.99	4.31±0.92	0.929
Triglyceride, mmol/L	1.94±0.62	2.00±1.31	0.754
LDL, mmol/L	2.74±0.84	2.50±0.83	0.253
HDL, mmol/L	0.92±0.11	0.94±0.16	0.499
Creatinine, μmol/L	74.03±11.96	72.59±14.98	0.687
CKMB, U/L	12.55±9.26	13.06±24.22	0.873
cTNT, ng/mL	0.13±0.27	0.10±0.46	0.695
EF	0.54±0.06	0.55±0.05	0.419
LAD, mm	38.70±5.43	36.22±4.54	0.067
PASP, mm Hg	35.85±11.97	33.89±6.09	0.483
LVEDV, mL	122.60±46.23	110.36±36.48	0.262
β-Blockers, %	18 (90.0)	92 (92.0)	0.882
ACEI, %	8 (40.0)	46 (46.0)	0.622
CCB, %	11 (55.0)	51 (51.0)	0.744
Statins, %	11 (55.0)	71 (71.0)	0.160

Data presented as mean±SD or as a ratio. ACEI indicates angiotensin-converting enzyme inhibitor; BMI, body mass index; CCB, calcium channel blocker; CKMB, creatine kinase MB; cTNT, cardiac troponin T; DM, diabetes mellitus; EF, ejection fraction; HDL, high-density lipoprotein; LAD, left atrial diameter; LDL, low-density lipoprotein; LVEDV, left ventricle end-diastolic volume; NYHA, New York Heart Association; PASP, pulmonary arterial systolic pressure; PoAF, postoperative atrial fibrillation.

Table 3. Demographic and Clinical Characteristics by Presence of PoAF in 15 Paired Patients

Characteristics	PoAF Group n=15	No PoAF Group n=15	*P* Value
Sex (male/female)	11/4	11/4	1.000
Age, y	61.00±10.05	61.13±8.50	0.969
BMI, kg/m^2	24.30±3.56	24.37±2.70	0.934
Smoking, %	12 (80.0)	12 (80.0)	1.000
Heart rate, beats/min	74.4±7.67	75.4±10.01	0.762
NYHA III–IV, %	0 (0)	0 (0)	1.000
Hypertension, %	13 (86.7)	13 (86.7)	1.000
DM, %	6 (40.0)	6 (40.0)	1.000
Pre–myocardial infarction, %	8 (46.7)	7 (53.3)	0.715
Blood glucose, mmol/L	6.12±1.81	6.02±1.58	0.820
Cholesterol, mmol/L	4.30±1.09	4.42±1.08	0.760
Triglyceride, mmol/L	1.63±0.97	1.62±0.60	0.978
LDL, mmol/L	2.65±1.12	2.838±0.97	0.692
HDL, mmol/L	0.89±0.18	0.92±0.19	0.752
Creatinine, μmol/L	73.14±12.62	75.18±18.91	0.731
EF	0.54±0.06	0.55±0.07	0.548
LAD, mm	38.70±5.34	38.5±5.28	0.883
PASP, mm Hg	35.8±9.80	36.8±6.67	0.640
LVEDV, mL	120.6±40.80	121.0±35.6	0.967
β-Blockers, %	14 (93.0)	14 (93.0)	1.000
ACEI, %	7 (46.7)	7 (46.7)	1.000
CCB, %	9 (60.0)	9 (60.0)	1.000
Statins, %	8 (53.3)	8 (53.3)	1.000

Data presented as mean±SD or as a ratio. ACEI indicates angiotensin-converting enzyme inhibitor; BMI, body mass index; CCB, calcium channel blocker; DM, diabetes mellitus; EF, ejection fraction; HDL, high-density lipoprotein; LAD, left atrial diameter; LDL, low-density lipoprotein; LVEDV, left ventricle end-diastolic volume; NYHA, New York Heart Association; PASP, pulmonary arterial systolic pressure; PoAF, postoperative atrial fibrillation.

were reverse transcribed with random primers. Specific circRNAs were amplified using the primers listed in Table 1. The assays for the following circRNAs were first performed on 365 samples from 9 candidates: hsa_circRNA_404686, hsa_circRNA_000367, hsa_circRNA_001729, hsa_circRNA_100790, hsa_circRNA_104270, hsa_circRNA_030162, hsa_circRNA_100789, hsa_circRNA_025016, and hsa_circRNA_102049. The expression level of 18S RNA was used as a stable endogenous control for normalization. All assays were performed in triplicate.

Annotation and Functional Prediction for hsa_circ_025016

To explore the targeted miRNA profile, the miRNA support vector regression algorithm was used to score and rank the efficiencies of the predicted miRNA targets. Hsa_circ_025016

was used as a seed to enrich a circRNA–miRNA–gene network according to analyses with TargetScan (http://www.targetscan.org/) combined with miRanda (http://www.microrna.org/). The biological pathways were defined with the Kyoto Encyclopedia of Genes and Genomes.

Statistical Analysis

In the discovery phase, paired *t* tests were performed to identify the circRNAs that were differentially expressed in the PoAF and non-PoAF groups. Multiple comparison corrections were performed by calculating the Benjamini–Hochberg false discovery rate. Filtering of all the circRNAs with fold changes ≥4 and false discovery rate values <0.05 yielded 9 circRNAs that were differentially expressed between the PoAF and non-PoAF groups. The training cohort (365) was divided into training data and validation data via 10-fold cross-validation

Table 4. Dysregulated circRNAs in Patients With PoAF Compared to Who Did Not Develop Af

P Value	FDR	FC (abs)	Gene Symbol	circRNA	Chrom
Upregulated circRNAs					
5.24085E-06	0.003754004	13.6095532	GPR137B	hsa_circRNA_404686	chr1
7.93744E-06	0.004796739	12.1794537	SIAE	hsa_circRNA_000367	chr11
0.000591413	0.035205611	5.3908059	ZNF646	hsa_circRNA_001729	chr16
4.69587E-05	0.010234685	4.5960716	CAPRIN1	hsa_circRNA_100790	chr11
1.88183E-05	0.007525187	4.3381437	FAM120B	hsa_circRNA_104270	chr6
0.000196313	0.021978096	4.1453242	TPT1	hsa_circRNA_030162	chr13
6.79548E-05	0.01284492	4.1117028	CAPRIN1	hsa_circRNA_100789	chr11
0.000257758	0.028857183	4.1002143	CACNA1C	hsa_circRNA_025016	chr12
4.64186E-05	0.010234685	4.0394495	TADA2A	hsa_circRNA_102049	chr17
6.88132E-05	0.01284492	3.6851138	CAPRIN1	hsa_circRNA_100787	chr11
4.5274E-05	0.010234685	3.6084518	TADA2A	hsa_circRNA_405571	chr17
0.001104905	0.047082396	2.7738857	TADA2A	hsa_circRNA_102051	chr17
0.000153536	0.020012406	2.4205083	RPL11	hsa_circRNA_010884	chr1
0.000732237	0.038163645	2.4201776	MTO1	hsa_circRNA_104135	chr6
0.000298757	0.026560557	2.3774407	RPS17L	hsa_circRNA_036567	chr15
4.04835E-05	0.010155248	2.3613541	EEF1A1	hsa_circRNA_077007	chr6
0.000141757	0.019506189	2.3492044	TRAPPC4	hsa_circRNA_400850	chr11
6.84306E-05	0.01284492	2.2456712	LYST	hsa_circRNA_005899	chr1
0.000037955	0.009704072	2.2092508	IFI30	hsa_circRNA_102484	chr19
3.8865E-07	0.002368837	2.2050474	R3HDM1	hsa_circRNA_056558	chr2
4.58024E-05	0.010234685	2.1796815	NONO	hsa_circRNA_091000	chrX
7.28006E-06	0.004608971	2.081796	TMSB10	hsa_circRNA_055387	chr2
1.63637E-05	0.007501896	2.0692549	RHOA	hsa_circRNA_065649	chr3
0.000308439	0.026756707	2.0369578	UBA52	hsa_circRNA_102488	chr19
Downregulated circRNAs					
0.000239906	0.023626342	2.6089515	EIF2S2	hsa_circRNA_402563	chr20
0.000429469	0.030594986	2.4898771	NHS	hsa_circRNA_104983	chrX
3.7363E-07	0.002368837	2.3873504	CARHSP1	hsa_circRNA_037798	chr16
0.000126081	0.018220113	2.2970696	MYH9	hsa_circRNA_400071	chr22
0.000580943	0.035205611	2.2293785	SNX29	hsa_circRNA_405439	chr16
0.000602971	0.035205611	2.1893188	FAM120A	hsa_circRNA_104826	chr9
0.000801696	0.040069723	2.1390998	CNKSR3	hsa_circRNA_104230	chr6

circRNAs indicates circular RNAs; FC (abs), Fold change (absolute ratio); FDR, false discovery rate.

method using the R package caret. The cutoff value, sensitivity, specificity, and area under the receiver operating characteristic curve of each training data were estimated by roc () function of R package *Daim*. The sensitivity and specificity of each validation data were calculated based on the corresponding cutoff of each training data, while the sensitivity and specificity of validation cohort (284) were calculated based on the mean cutoff of training data, both with a diagnostic test 4-fold table. Based on the expression of hsa_circ_025016 and the incidence of PoAF (16.7%) in discovery phage, at an α level of 5% and a power of 90%, 15 patients of PoAF and 75 patients of no-PoAF would be required. Therefore, sample size in training and validation cohorts was sufficient. All statistical calculations were

Table 5. Demographic and Clinical Characteristics by Presence of PoAF in Training Cohorts (365)

Characteristics	PoAF Group n=75	No PoAF Group n=290	P Value
Sex (male/female)	60/15	209/81	0.214
Age, y	63.55±8.05	61.86±8.56	0.112
BMI, kg/m^2	25.23±2.41	24.68±2.88	0.166
Smoking, %	52 (69.3)	180 (62.2)	0.244
Heart rate, beats/min	74.01±9.86	72.9±11.04	0.398
NYHA III–IV, %	11 (14.6)	40 (13.8)	0.139
Hypertension, %	49 (65.3)	190 (65.5)	0.152
DM, %	32 (42.6)	116 (40.0)	0.675
Pre–myocardial infarction, %	45 (60.0)	140 (48.3)	0.009[†]
Blood glucose, mmol/L	6.60±3.29	6.27±2.25	0.414
Cholesterol, mmol/L	4.38±1.30	4.27±1.59	0.535
Triglyceride, mmol/L	2.16±2.59	2.03±1.75	0.682
LDL, mmol/L	2.80±0.84	2.63±0.92	0.128
HDL, mmol/L	0.93±0.16	0.95±0.18	0.349
Creatinine, μmol/L	73.01±12.59	73.28±16.13	0.876
CKMB, U/L	9.96±7.61	9.24±9.65	0.492
cTNT, ng/mL	0.10±0.44	0.07±0.47	0.605
EF	0.54±0.05	0.55±0.05	0.216
LAD, mm	38.01±4.24	36.96±4.23	0.063
PASP, mm Hg	35.18±3.43	34.18±4.47	0.128
LVEDV, mL	120.62±40.79	110.44±32.66	0.048[†]
β-Blockers, %	68 (90.6)	270 (93.1)	0.472
ACEI, %	34 (45.3)	133 (45.8)	0.935
CCB, %	40 (53.3)	145 (50.0)	0.607
Statins, %	42 (56.0)	205 (70.7)	0.015[†]

Data presented as mean±SD or as a ratio. ACEI indicates angiotensin-converting enzyme inhibitor; BMI, body mass index; CCB, calcium channel blocker; CKMB, creatine kinase MB; cTNT, cardiac troponin T; DM, diabetes mellitus; EF, ejection fraction; HDL, high-density lipoprotein; LAD, left atrial diameter; LDL, low-density lipoprotein; LVEDV, left ventricle end-diastolic volume; NYHA, New York Heart Association; PASP, pulmonary arterial systolic pressure; PoAF, postoperative atrial fibrillation.
[†]$P<0.05$.

Table 6. Demographic and Clinical Characteristics by Presence of PoAF in Validation Cohorts (284)

| Characteristics	PoAF Group n=68	No PoAF Group n=216	P Value
Sex (male/female)	55/13	159/57	0.225
Age, y	61.97±6.08	62.05±10.16	0.951
BMI, kg/m^2	23.75±5.83	24.88±3.61	0.127
Smoking, %	42 (61.7)	128 (59.2)	0.713
Heart rate, beats/min	76.43±11.72	74.32±9.84	0.113
NYHA III–IV, %	7 (10.2)	31 (14.3)	0.607
Hypertension, %	47 (69.1)	157 (72.6)	0.568
DM, %	22 (32.4)	80 (37.0)	0.483
Pre–myocardial infarction, %	33 (48.5)	92 (42.5)	0.337
Blood glucose, mmol/L	6.89±2.68	6.44±2.47	0.242
Cholesterol, mmol/L	4.69±1.33	4.30±1.05	0.067
Triglyceride, mmol/L	1.90±0.71	1.95±1.40	0.837
LDL, mmol/L	3.00±1.05	2.73±0.83	0.108
HDL, mmol/L	1.00±0.18	0.95±0.17	0.094
Creatinine, μmol/L	77.37±17.52	79.40±20.37	0.469
CKMB, U/L	20.18±42.48	17.04±25.16	0.477
cTNT, ng/mL	0.28±1.24	0.23±0.86	0.679
EF	0.55±0.05	0.55±0.04	0.699
LAD, mm	38.12±4.49	36.73±4.79	0.081
PASP, mm Hg	34.53±9.73	33.72±5.79	0.489
LVEDV, mL	117.58±32.08	107.14±31.54	0.020[†]
β-Blockers, %	62 (91.1)	204 (94.4)	0.335
ACEI, %	23 (33.8)	88 (40.7)	0.335
CCB, %	48 (70.0)	131 (60.6)	0.139
Statins, %	29 (42.6)	132 (61.1)	0.007[†]

Data presented as mean±SD or as a ratio. ACEI indicates angiotensin-converting enzyme inhibitor; BMI, body mass index; CCB, calcium channel blocker; CKMB, creatine kinase MB; cTNT, cardiac troponin T; DM, diabetes mellitus; EF, ejection fraction; HDL, high-density lipoprotein; LAD, left atrial diameter; LDL, low-density lipoprotein; LVEDV, left ventricle end-diastolic volume; NYHA, New York Heart Association; PASP, pulmonary arterial systolic pressure; PoAF, postoperative atrial fibrillation.
[†]$P<0.05$.

performed using SPSS 18.0 software (IBM, Chicago, IL). In all statistical analyses, the circRNAs levels were log-transformed using the base 2 logarithm to account for the skewnesses of their distributions. Continuous variables were compared using unpaired Student t tests. Categorical variables were compared using χ^2 tests.[8] A logistic regression was used to select the diagnostic circRNA markers based on the training data set. $P<0.05$ was considered significant in all comparisons.

Results

Levels of Circulating circRNAs Were Altered in Patients Who Developed PoAF

The characteristics of the 120 patients included in this study are summarized in Table 2. Because of the relatively small size of the cohort and low incidence of AF after surgery, none of the characteristics were found to be associated with PoAF. In consideration of our previous study and other clinical
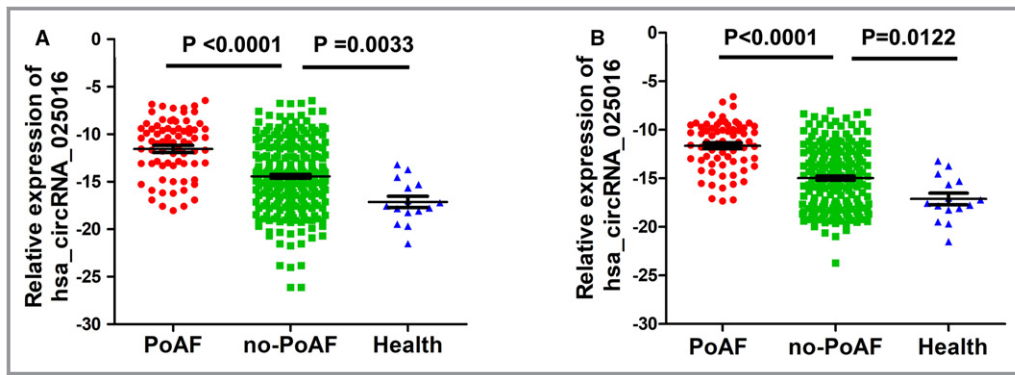
Figure 2. Hsa_circRNA_025016 as a prognostic indicator of PoAF. A and B, Comparison of the hsa_circRNA_025016 levels in the healthy controls, patients with PoAF, and patients without PoAF in the training (A) and validation (B) cohorts. Unpaired Student *t* tests were used to determine the differences in hsa_circRNA_025016 levels between the groups (the error bars represent the SEM). PoAF indicates postoperative atrial fibrillation.

studies, patients with and without PoAF were matched for age, sex, smoking, left atrial diameter, left ventricle end-diastolic volume, and the use of statins before surgery. The characteristics of the 30 patients are presented in Table 3. In an attempt to study alterations in the circulating circRNAs in the PoAF patients, microarray analyses were performed on the RNA from the plasma of these 30 patients.

As illustrated in Figure 1B, 31 circRNAs were specifically dysregulated (24 circRNAs were upregulated, and 7 circRNAs were downregulated with the fold changes ≥2 and FDR (false discovery rate) <0.05, Table 4) in patients with PoAF compared with who did not develop AF. Filtering of all of the differentially expressed circRNAs for changes ≥4-fold yielded 9 circRNA candidates (hsa_circRNA_404686, hsa_circRNA_000367, hsa_circRNA_001729, hsa_circRNA_100790, hsa_circRNA_104270, hsa_circRNA_030162, hsa_circRNA_100789, hsa_circRNA_025016, and hsa_circRNA_102049) for further testing via quantitative real-time polymerase chain reaction.

Hsa_circRNA_025016 Was Elevated in Patients With PoAF

The characteristics of the training and validation cohorts' participants are presented in Tables 5 and 6. The 9 candidate

circRNAs discovered in the microarray analyses were first tested with quantitative real-time polymerase chain reaction using an independent cohort (training cohort) of 365 plasma samples. Six of the 9 circRNAs were stably detected in the plasma samples. Only 2 circRNAs—hsa_circRNA_025016 and hsa_circRNA_404686—were significantly upregulated and predicted PoAF in patients after CABG (odds ratio, 1.355 95% CI [1.250–1.468], *P*<0.0001 and 1.098 95% CI [1.009–1.195], *P*=0.03, Figure 1C). Because the levels of hsa_circRNA_404686 were positively correlated with hsa_circRNA_025016 (Figure 1D), and hsa_circRNA_025016 exhibited the strongest association with PoAF, hsa_circRNA_025016 was selected for further analyses.

Next, we investigated the expression of hsa_circRNA_025016 in the training and validation cohorts. We found that hsa_circRNA_025016 was elevated in patients with coronary heart disease compared with the healthy controls. Additionally, an increase in the hsa_circRNA_025016 level was observed in patients with PoAF compared with those without PoAF (Figure 2A and 2B).

Hsa_circRNA_025016 as a Prognostic Indicator for PoAF

The means and 95% CIs of the cutoff, sensitivity, and specificity were calculated via a 10-fold cross-validation

Table 7. Cutoff and Prediction Performance of hsa_circRNA_025016 in PoAF in Training Cohort (n=365)

	Training Data	Validation Data
Cutoff	−12.118 (−12.334, −11.902)	⋯
AUC	0.802 (0.798, 0.806)	⋯
Sensitivity	0.794 (0.777, 0.811)	0.766 (0.644, 0.889)
Specificity	0.776 (0.763, 0.789)	0.784 (0.749, 0.819)

AUC indicates area under the receiver operating characteristic curve; PoAF, postoperative atrial fibrillation.

Table 8. hsa_circRNA_025016 and PoAF in Validation Cohort (n=284)

	hsa_circRNA_025016		
	High (> −12.118)	Low (< −12.118)	*P* Value
PoAF	50	18	<0.0001*
No PoAF	49	167	

Sensitivity=73.52%, specificity=77.30%. PoAF indicates postoperative atrial fibrillation.
**P*<0.0001.
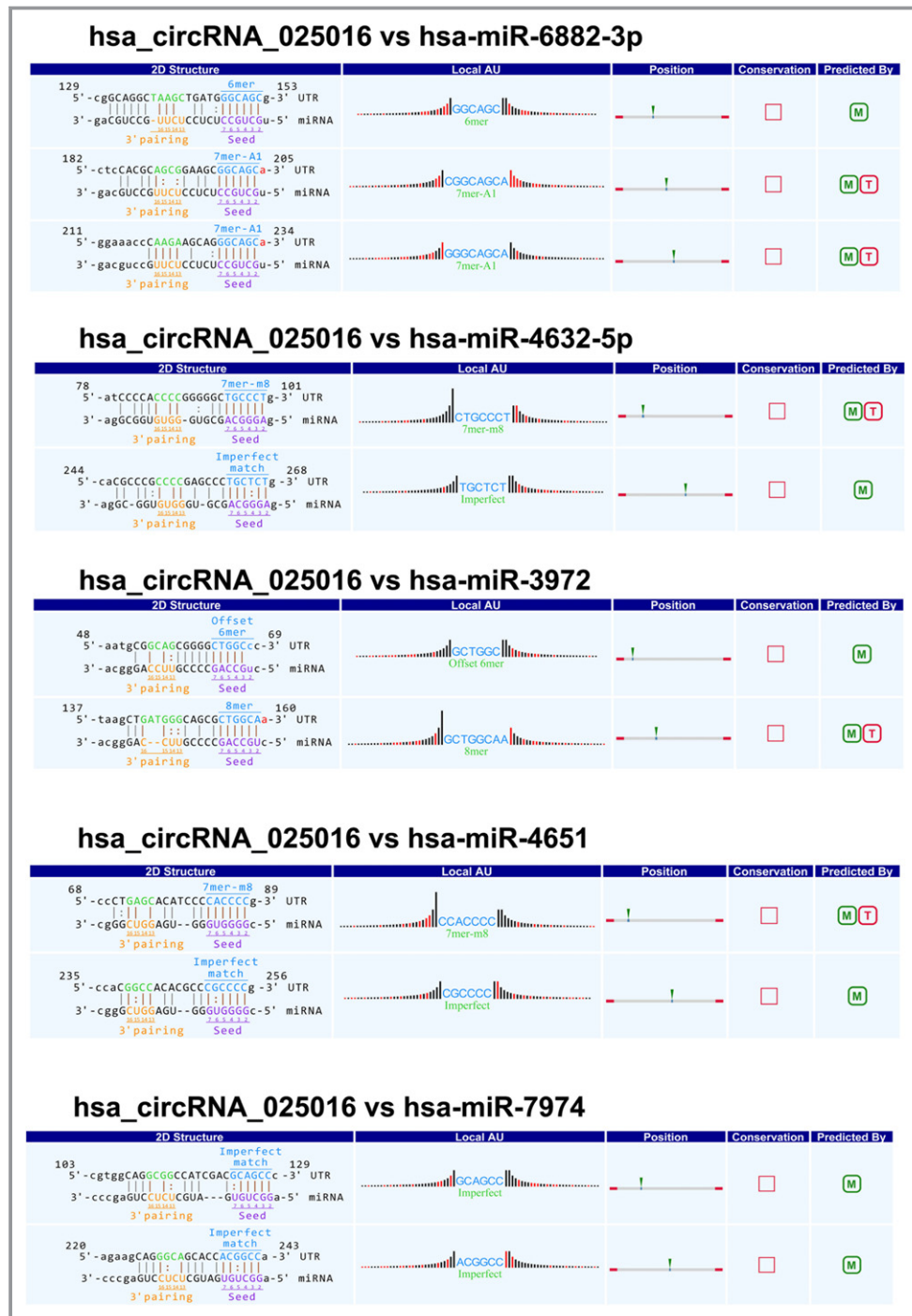
Figure 3. Prediction of the miRNAs targeted by hsa_circRNA_025016.

method using the R package caret. The mean cutoff for 10 training data set was −12.118, and we considered this to be the optimum cutoff. The area under the receiver operating characteristic curve was 0.802, and the 95% confidence interval was 0.798 to 0.806. For the training data, the sensitivity was 0.794% (0.777–0.811%), and the specificity was 0.776% (0.763–0.789%); these results indicated a good predictive effect (Table 7).

The mean cutoff of −12.118 was used to evaluate the predictive effect in an independent validation cohort set (284 plasma samples). In the validation cohort, patients were divided into a high hsa_circRNA_025016 expression group and a low hsa_circRNA_025016 expression group according to the cutoff value. The predicted probability was evaluated with the χ^2 test. This cutoff value was able to predict PoAF in the independent cohort (Pearson χ^2=56.6,
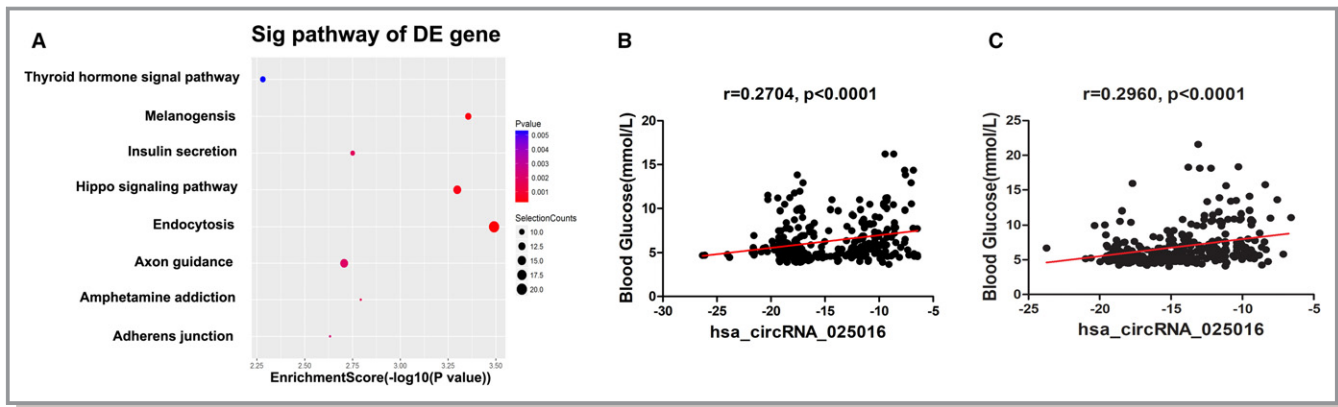
Figure 4. Prediction and annotation of the targets of hsa_circRNA_025016 in the miRNA-mRNA network. A, Kyoto Encyclopedia of Genes and Genomes analysis based on the hsa_circRNA_025016-miRNA-mRNAs network. B and C, The relationships between blood glucose and hsa_circRNA_025016 in the training (B) (r=0.2704, P<0.0001) and validation cohorts (C) (r=0.2960, P<0.0001).

P<0.0001, sensitivity=73.52% and specificity=77.83%, Table 8).

Prediction and Annotation of the hsa_circRNA_025016 Targeted miRNA-mRNA Network

We assumed that hsa_circRNA_025016 exerts its effects by acting as a miRNA sponge. The miRNA support vector regression algorithm was used to score and rank the efficiency of the predicted miRNA targets. Five miRNAs were identified as conserved between the TargetScan and miRanda predictions, including hsa-miR-6882-3p, hsa-miR-4632-5p, hsa-miR-3972, hsa-miR-4651, and hsa-miR-7974 (Figure 3). To explore the possible mechanism of hsa_circRNA_025016, Kyoto Encyclopedia of Genes and Genomes pathway analysis was utilized based on the predicted results. The results suggested that melanogenesis, insulin secretion, and the thyroid hormone signaling pathway may be modulated by hsa_-circRNA_025016 (Figure 4A).

To explore the effect of hsa_circRNA_025016 on the insulin secretion pathway, the fasting blood glucose levels were compared according to the hsa_circRNA_025016 expression levels. As presented in Tables 9 and 10, the fasting blood glucose levels were significantly higher in patients with high levels of hsa_circRNA_025016 expression in both cohorts. Moreover, hsa_circRNA_025016 exhibited significant linear correlations with fasting blood glucose in the training and validation cohorts (Figure 4B and 4C).

Discussion

PoAF is related to increased morbidity and mortality after cardiac surgery and is an independent risk factor for poor long-term outcomes.[9] However, poor understandings of the underlying mechanism and predisposing factors have limited the possible strategies for preventive preoperative measures. In the present study, we performed a wide screen to identify circRNAs that are differentially present in the plasma of patients with and without PoAF. Fifteen of the circRNAs were distinguished through hierarchical clustering analysis. When tested in the training cohort, at least 3 of these circRNAs candidates were found to be significantly upregulated in PoAF patients, and the strongest association was found for hsa_circRNA_025016. Surprisingly, the cutoff value for hsa_circRNA_025016 obtained from the training data resulted in high accuracy in the diagnosis of PoAF in the independent validation cohort set.

The study by Harling et al[7] demonstrated that the levels of circulating and atrial tissue microRNAs are potential markers of PoAF. Other studies have found dysregulations of microRNAs in AF.[10,11] However, one of the main mechanisms of circRNAs is acting as inhibitors of miRNA that function by binding ("sponging") a specific miRNA. Furthermore, circRNAs are resistant to the exonuclease RNase R because of the lack of free ends; thus, circRNAs possess distinct advantages as new biomarkers for diagnoses, prognoses, and therapeutic response predictions. Moreover, circRNAs are a special class of noncoding RNAs that are abundant in body fluids. Therefore, we believe that circRNAs represent a class of highly stable biomarkers for the prediction of PoAF.

Hsa_circ_025016 was selected and further tested in 2 independent larger cohorts to demonstrate its high diagnostic value as a biomarker for PoAF. However, the mechanism of hsa_circ_025016 has not yet been demonstrated. To our surprise, the host gene symbol is calcium voltage-gated channel subunit α1 C (CACNA1C), which encodes an α-1 subunit of a voltage-dependent calcium channel. Loss-of-function mutations in CACNA1C have been reported in short QT syndrome.[12]

Table 9. Demographic and Clinical Characteristics by the Expression of hsa_circRNA_025016 in Training Cohort (n=365)

| Characteristics | hsa_circRNA_025016 | | |
	High (> −12.118, n=123)	Low (< −12.118, n=242)	P Value
Sex (male/female)	90/33	179/63	0.870
Age, y	62.60±9.35	62.00±9.06	0.554
BMI, kg/m^2	25.11±2.66	24.63±2.34	0.078
Smoking, %	79 (64.2)	153 (63.2)	0.850
Heart rate, beats/min	73.91±10.99	72.7±10.07	0.294
NYHA III–IV, %	14 (11.4)	37 (15.2)	0.308
Hypertension, %	78 (63.4)	161 (66.5)	0.554
DM, %	48 (39.0)	101 (41.7)	0.618
Pre–myocardial infarction, %	60 (48.7)	125 (51.6)	0.603
Blood glucose, mmol/L	6.86±2.89	6.10±3.57	0.042[†]
Cholesterol, mmol/L	4.45±1.49	4.20±1.78	0.182
Triglyceride, mmol/L	2.19±2.28	1.99±2.58	0.456
LDL, mmol/L	2.76±0.99	2.61±0.96	0.164
HDL, mmol/L	0.96±0.28	0.93±0.18	0.216
Creatinine, μmol/L	75.09±14.87	72.27±14.56	0.083
CKMB, U/L	9.87±7.71	9.14±9.46	0.459
cTNT, ng/mL	0.10±0.30	0.06±0.45	0.374
EF	0.55±0.05	0.54±0.06	0.873
LAD, mm	37.45±4.44	37.05±4.47	0.419
PASP, mm Hg	34.89±4.45	34.13±4.80	0.144
LVEDV, mL	115.54±35.21	111.00±43.99	0.321
β-Blockers, %	116 (94.3)	222 (91.7)	0.374
ACEI, %	54 (43.9)	113 (46.7)	0.613
CCB, %	64 (52.0)	121 (50.0)	0.713
Statins, %	79 (64.2)	168 (69.4)	0.316

Data presented as mean±SD or as a ratio. ACEI indicates angiotensin converting enzyme inhibitor; BMI, body mass index; CCB, calcium channel blocker; CKMB, creatine kinase MB; cTNT, cardiac troponin T; DM, diabetes mellitus; EF, ejection fraction; HDL, high-density lipoprotein; LAD, left atrial diameter; LDL, low-density lipoprotein; LVEDV, left ventricle end-diastolic volume; NYHA, New York Heart Association; PASP, pulmonary arterial systolic pressure.
[†]*P*<0.05.

Table 10. Demographic and Clinical Characteristics by the Expression of hsa_circRNA_025016 in Validation Cohort (n=284)

| Characteristics | hsa_circRNA_025016 | | |
	High (> −12.118, n=99)	Low (< −12.118, n=185)	P Value
Sex (male/female)	77/22	137/48	0.487
Age, y	62.71±7.08	61.66±10.01	0.355
BMI, kg/m^2	24.62±4.98	24.57±3.69	0.924
Smoking, %	56 (56.5)	114 (61.6)	0.407
Heart rate, beats/min	75.17±10.65	74.64±11.05	0.697
NYHA III–IV, %	13 (13.1)	25 (13.5)	0.928
Hypertension, %	65 (66.6)	139 (75.1)	0.091
DM, %	36 (36.4)	66 (35.6)	0.908
Pre–myocardial infarction, %	44 (44.4)	81 (43.7)	0.915
Blood glucose, mmol/L	7.02±2.15	6.29±2.71	0.021[†]
Cholesterol, mmol/L	4.53±1.25	4.31±1.02	0.111
Triglyceride, mmol/L	2.09±1.24	1.83±0.77	0.052
LDL, mmol/L	2.89±0.904	2.73±0.781	0.127
HDL, mmol/L	0.99±0.17	0.95±0.18	0.070
Creatinine, μmol/L	78.06±17.28	79.35±20.02	0.588
CKMB, U/L	18.87±38.76	17.25±25.38	0.712
cTNT, ng/mL	0.26±1.16	0.23±0.87	0.806
EF	0.55±0.05	0.55±0.03	0.690
LAD, mm	37.85±4.55	36.66±4.83	0.045[†]
PASP, mm Hg	33.98±5.76	33.84±7.02	0.865
LVEDV, mL	112.76±28.90	107.97±35.56	0.250
β-Blockers, %	91 (91.9)	174 (94.0)	0.492
ACEI, %	32 (32.3)	79 (42.7)	0.087
CCB, %	62 (62.9)	117 (63.2)	0.918
Statins, %	49 (49.5)	112 (60.5)	0.073

Data presented as mean±SD or as a ratio. ACEI indicates angiotensin-converting enzyme inhibitor; BMI, body mass index; CCB, calcium channel blocker; CKMB, creatine kinase MB; cTNT, cardiac troponin T; DM, diabetes mellitus; EF, ejection fraction; HDL, high-density lipoprotein; LAD, left atrial diameter; LDL, low-density lipoprotein; LVEDV, left ventricle end-diastolic volume; NYHA, New York Heart Association; PASP, pulmonary arterial systolic pressure.
[†]*P*<0.05.

Another mechanism of circRNAs is acting as miRNA sponges and regulating the circRNA-miRNA-mRNA network. Therefore, we predicted the top 5 candidate miRNA binding targets according to the miRNA support vector regression scores and gained further insight into the functions of hsa_circ_025016. Kyoto Encyclopedia of Genes and Genomes pathway analysis was utilized based on the predicted results from the TargetScan and miRanda analyses.

Interestingly, melanogenesis, insulin secretion, and the thyroid hormone signaling pathways were found to be the potentially affected pathways. Tatsuishi et al reported that blood glucose is a very strong predictor of PoAF after CABG.[13] More importantly, we found that fasting blood glucose was increased in patients with high hsa_circ_025016 expression levels. Moreover, hsa_circ_025016 expression levels were positively correlated with fasting

blood glucose in patients before surgery. These findings suggest that modulating insulin secretion may be one of the functions of hsa_circ_025016. Kirchhof et al[14] found that the dysregulation of PITX2 expression could be responsible for susceptibility to AF. Expression array analyses have also suggested that melanogenesis is affected by the reduced expression of Pitx2c. Intriguingly, melanocyte-like cells are present in mouse and human hearts that are predisposed to AF.[15] Additionally, melanocortin can exert electrophysiological effects and stimulate the expression of interleukins.[16] A recent review demonstrated that subclinical hyperthyroidism is associated with increased coronary heart disease events, mortality, and the incidence of AF.[17,18] Therefore, thyroid hormone may be another potential target that is affected by hsa_circ_025016. However, the underlying mechanisms by which hsa_circ_025016 regulates AF via affecting melanogenesis, insulin secretion, and the thyroid hormone signaling pathway need to be explored in future studies.

Conclusions

In conclusion, we found a plasma circRNA in a large number of participants that is unregulated in patients who developed PoAF when compared with non-PoAF patients with a high degree of accuracy. Our study demonstrates that this plasma hsa_circ_025016 holds potential as a biomarker for the prediction of PoAF and could enable us to more accurately target patients who are at risk and thereby improve the effects of prophylactic treatments.

Disclosures

None.

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