Downregulation of autophagy-related circular RNA (ACR) is correlated with poor survival of patients with chronic heart failure

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ABSTRACT

Autophagy-related circular RNA (ACR) has been reported to protect myocardial tissues from injury and participate in chronic heart failure (CHF), while its role in CHF is unknown. This study aimed to study the role of ACR in CHF. ACR and miR-532 levels in CHF (ischemic-origin, n = 60) patients and healthy controls (n = 60) were analyzed by RT-qPCR. The prognostic value of ACR was analyzed by survival curve analysis. ACR was overexpressed in cardiomyocytes, and the effects of ACR overexpression on the expression of miR-532 and the methylation of miR-532 gene were analyzed using RT-qPCR and methylation-specific PCR (MSP). Cardiomyocyte apoptosis under hypoxic conditions was analyzed with cell apoptosis assay. It was observed that ACR expression was downregulated in CHF. Kaplan-Meier and multivariate Cox regression analysis suggested that low ACR predicted overall survival of CHF patients and ACR was inversely correlated with miR-532 across plasma samples. In cardiomyocytes, ACR increased miR-532 gene methylation to decrease its expression. Cell apoptosis analysis showed that ACR overexpression reduced the enhancing effects of miR-532 overexpression on cardiomyocyte apoptosis under hypoxic conditions. Therefore, ACR is downregulated in CHF and may suppress hypoxia-induced cardiomyocytes by downregulating miR-532 via methylation.

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Highlights

- ACR is downregulated in CHF.
- Low ACR expression predicts poor survival of CHF.
- ACR downregulates miR-532 through methylation.

Introduction

Chronic heart failure (CHF) is a consequence of the reduced ability of heart muscles to pump blood [1]. CHF is mainly caused by hypertension, coronary artery disease, and valve conditions [2]. Besides that, the prevalence of CHF is higher in patients with diabetes or obesity than in normal populations [3,4]. Due to the reduced blood and oxygen supply, patients with CHF may develop severe hypoxemia and even hypoxia, leading to a high mortality rate [5,6]. It is estimated that even with active treatment, 10% of CHF patients will die within 30 days of diagnosis, and only fewer than 50% of CHF patients can survive more than 5 years after the initial diagnosis.

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At present, CHF patients may be treated with angiotensin to slowdown disease progression [7]. However, there is no cure for CHF at present. Certain molecular players may be targeted to treat CHF [8]. However, effective and safe targets remain lacking. CircRNAs participate in human diseases, including CHF, mainly by indirectly affecting protein accumulation [9,10]. Therefore, circRNAs may be targeted for CHF treatment. However, the function circRNAs in CHF is largely unknown. A recent study showed that autophagy-related circular RNA (ACR) protects myocardial ischemia/reperfusion injury [11]. Our preliminary microarray analysis revealed that ACR is down-regulated in CHF and inversely correlated with miR-532 (data not shown), which can promote cardiomyocyte apoptosis in diabetic hearts [12]. Therefore, ACR and miR-532 may interact with each other to participate in CHF. Therefore, this study was carried out to investigate their interactions in CHF.

Materials and methods

**CHF patients and healthy controls**

We enrolled a total of 60 CHF patients (32 males and 28 females) at The Affiliated Hospital of Liaoning University of Traditional Chinese Medicine between January 2014 and June 2015. All patients were of ischemic origin. All CHF patients were newly diagnosed cases and not treated within 3 months prior to admission. All subjects (1) were over 18 years old and had been treated for heart failure for more than a month and (2) met the diagnostic criteria for CHF or had left ventricular enlargement and decreased left ventricular ejection fraction. Patients who had i) infection, ii) cancer, iii) history of surgery within 1 year, iv) history of cerebral vascular events within 6 months, v) heart assist devices, or vi) liver or renal failure were excluded from the study. In addition, 60 healthy volunteers (31 males and 29 females) were included as the control. All CHF patients and control subjects were at the age between 49 and 71 years, with a median age of 60 years. All participants signed informed consent. The study was approved by the Ethics Committee of our hospital. Table 1 shows the clinical data of all patients and controls.

**Blood extraction and follow-up**

Fasting blood (2 ml) was extracted in a tube with 10x citric acid and centrifugated for 15 min at 1200 g to separate plasma samples. After admission, the CHF 60 patients were followed every month for a total of 5 years to monitor their survival.

**Cardiomyocytes and transient transfections**

Human cardiomyocyte cell-line AC16 (EMD Millipore) was cultured in DMEM supplemented with 2 mM L-glutamine, 12% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For hypoxia

| Feature            | Controls (n = 60) | CHF (n = 60) | P-value  |
|--------------------|------------------|-------------|----------|
| Age (years)        | 66.52 ± 0.69     | 67.53 ± 0.51| 0.09     |
| Sex (male/female)  | 31/29            | 32/28       | 0.423    |
| BMI (kg/m²)        | 25.13 ± 0.38     | 25.69 ± 0.39| 0.256    |
| Smoking history (never/ever) | 29/31 | 28/32 | 0.421 |
| Drinking history (never/ever) | 27/33 | 29/31 | 0.425 |
| TC (nM)            | 4.77 ± 0.19      | 4.73 ± 0.16 | 0.695 |
| TG (nM)            | 1.39 ± 0.69      | 1.40 ± 0.58 | 0.452 |
| LDL-C (nM)         | 2.85 ± 0.14      | 3.05 ± 0.19 | 0.152 |
| HDL-C (nM)         | 1.21 ± 0.42      | 1.22 ± 0.05 | 0.566 |
| UA (µM)            | 354.86 ± 15.35   | 356.42 ± 13.70 | 0.333 |
| BNP (ng/l)         | 67.26 ± 20.79    | 1,522.84 ± 853.85 | <0.001 |
| LVEF (%)           | 59.49 ± 0.58     | 32.45 ± 3.63 | <0.001 |
| Complication (no/yes) | 25/35 | 26/34 | 0.266 |
| Hypertension       | 25/35            | 26/34       | 0.266    |
| Diabetes           | 29/31            | 27/33       | 0.152    |

Values were presented as mean ±SD. P < 0.05 was considered to be statistically significant. BMI, body mass index; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; UA, uric acid; BNP, brain natriuretic peptide; LVEF, left ventricle ejection fraction.
treatment, cells were cultured in an incubator with 1%O₂/94%N₂/5%CO₂ for 48 h. Hypoxic and normoxic media were prepared by bubbling with gas. Medium with normal O₂ concentration was used for normal cell culture.

ACR expression vector (pcDNA3.1) was constructed. Transfection of AC16 cells was performed by addition of 1 µg ACR expression vector or 50 nM miR-532 mimic with Lipofectamine 2000 (Thermo Fisher Scientific) into 10⁵ cells for 6 h. After washed with fresh medium, cells were cultured in fresh medium for 48 h prior to the subsequent experiments. Control (C, untransfected cells) and NC (empty vector- or miRNA NC-transfected cells) experiments were also included.

**RNA sample preparations**

Total RNAs were isolated from AC16 cells and plasma samples using RiboZol™ RNA Extraction Reagent (VWR) and treated with DNase I (Invitrogen) for 2 h at 37°C to remove genomic DNAs.

**RT-qPCRs**

Following the preparation of cDNA samples with SS-IV-RT system (Invitrogen), ACR and miR-532 were analyzed by qPCR with 18S rRNA or U6 as the internal control. QPCR mixture was prepared using SYBR® Green Quantitative RT-qPCR Kit (Sigma-Aldrich). QPCR reactions were performed on CFX Opus 96 Real-Time PCR System (Bio-Rad) at the conditions of 1 min at 95°C, followed by 40 cycles of 10 s at 95°C and 55 s at 58°C. The relative gene levels were calculated using the 2⁻ΔΔCT method [13]. QPCR primers were 5’-GAAGTTGCTTTATGTTCTGG-3’ (forward) and 5’-TGATGTTGTTTATGTTGTTTTCGAGG-3’ (reverse) for ACR; 5’-GTAACCGGGGTAACCCCGG-3’ (forward) and 5’-CATGCTGCTGCTGCTGCTG-3’ (reverse) for 18S rRNA; 5’-CATGCTGCTGCTGCTGCTG-3’ (reverse) for 18S rRNA;
GTGTAAGGAC-3' (forward) for miR-532. U6 primer and universal reverse primer were from the kit.

**Methylation-specific PCR (MSP)**

Methylation-specific PCR (MSP) was performed as previously reported [14]. Briefly, genomic DNA was isolated, converted using EZ DNA Methylation-Gold Kits (ZYMOR RESEARCH), and subjected to MSP and routine PCRs using Titanium Taq PCR kit (Takara Bio) to analyze the methylation of miR-532 gene. PCR products were analyzed on 1% agarose gel electrophoresis, stained with ethidium bromide, and photographed using MyECL imager (Thermo Fisher Scientific). Primers were 5'-CTTCTAATGACCTGCATGCC-3' and 5'-AGACATGCTGTAATGAGAAGGTG-3' for routine PCR and 5'-TTTTTTAATGATTTGTATGTT-3' and 5'-AAACATACTATAATAAAAAATA-3' for MSP. These primers were targeted to the promoter region of miR-532 (from –70 to –370).

**Cell apoptosis assay**

AC16 cells were resuspended as 20,000 cells per ml in a tube and cultured under hypoxic conditions (1%O₂/94%N₂/5%CO₂) or normal conditions for 48 h with three replicates for each experiment. Cells were stained with PI and Annexin V-FITC (Annexin V-FITC Apoptosis Detection Kit, Sigma-Aldrich) and sorted by flow cytometry using ZE5 Cell Analyzer (Bio-Rad).

**TUNEL analysis**

Cells were cultured as described above, collected after transfection, and washed with PBS. After fixed in 4% paraformaldehyde fixation, apoptotic cells were detected in situ using Cell Death Detection Kit (Promega, USA). TUNEL-positive cells were considered apoptotic cells and photographed using a fluorescence microscope (Axio Vert A1, Zeiss).

**Western blot**

Proteins were isolated using RIPA lysis buffer from cells at 48 h after transfection, denatured, separated on 10% SDS-PAGE, and transferred onto PVDF membrane (Sigma-Aldrich). The membranes were blocked in 5% fat-free milk and incubated with rabbit antibodies against Bax (1:500, ab54154, Abcam, Hong Kong), Bcl2 (1:500, ab182858, Abcam, Hong Kong), and GAPDH (1:500, ab37168, Abcam, Hong Kong). After incubating with secondary antibodies, signals were detected using ECL detecting system (Applygen, Beijing, China) and photographed using MyECL imager (Thermo Fisher Scientific).

**Statistical analysis**

Student’s t-test was applied to compare two groups. Patients were assigned to high and low ACR groups (n = 30) with median ACR expression level in CHF plasma samples as the cutoff value. Survival curves were compared by log-rank test. P values <0.05 were considered statistically significant.

**Results**

**ACR and miR-532 expression was altered in CHF patients and hypoxia-treated AC16 cells**

Our preliminary sequencing analysis revealed the altered ACR and miR-532 expression in CHF, suggesting their potential involvement in CHF. To further confirm their expression in CHF, both ACR and miR-532 levels in plasma samples collected from CHF patients (n = 60) and controls (n = 60) were detected using RT-qPCR. The results revealed that ACR was significantly under-expressed in the CHF group compared with the control group (P = 0.0090, Hazard Ratio = 3.077).

**Figure 2.** Low ACR levels were closely correlated with poor survival of CHF patients. Survival curves were plotted for both high and low ACR groups and compared by log-rank test. Data used here were the average values of three qPCR technical replicates presented in Figure 1.
Table 2. Multivariate Cox regression analysis for patients with chronic heart failure.

| Feature | Univariate analysis | Multivariate analysis |
|---------|---------------------|-----------------------|
|         | HR | 95% CI | P-value | HR | 95% CI | P-value |
| Age (≥67 years vs. <67 years) | 1.322 | 0.712-2.223 | 0.445 | 1.285 | 0.654-2.125 | 0.552 |
| Sex (male vs. female) | 1.248 | 0.592-2.097 | 0.664 | 1.206 | 0.536-1.711 | 0.735 |
| BMI (≥24 kg/m² vs. <24 kg/m²) | 1.569 | 0.779-2.405 | 0.333 | 1.655 | 0.693-2.845 | 0.254 |
| Smoking (ever vs. never) | 1.425 | 0.731-1.762 | 0.389 | 1.421 | 0.658-2.356 | 0.406 |
| Drinking (ever vs. never) | 1.333 | 0.695-2.197 | 0.466 | 1.311 | 0.684-2.254 | 0.258 |
| TC (high vs. low) | 1.409 | 0.765-2.335 | 0.433 | 1.654 | 0.654-2.125 | 0.356 |
| TG (high vs. low) | 1.619 | 0.733-2.565 | 0.289 | 1.836 | 0.744-2.844 | 0.258 |
| LDL-C (high vs. low) | 1.836 | 0.949-3.843 | 0.063 | 2.025 | 0.913-3.894 | 0.055 |
| HDL-C (low vs. high) | 1.955 | 0.914-2.819 | 0.117 | 1.936 | 0.856-3.151 | 0.131 |
| UA (high vs. low) | 2.038 | 0.986-4.004 | 0.059 | 1.955 | 0.859-3.256 | 0.069 |
| BNP (high vs. low) | 4.156 | 1.855-6.307 | 0.013 | 3.456 | 1.72-7.100 | 0.017 |
| LVEF (low vs. high) | 2.033 | 1.613-2.425 | 0.038 | 2.441 | 0.982-3.568 | 0.066 |
| ACR (low vs. high) | 3.256 | 1.842-6.498 | 0.004 | 3.596 | 1.824-6.458 | 0.015 |

BMI, body mass index; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; UA, uric acid; BNP, brain natriuretic peptide; LVEF, left ventricle ejection fraction.

Figure 3. ACR overexpression downregulated miR-532 in AC16 cells through methylation. Correlations between ACR and miR-532 across CHF (A) and control (B) samples were studied. AC16 cells were overexpressed with ACR or miR-532 (C). The involvement of ACR in regulating miR-532 accumulation (D) and the involvement of miR-532 in ACR accumulation (E) was also analyzed by RT-qPCRs. MSP was performed to analyze the involvement of ACR in miR-532 methylation (F). Three biological replicates were used in each experiments and mean ± SD values were presented. * p < 0.05.
group (Figure 1a) while miR-532 was significantly overexpressed (Figure 1b) in CHF than in the control group (p < 0.01). Hypoxia treatment of AC16 cells in 1%O₂/94%N₂/5%CO₂ for 48 h significantly decreased ACR accumulation (Figure 1c, p<0.05) and increased miR-532 accumulation (Figure 1d, p<0.05). Therefore, hypoxic conditions in CHF may alter ACR and miR-532 expression levels.

Low ACR levels predicted poor survival

CHF is related to high mortality. The survival analysis was performed to explore the role of ACR in predicting the survival of CHF patients. The results showed that patients in the low ACR group experienced a significantly lower overall survival rate than patients in the high ACR group (Figure 2). Multivariate Cox analysis suggested that ACR may serve as an independent prognostic factor for CHF patients’ survival [hazard ratio (HR) = 3.596, 95% CI = 1.824–6.458, P = 0.015] (Table 2). Therefore, decreased ACR accumulation in CHF patients may be involved in the death of CHF patients.

ACR overexpression downregulated miR-532 in AC16 cells through methylation under both hypoxic and normal conditions

Correlation analysis showed that ACR was inversely correlated with miR-532 across CHF (Figure 3a) and control (Figure 3b) samples, suggesting their potential interaction. Overexpression of AC16 cells with ACR or miR-532 significantly increased ACR and miR-532 levels between 48 h and 96 h under both hypoxic and normal conditions (Figure 3c, p<0.05). ACR overexpression decreased miR-532 expression between 48 h and 96 h under both hypoxic and normal conditions (Figure 3d, p<0.05), while miR-532 showed no regulatory role in ACR expression (Figure 3e). MSP analysis showed that ACR overexpression increased miR-532 gene methylation compared to the empty vector (figure 3f). Therefore, ACR overexpression may downregulate miR-532 in AC16 cells through methylation.

ACR and miR-532 participated in AC16 cell apoptosis induced by hypoxic conditions

Cell apoptosis contributes to the progression of CHF. Therefore, we performed both cell apoptosis (Figure 4) and TUNEL staining analysis (Figure 5) to analyze AC16 cell apoptosis under both hypoxia conditions (treatment for 48 h) and normal conditions. Cell apoptosis assay showed that ACR overexpression decreased cell apoptosis while miR-532 increased cell apoptosis under hypoxia conditions and ACR inhibited the role of miR-532 in AC16 cell apoptosis under hypoxic conditions (Figure 4a, p<0.05). In contrast, ACR and miR-532 overexpression failed to significantly affect AC16 apoptosis under...
normal conditions (Figure 4b). Similarly, TUNEL staining analysis also showed that ACR inhibited the inhibitory effect of miR-532 on AC16 cell apoptosis under hypoxic conditions (Figure 5a, p<0.05). In contrast, ACR and miR-532 overexpression failed to significantly affect AC16 apoptosis under normal conditions (Figure 5b). Western blot was performed to detect Bax and Bcl2, and Bax/Bcl2 ratios. Under hypoxic conditions, ACR overexpression increased Bax/Bcl2 ratio while miR-532 decreased Bax/Bcl2 ratio. In contrast, Bax/Bcl2 ratio was not affected by ACR and miR-532 under normal condition (Figure 5c, p<0.05). Therefore, ACR may inhibit hypoxia-induced cell apoptosis in CHF through miR-532.
Discussion

The study explored the involvement of ACR in CHF and its crosstalk with miR-532. We found that ACR was significantly downregulated in CHF. In addition, ACR may regulate miR-532 gene methylation to decrease its accumulation, thereby suppressing cardiomyocyte apoptosis under hypoxic conditions.

Cardiac ischemia is a major cause of CHF, especially in older people [15]. Zhou et al. reported that ACR modulates the Pink1/FAM65B pathway and suppresses autophagy to protect myocardial tissues from ischemia/reperfusion injury [11], suggesting the potential involvement of ACR in CHF. In this study, all CHF patients enrolled were of ischemic origin. ACR accumulation was decreased in CHF patients compared to healthy controls. In addition, hypoxia treatment also significantly decreased ACR expression in AC16 cells. Future studies may focus on the correlation between ACR downregulation in CHF and hypoxia condition.

MiR-532 is upregulated in diabetic hearts and promotes cardiomyocyte apoptosis to aggregate disease conditions [12]. We reported that miR-532 is more accumulated in CHF and promotes cardiomyocyte apoptosis under hypoxic conditions. Therefore, miR-532 has a similar expression pattern and function in diabetic heart and CHF, which may be explained by the fact that both types of heart disease are related to hypoxia.

MiRNAs target gene expression at different levels [16,17], while circRNAs may suppress the role of miRNAs by absorbing them [18]. Interestingly, our study showed that ACR overexpression increases miR-532 promoter methylation. It is known that promoter methylation significantly affects gene expression. Therefore, ACR may downregulate miR-532 expression by increasing its promoter methylation under both hypoxic and normal conditions. Interestingly, ACR and miR-532 interact with each other under both hypoxic and normal conditions and are correlated with each other across plasma samples from both CHF patients and controls. However, they only regulate AC16 cell apoptosis under hypoxic conditions but not normal conditions, suggesting the involvement of hypoxia-related factors in regulating AC16 cell apoptosis induced by hypoxia.

Conclusion

ACR is less accumulated in CHF and regulates miR-532 gene methylation to downregulate its expression, thereby reducing cardiomyocyte apoptosis under hypoxic conditions.

Ethical Approval and Consent to participate

Informed consent was obtained from all individual participants included in the study. All producers were approved by the Ethics Committee of The Affiliated Hospital of Liaoning University of Traditional Chinese Medicine and operated in keeping with the standards set out in the Announcement of Helsinki and laboratory guidelines of research in China.

Consent to publish

Not Applicable.

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Not Applicable.

Availability of supporting data

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Disclosure statement

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Authors’ contributions

Haihui Yan and Miao Tian: concept, manuscript writing, editing, and review
Dan Du and Chen Wang: data collection and analysis, manuscript preparation
All authors have read and approved the submission of the manuscript.

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