Differentially expressed Inc - NOS2P3-miR-939-5p axis in chronic heart failure inhibits myocardial and endothelial cells apoptosis via iNOS/TNFα pathway

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Abstract

Background: Inflammatory cytokines induced cells apoptosis is important for initiation and progression of chronic heart failure (CHF). Long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) were critical in this pathogenesis. However, their roles in inflammation and apoptosis of CHF remain unclear.

Methods: A total of 75 CHF and 36 non-CHF control patients were collected and the level of miR-939-5p was detected. Bioinformatics analysis and luciferase reporter assay was used to predict and verify pairs of lncRNA-miRNA-mRNA. RT-qPCR was used to evaluate the expression of lncRNA, miRNAs and mRNAs. CCK8 and flow cytometry were applied to determine cells vitality and apoptosis. Western blot was used for protein level analysis.

Results: CHF patients had elevated serum miR-939-5p, with greater increase in NYHA I-II patients than in NYHA III-IV. Moreover, miR-939-5p was positively correlated with BNP in NYHA III-IV patients, while not in NYHA I-II, suggesting that miR-939-5p might be an additional supplement for diagnosis of heart failure. Further study showed miR-939-5p mimic promoted proliferation and inhibited inflammatory cytokines induced apoptosis of HUVECs and H9C2, while inhibition of endogenous miR-939-5p by antagomir produced the opposite effects. INOS and TNFα were identified and confirmed as target genes of miR-939-5p. Moreover, we identified lncRNA-NOS2P3 as a sponge RNA to inhibit miR-939-5p expression, regulate the expression of iNOS/TNFα, and control inflammation induced myocardial and endothelial cells apoptosis.

Conclusions: Together, CHF patients exhibited elevated serum miR-939-5p especially in NYHA I-II grades. Lnc-NOS2P3-miR-939-5p-iNOS/TNFα pathway played an important role in regulating inflammatory cytokines induced cells apoptosis and provided a promising strategy for diagnosis and treatment of CHF.

Full Text

Due to technical limitations, full-text HTML conversion of this manuscript could not be completed.

However, the manuscript can be downloaded and accessed as a PDF.

Figures
The serum expression of miR-939-5p was higher in chronic heart failure patients than normal people. (A), The relative serum miR-939-5p expression was detected and compared in NYHA I-II grade, NYHA III-IV grade CHF patients and normal people by qRT-PCR, adding cel-39-3p as external control. Data are mean ± S.E.M.*p<0.05 and **p<0.01. B and C, The correlation between BNP and miR-939-5p level in CHF patients were analysed by Spearman’s correlation. (B), there is no obvious correlation in CHF NYHA I-II patients, (n=41) p>0.05. (C), miR-939-5p expression in CHF NYHA III-IV patients was significantly correlated with BNP level, (n=24) r=0.701, p<0.01.
MiR-939-5p dose-dependently regulated inflammation induced apoptosis in HUVECs. A-D,
miR-939-5p mimic or antagomir were transfected into HUVECs cells separately in 50nM, 100nM and 150nM, treated with or without cytokines mix (CM) of 2ng/ml IL-1β and 500IU/ml IFNγ. (A), proliferation by CCK8 was dose-dependently upregulated by miR-939-5p mimics in blank or CM treatment, mimic control as negative control. (B), proliferation was dose-dependently down-regulated by miR-939-5p antagomir compared to antagomir control. (C), apoptosis proteins by western blot with miR-939-5p mimic and control. (D), apoptosis proteins by western blot with miR-939-5p antagomir and control. E and F, effect of miR-939-5p on apoptosis by flow chart at different time points, 0h, 24h, 48h and 60h. (E), miR-939-5p mimic vs. mimic control. (F), miR-939-5p antagomir vs. antagomir control. Data are mean ±S.E.M.*p<0.05 and **p<0.01.
Figure 3

INOS was the validated target gene of miR-939-5p. A-F, 50, 100 and 150nM miR-939-5p mimics or antagonirs were transfected into HUVECs with controls. The mRNA levels of iNOS by RT-qPCR in blank or CM treatment, with miR-939-5p mimics and control, GAPDH as internal control (A), or with miR-939-5p antagonirs and control (B). C and D, protein levels of iNOS with dose-dependently expression of miR-939-5p mimic or antagonir and controls,
β-actin as internal control. (C), miR-939-5p mimic dose-dependently reduced protein level of iNOS. (D), knockdown of endogenous miR-939-5p dose dependently increased iNOS protein. E and F, the concentration of nitric oxide in culture supernatant was detected using Griess assay (OD 540nm), (E), nitric oxide level elevated with dose-dependent expression of miR-939-5p mimics vs. control. (F), miR-939-5p antagomir reduced nitric oxide level. (G), nitric oxide level by Griess assay in H9C2 cells was inhibited by miR-939-5p. (H), miR-939-5p antagomir raise the nitric oxide level in H9C2 cells. Data are mean ±S.E.M.*p<0.05 and**p<0.01.
TNFα was another target gene of miR-939-5p. (A), putative miR-939-5p binding sites in the 3'UTR region of TNFα analyzed by TargetScan program. Mutated miR-939-5p binding sites (TNFα-3'UTR-MUT) is shown. (B), luciferase constructs of TNFα-3’UTR-WT or TNFα-3’UTR-MUT were co-transfected into HUVECs with miR-939-5p mimic or control. PGL3 served as a control. The luciferase activity was analyzed. C and D, different concentration from 50-150 nM of miR-939-5p mimics 32 or antagonirs were transfected into HUVECs. (C), miR-939-5p mimic reduced mRNA level of TNFα. (D), miR-939-5p antagonim upregulated mRNA level of TNFα. E and F, TNFα in the cell culture supernatant was detected by ELISA. (E), miR-939-5p mimic inhibited the level of TNFα vs. mimic control. (F), knockdown of endogenous miR-939-5p promoted TNFα secretion. Data are mean ±S.E.M.*p<0.05and**p<0.01.
Selection of candidate IncRNAs interacted with miR-939-5p. (A), 10 potential candidate IncRNAs from IncRNA-databases filtration of differentially expressed genes in IncRNA microarray. 100nM miR-939-5p mimic or antagonir and their controls were transfected into HUVECs. RNA was isolated and assayed by RT-qPCR, results are presented relative to GAPDH (2-\(\Delta\Delta\)Ct). B and C, 100nM smart silencers of 3 IncRNAs (si-Inc) were transfected into HUVECs. (B), the interference effects were quantified by qRT-PCR using IncRNAs specific primers from Ribobio, Guangzhou. (C), MiR-939-5p was measured by qRT-PCR, U6 as internal control. D and E, 50, 100, 150nM smart silencers of lnc-NO2P3 (si-lnc-NOS2P3) were transfected into HUVECs. (D), The level of lnc-NOS2P3 was determined. (E), Relevant miR-939-5p levels were detected versus U6. (F), lnc-NOS2P3 expression plasmid was constructed into pIRES2-EGFP. Lnc-NOS2P3-mut to two binding sites of miR-939-5p was formed. (G), 400ng and 800ng Inc-NO2P3-WT or Inc-NO2P3-mut and negative control were separately transfected into HUVECs, miR-939-5p levels were determined versus to U6. Data are mean 33 ±S.E.M.*p<0.05 and **p<0.01.
Lnc-NOS2P3 was confirmed to regulate miR-939-5p and its target gene as sponge RNA. (A), Inc-NOS2P3 wide type and its mutant to miR-939-5p binding sites were cloned into
luciferase carrier GV272 as Inc-NOS2P3-WT-luc and Inc-NOS2P3-mut-luc. (B), they were co-transfected into HUVECs with 100nM miR-939-5p mimic and control. Relative luciferase was obtained. (C), TNFα mRNA was obviously reduced after transfection of 100nM smart silencer of Inc-NOS2P3. D-F, 400ng and 800ng Inc-NOS2P3-WT or Inc-NOS2P3-mut GFP plasmid and their negative control were transfected into 24 plates with or without CM. (D), the increasing expression of Inc-NOS2P3 and its mutant were detected by Inc-NOS2P3 by RT-qPCR. GAPDH acted as control. (E), Relative TNFα mRNA level was measured 48 hours after treated with or without CM. (F), Relative iNOS mRNA level was obtained 48 hours later. Data are mean ±S.E.M.*p<0.05and**p<0.01.
Knockdown of endogenous Inc-NO2P3 inhibited the inflammation induced apoptosis which could be rescued by miR-939-5p antagonir. (A), 50nM or 100nM si-Inc-NOS2P3, 50nM or 100nM miR-939-5p an tagomir (miR-939-5p an), si-negative control (si-N ctrl.), and
antagomir control (An ctrl.) were transfected into HUVECs as groups shown. Cells proliferation was got by CCK8 assay. (B), Apoptosis cells were detected by flow cytometry in three replicate samples. The experiment was repeated twice and data were analyzed statistically. (C), Apoptosis related proteins 34 were detected by western blot in various groups. Data are mean ±S.E.M.*p<0.05and**p<0.01.
Figure 8
Overexpression of Inc-NOS2P3 promoted inflammation induced apoptosis which was rescued by miR-939-5p mimic. (A), 800ng Inc-NOS2P3-WT or Inc-NOS2P3-mut and vector control were trasfected into HUVECs with 100nM miR-939-5p or mimic control. CCK8 assay showed its proliferation 48 hours later. B and C, 800ng Inc-NOS2P3-WT (WT) or Inc-NOS2P3-mut (Mut) and their vector control (NC) were transfected with 100nM miR-939-5p mimic (939mm) or its mimic control (mm). (B), Flow cytometry showed the apoptosis cells ratios, and data were analyzed statistically. (C), Apoptosis related proteins were measured by western blot. Data are mean ±S.E.M.*p<0.05and**p<0.01.

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