Long Noncoding RNA DANCR Suppressed Lipopolysaccharide-Induced Septic Acute Kidney Injury by Regulating miR-214 in HK-2 Cells

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Background: Acute kidney injury (AKI) is one of the most important causes of death in sepsis patients. Here, we first measured the level of DANCR (differentiation antagonizing nonprotein coding RNA) expression in AKI, and the potential mechanism was further elucidated.

Material/Methods: We used qRT-PCR to examine the level of DANCR in patient blood serum samples and in HK-2 cells. In addition, DANCR overexpression was established using lentiviral transfection in HK-2 cells. Subsequently, Cell Counting Kit-8 (CCK-8) assay and flow cytometry were applied to evaluate the role of DANCR in HK-2 cells treated with lipopolysaccharide (LPS). Enzyme linked immunosorbent assay (ELISA), western blot and recovery experiments were performed to elucidate the further mechanism.

Results: We found that DANCR was decreased in the serum of AKI patients and HK-2 cells treated with LPS. Additionally, DANCR promoted cell viability and suppressed cell apoptosis and cytokine production in LPS-treated HK-2 cells. Bioinformatics analysis showed that DANCR served as a sponge for miR-214. Furthermore, DANCR inhibited the expression of Krüppel-like factor 6 (KLF6).

Conclusions: Our study suggests that AKI development could be alleviated by sponging miR-214 and regulating KLF6 expression, which provides a novel potential mechanism involved in the diagnosis and treatment of sepsis-induced AKI patients.

MeSH Keywords: Acute Kidney Injury • MicroRNAs • RNA, Long Noncoding • Sepsis

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Background

Sepsis is a systemic inflammatory response syndrome caused by infectious factors that can lead to organ dysfunction and/or circulatory disorders in severe cases [1]. It is a common complication of severe trauma, burns, shock, infection and major surgery [2]. It has been reported that more than 30% of the mortality in intensive care units is related to sepsis-induced AKI [3]. Hence, improving the prevention and treatment of AKI in patients with sepsis is of great significance.

Long noncoding RNAs (lncRNAs), greater than 200 nucleotides in length, have been verified to modulate diverse cellular processes, including the cell cycle, autophagy and apoptosis [4]. Notably, accumulated evidence has revealed that lncRNAs are involved in sepsis-induced AKI development. For example, TapSAKI was observed to promote HK-2 cell apoptosis and the inflammatory response in sepsis-induced AKI [5], and HOTAIR may accelerate kidney injury induced by urine-derived sepsis [6]. As a novel lncRNA, DANCR (differentiation antagonizing nonprotein coding RNA) was first to be reported to repress cell differentiation [7] and has been confirmed to act as an important role in cancers and orthopedic disorders [8,9]. However, the molecular function of DANCR in other diseases has yet to be fully explored.

Material and Methods

Sample collection

Between December 2016 and December 2017, 20 patients (18 to 60 years old) diagnosed with sepsis-induced AKI at The Second Hospital of Tianjin Medical University were recruited in this study. During the same period, 20 healthy volunteers were enrolled as controls. The characteristics of the participants enrolled in the study are shown in Table 1. The diagnostic criteria for sepsis were according to the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM), and the criteria for AKI were based on the Acute Kidney Injury Network (AKIN) classification. Patients were included if they 1) were over 50 years old; 2) conformed to the diagnostic criteria of ACCP/SCCM; and 3) conformed to the diagnostic criteria of AKIN. The patients were excluded if they 1) were diagnosed with sepsis; 2) had concurrent viral myocarditis; 3) had concurrent severe hepatitis or cirrhosis; 4) did not have complete clinical records; or 5) had poor adherence. All participants signed informed consent, and this study was approved by the ethics committee of Tianjin Medical University. The 5 mL serum samples from the participants were centrifuged at 12 000 g for 3 minutes, and the supernatant was frozen at −80°C immediately for further analysis.

Cell culture and transfection

The human renal tubular epithelial cell line HK-2 (obtained from the Cell Bank of the Chinese Academy) was cultured in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (1: 1, Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin with humidified air and 5% CO₂ at 37°C. Until approximately 60% confluence, HK-2 cells were treated with 100 ng/mL lipopolysaccharide (LPS) for 24 hours to mimic inflammation. Cells were transfected with DANCR mimic, miR-214 mimic or the corresponding controls mixed with Lipofectamine 2000 reagent according to the manufacturer’s protocols.

Table 1. Baseline characteristics of case (patients with sepsis-induced AKI) and control (healthy individuals) group.

| Baseline characteristics | AKI            | Control        | P value |
|--------------------------|----------------|----------------|---------|
| Age (years)              | 65.25±7.21     | 64.78±6.54     | 0.725   |
| Gender                   |                |                |         |
| Male                     | 12             | 11             | 0.749   |
| Female                   | 8              | 9              |         |
| Body mass (kg/m²)        | 26.23±7.56     | 25.98±9.24     | 0.695   |
| BUN (mmol/L)             | 10.25±1.56     | 5.23±1.78      | 0.012*  |
| Cr (μmol/L)              | 260.23±20.56   | 46.75±16.13    | 0.006*  |

* P<0.05.
Cell viability

HK-2 cells (1×10^4 cells/well) with different transfections were seeded in 96-well plates and cultured for 0, 24, 48, 72, and 96 hours. Then 10 µL of Cell Counting Kit-8 (CCK-8) solution was then added to the cells for 3 hours of incubation. The absorbance at 450 nm (OD450) was calculated using a Microplate Reader.

Cell apoptosis

The cells were suspended in the binding buffer, placed in ice-cold 70% ethanol, and stained with FITC/Annexin V and propidium iodide (PI) in the dark at room temperature for 15 minutes. Apoptosis detection was immediately performed using a FACScan (Beckman Coulter, Fullerton, CA, USA) and analyzed using CellQuest software.

Enzyme linked immunosorbent assay (ELISA)

The concentrations of inflammatory cytokines (tumor necrosis factor [TNF]-α, monocyte chemotactic protein [MCP]-1, interleukin [IL]-1β, and IL-6) were measured in 1:50 serum samples from all participants using ELISA kits (Shanghai, China) and inserted into the pGL3 Basic vector. Cells were seeded into 24-well plates and co-transfected with the luciferase reporter constructs, miR-630 mimics, and Renilla luciferase construct (Promega) and incubated for 24 hours. The relative luciferase activities were measured using the Dual-Luciferase Reporter System (Promega).

Statistical analysis

All measurement data were expressed as the mean±standard deviation. Differences were calculated with Student’s t-test or one-way ANOVA. Pearson’s correlation analysis was used to evaluate the expression correlation. All statistical analyses were calculated using SPSS 17.0 software and GraphPad Prism 6. *P*<0.05 indicated a statistically significant difference.

Results

The level of DANCR was decreased in the serum of AKI patients and HK-2 cells

To evaluate the role of DANCR in sepsis-induced AKI, we initially measured DANCR expression levels in 20 patients and healthy volunteers using RT-qPCR. The results demonstrated that DANCR expression was remarkably lower in serum of patients than healthy controls (Figure 1A). We observed that the concentrations of inflammatory cytokines (TNF-α, MCP-1, IL-1β, and IL-6) in patients were significantly increased (Figure 1B). In addition, the DANCR level was notably decreased in HK-2 cells treated with LPS (Figure 1C).

DANCR promoted cell viability and suppressed cell apoptosis and cytokine production in LPS-treated HK-2 cells

Subsequently, we explored the role of DANCR in LPS-treated HK-2 cells. First, we established a DANCR overexpression model in LPS-treated HK-2 cells by transfection (Figure 2A). The CCK-8 assay showed that DANCR overexpression significantly reduced the proliferation of LPS-treated HK-2 cells (Figure 2B). In addition, flow cytometry analysis demonstrated that the cell apoptosis rate was lower in LPS-treated HK-2 cells after transfection.
with DANCR mimic (Figure 2C). Furthermore, DANCR overexpression obviously reduced the expression of related cytokines (Figure 2D).

**DANCR served as a sponge for miR-214**

Given that IncRNAs serve as molecular sponges for miRNAs in many diseases [4], we used starBase v2.0 to predict the candidate target miRNAs binding with DANCR [13]. As shown in Figure 3A, miR-214 was observed to have a potential binding site with DANCR. A luciferase reporter assay verified the binding with decreased fluorescence in cells transfected with the miR-214 mimic and DANCR wild type construct (Figure 3B). In addition, the miR-214 expression level was significantly upregulated in patient sera and LPS-treated HK-2 cells (Figure 3C). Under LPS-stimulated conditions, DANCR overexpression significantly decreased miR-214 levels in HK-2 cells, which was reversed by the miR-214 mimic (Figure 3D). The CCK-8 assay revealed that the miR-214 mimic reversed the effect of DANCR overexpression on the proliferation of HK-2 cells treated with LPS at 96 hours (Figure 3E). In addition, the miR-214 mimic increased the apoptosis rate of LPS-treated HK-2 cells transfected with the DANCR mimic (Figure 3F). As expected, the miR-214 mimic obviously promoted cytokine production in cells transfected with the DANCR mimic (Figure 3G).

**DANCR could suppress KLF6 expression**

KLF6 has been verified to take part in the inflammation occurrence [14,15]. Thus, we explored the effect of DANCR on the expression of KLF6 in LPS-treated HK-2 cells. We noticed that KLF6 expression was significantly elevated in the serum of patients and LPS-treated HK-2 cells (Figure 4A). After transfection with DANCR, the expression level of KLF6 was downregulated significantly in LPS-treated HK-2 cells, which was rescued by the miR-214 mimic (Figure 4B).

**Discussion**

Acute kidney injury (AKI) is a common critical disease in the clinic, and its main characteristics are acute decline in renal function caused by various etiologies [16]. Previous research has shown that more than 50% of sepsis patients often develop AKI as a complication during treatment [17]. So far, accumulating evidence has revealed that abnormally expressed noncoding
RNAs are closely related to the development of AKI [18]. As a novel lncRNA, DANCR has been observed to regulate the inflammatory phenotype of breast cancer cells in breast cancer progression [19]. DANCR also regulates the proliferation and apoptosis of chondrocytes in osteoarthritis [20]. Here, we initially found that the level of DANCR expression was notably downregulated in the serum of patients and HK-2 cells treated with LPS. Then, we observed that DANCR promoted cell viability and inhibited cell apoptosis and inflammatory cytokine production in LPS-treated HK-2 cells. These data implied that DANCR may repress the biological behavior of HK-2 cells under LPS-stimulated conditions.

It is accepted that lncRNAs may exert their regulatory function in gene expression by sponging miRNAs [21]. Bioinformatics analysis showed that DANCR could functionally act as a sponge for miR-214. Some studies have reported that miR-214 silencing attenuated inflammation progression and suppressed the biological behavior of immune cells.

**Figure 2.** DANCR promoted cell viability and suppressed cell apoptosis and cytokine production in LPS-treated HK-2 cells. (A) DANCR overexpression was established in LPS-treated HK-2 cells. (B) DANCR overexpression significantly reduced the proliferation of LPS-treated HK-2 cells. (C) Flow cytometry analysis demonstrated that the cell apoptosis rate was lower in LPS-treated HK-2 cells after transfection with DANCR mimic. (D) DANCR overexpression obviously reduced the expression of related cytokines (TNF-α, MCP-1, IL-1β, and IL-6). * P<0.05 compared to the control group; # P<0.05 compared to the LPS group; ** P<0.01 compared to the control group; *** P<0.01 compared to the LPS group. DANCR – differentiation antagonizing nonprotein coding RNA; LPS – lipopolysaccharide; TNF – tumor necrosis factor, IL – interleukin; MCP – monocyte chemoattractant protein.
Figure 3. DANCR served as a sponge for miR-214. (A) Bioinformatics analysis showed that miR-214 has a potential binding site with DANCR. (B) Luciferase reporter assay revealed that miR-214 overexpression repressed the luciferase activity of only the DANCR wild type construct in HK-2 cells. (C) The miR-214 level was significantly upregulated in patient sera and LPS-treated HK-2 cells. (D) DANCR overexpression significantly decreased miR-214 levels in HK-2 cells, which was reversed by the miR-214 mimic. (E) miR-214 mimic could reverse the effect of DANCR overexpression on the proliferation of HK-2 cells treated with LPS at 96 hours. (F) miR-214 mimic increased the apoptosis rate of LPS-treated HK-2 cells transfected with DANCR mimic. (G) The miR-214 mimic obviously promoted cytokine production in cells transfected with the DANCR mimic.

* P<0.05 compared to the control group; ** P<0.01 compared to the control group.
DANCR – differentiation antagonizing nonprotein coding RNA; LPS – lipopolysaccharide.
the secretion of proinflammatory cytokines in some diseases [22,23]. Similarly, we observed that the miR-214 expression level was significantly upregulated in the blood serum of patients and LPS-treated HK-2 cells. In addition, miR-214 could rescue the suppressive effect of DANCR overexpression on the proliferation of HK-2 cells and increase cell apoptosis and cytokine production.

Kruppel-like factors (KLFs) are highly conserved zinc-finger proteins that regulate the cellular transcription machinery [24]. In light of the evidence that KLF6 promotes proinflammatory gene expression in kidney diseases [25], we investigated the effect of DANCR on this molecule. After transfection with DANCR, the level of KLF6 expression was remarkably downregulated in HK-2 cells treated with LPS. miR-214 reversed the inhibitory effect of DANCR on KLF6 expression. These data implied that DANCR may suppress KLF6 expression in LPS-induced septic AKI.

**Conclusions**

Overall, we first identified and confirmed that DANCR was downregulated in AKI. DANCR overexpression could alleviate AKI development by sponging miR-214 and inhibiting KLF6 expression, which provides a novel potential mechanism involved in the diagnosis and treatment of sepsis-induced AKI.

**Conflict of interest**

None.
References:

1. Singer M, Deutschman CS, Seymour CW et al: The third international consensus definitions for sepsis and septic shock (Sepsis-3). JAMA, 2016; 315: 801–10

2. Maren EA, Baun MM, Meiningier JC, Wade CE: Comparison of mortality associated with sepsis in the burn, trauma, and general intensive care unit patient: A systematic review of the literature. Shock, 2012; 37: 4–16

3. Lopes IA, Fernandes P, Jorge S et al: Long-term risk of mortality after acute kidney injury in patients with sepsis: A contemporary analysis. BMC Nephrol, 2010; 11: 9

4. Mercer TR, Dinger ME, Mattick JS: Long non-coding RNAs: Insights into functions. Nat Rev Genet, 2009; 10: 155–59

5. Shen J, Liu L, Zhang F et al: LncRNA TapSAKI promotes inflammation injury in HK-2 cells and urine derived sepsis-induced kidney injury. J Pharm Pharmacol, 2019; 71: 839–48

6. Shen J, Zhang J, Jiang X et al: LncRNA HOX transcript antisense RNA accele rated kidney injury induced by urine-derived sepsis through the miR-22/ high mobility group box 1 pathway. Life Sci, 2018; 210: 185–91

7. Kretz M, Webster DE, Flockhart RJ et al: Suppression of progenitor differentiation requires the long noncoding RNA ANCR. Genes Dev, 2012; 26: 338–43

8. Guo D, Li Y, Chen Y et al: DANCR promotes HCC progression and regulates EMT by sponging miR-27a-3p via ROCK1/LIMK1/COFILIN1 pathway. Cell Prolif, 2019; 52: e12628

9. Lu Y, Hu Z, Mangala LS et al: MYC targeted long noncoding RNA DANCR promotes cancer in part by reducing p21 levels. Cancer Res, 2018; 78: 64–74

10. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell, 2004; 116: 281–97

11. Denby L, Ramdas V, Lu R et al: MicroRNA-234 antagonism protects against renal fibrosis. J Am Soc Nephrol, 2014; 25: 65–80

12. Zhu X, Li W, Li H: miR-214 ameliorates acute kidney injury via targeting DKK3 and activating of Wnt/beta-catenin signaling pathway. Biol Res, 2018; 51: 31

13. Li JH, Liu S, Zhou H et al: starBase v2.0: decoding miRNA-ceRNA, miRNA-nrRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. Nucleic Acids Res, 2014; 42: D92–97

14. Zhang Y, Lei CQ, Hu YH et al: Kruppel-like factor 6 is a co-activator of NF-kappaB that mediates p65-dependent transcription of selected downstream genes. J Biol Chem, 2014; 289: 12876–85

15. Date D, Das R, Narla G et al: Kruppel-like transcription factor 6 regulates inflammatory macrophage polarization. J Biol Chem, 2014; 289: 10318–29

16. Levey AS, James MT: Acute kidney injury. Ann Intern Med, 2017; 167: ITC66–80

17. Zarjou A, Agarwal A: Sepsis and acute kidney injury. J Am Soc Nephrol, 2011; 22: 999–1006

18. Brandenburger T, Salgado Somoza A, Devaux Y, LorenzoJM: Noncoding RNAs in acute kidney injury. Kidney Int, 2018; 94: 870–81

19. Zhang KJ, Tan XL, Guo L: The long non-coding RNA DANCR regulates the inflammatory phenotype of breast cancer cells and promotes breast cancer progression via EZH2-dependent suppression of SOCS3 transcription. Mol Oncol, 2019 [Epub ahead of print]

20. Zhang L, Zhang P, Sun X et al: Long non-coding RNA DANCR regulates proliferation and apoptosis of chondrocytes in osteoarthritis via miR-216a-SpJAK2-STAT3 axis. Biosci Rep, 2018; 38(6): pii: BSR20181228

21. Tay Y, Rinn J, Pandolfi PP: The multilayered complexity of ceRNA crosstalk and competition. Nature, 2014; 505: 344–52

22. Bai M, Chen H, Ding D et al: MicroRNA-214 promotes chronic kidney disease by disrupting mitochondrial oxidative phosphorylation. Kidney Int, 2019; 95: 1389–404

23. Zheng D, Zang Y, Xu H et al: MicroRNA-214 promotes the calcification of human aortic valve interstitial cells through the acceleration of inflammatory reactions with activated MyD88/NF-kappaB signaling. Clin Res Cardiol, 2019; 108: 691–702

24. Kaczynski J, Cook T, Urrutia R: Sp1- and Kruppel-like transcription factors. Genome Biol, 2003; 4: 206

25. Rane MJ, Zhao Y, Cai L: Kruppsilonppel-like factors (KLFs) in renal physiolo gy and disease. EBioMedicine, 2019; 40: 743–50