miR-124/MCP-1 signaling pathway modulates the protective effect of itraconazole on acute kidney injury in a mouse model of disseminated candidiasis

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Abstract. Previous studies have indicated that monocyte chemoattractant protein-1 (MCP-1), also referred to as C-C motif chemokine ligand 2, has a significant role in the pathogenesis of sepsis, however, how microRNAs (miRs) contribute to this process remains to be fully elucidated. In the present study, using a mouse model of disseminated candidiasis, the renoprotective effect of itraconazole (ITR) and adenovirus-delivered miR-124 was investigated. The mice were treated with ITR (50 mg/kg) or transfected with miR-124 mimics via tail-vein injection 7 days prior to Candida albicans infection. The survival outcome was monitored following candidiasis-induced sepsis with ITR or miR-124 mimics treatment. The levels of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, were determined using enzyme-linked immunosorbent assays. The mRNA and protein levels were assayed using reverse transcription-quantitative polymerase chain reaction and western blot analyses, respectively. The results showed that ITR and miR-124 mimics improved the survival outcome in candidiasis-induced septic mice. The findings also indicated a significant downregulation in the serum levels of TNF-α, IL-1β and IL-6 in the septic mice treated with ITR or miR-124 mimics. Of note, ITR treatment significantly increased the expression of miR-124 and decreased the levels of MCP-1 in the kidneys of the septic mice. It was also shown that the overexpression of miR-124 reduced the expression of MCP-1 and attenuated candidiasis-induced acute kidney injury (AKI) in septic mice. Transfection with miR-124 mimics was equivalent to ITR in reducing the excessive inflammatory response and renal lesions in septic mice. These results provided evidence supporting the use of miR-124 mimics as a therapeutic approach for attenuating candidiasis-induced AKI.

Introduction

Sepsis is a serious pathogenic infection and can deteriorate in a generalized immune response to systemic infection, which is a leading contributor to mortality rates in intensive care units (1). Candidiasis, particularly Candida albicans (C. albicans), is one of the leading worldwide nosocomial infections occurring following the onset of sepsis, and the mortality rate attributable to candidemia is ~40% despite the use of antifungal agents (2,3). In order to improve the treatment of candidemia-induced multiple organ failure, it is important to first understand its pathogenesis. A mouse model of disseminated C. albicans infection has been used extensively to examine the pathological process, and the kidney is the primary target organ (4). There is increasing evidence suggesting that the activation of the pro-inflammatory mechanism is involved in candidiasis-induced acute kidney injury (AKI) (5,6). Interleukin-1α (IL-1α), IL-1β, IL-6, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) have been shown previously to be important in the pathogenesis of candidemia-induced renal injury (7,8). In addition, chemokines and their receptors are reportedly induced in a fatal mouse model of invasive candidiasis (5,7). These findings suggest that reducing the excessive production of inflammatory cytokines during candidemia is beneficial for attenuating AKI.

Itraconazole (ITR) is anazole broad-spectrum antifungal agent, and is orally administered for C. parapsilosis, C. albicans and C. glabrata infections (9-11). Generally, the treatment of fungal infections with ITR may be due not only to its direct antifungal effect, but also due to the induction of an immunomodulatory effect on the host defense...
systems via the regulation of cytokine production (12). ITR has an anti-inflammatory effect in vivo and in vitro (13,14). In human lymphoid cells, ITR suppresses the accumulation of TNF-α and IFN-γ messenger RNAs (mRNAs) in the presence of polyhydroxyalkanoate (15). In addition, ITR inhibits the TNF-α-induced expression of CXC-10 in oral fibroblasts (16). In a murine model of chronic pulmonary paracoccidioidomycosis, ITR improves tissue lesions and immunomodulation through the regulation of pro-inflammatory cytokine levels (17). However, there has been no report on the protective effect of ITR in mice with renal injury.

A class of small non-coding RNAs (18-25 nucleotides) known as microRNAs (miRs), which regulate the translation of target mRNAs by binding to 3'-untranslated regions (3'-UTRs), have emerged as multifunctional post-translational modulators correlated with several diseases (18,19). The association between sepsis and differentially expressed miRs has become a focus of investigations (20). C. albicans infection or bacterial cell surface lipopolysaccharide (LPS) treatment can induce the upregulation of miRs, including miR-146, miR-155, miR-455 and miR-125a, in macrophages (21). In our previous study, it was demonstrated that miR-204 and miR-211 were down-regulated in the kidneys of septic mice, however, intravenous injection of miR-204 and miR-211 reversed sepsis-induced renal injury (22). Therefore, the identification of novel therapeutic targets based on an improved understanding of the molecular pathogenesis is required.

In the present study, ITR was identified as an antifungal agent against candidiasis-induced AKI through the inhibition of monocyte chemoattractant protein-1 (MCP-1). However, information on the effect of post-translational modulators on the nephroprotection of ITR in septic mice remains limited. Utilizing online prediction algorithms in the present study, MCP-1 was identified as a direct target of miR-124. It was recognized that miR-124/MCP-1 was closely associated with renal dysfunction.

Materials and methods

Cell culture. 293T cells (American Type Culture Collection, Manassas, VA, USA) were incubated in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and supplemented with 10% FBS, 100 µg/ml streptomycin and 100 U/ml penicillin (all from Sigma-Aldrich; Merck Millipore), and were fixed with 4% formalin at room temperature for 24 h and paraffin-embedded. The tissues were then cut into ~5-µm-thick sections, which were stained with H&E at room temperature for 1-2 min and visualized under a microscope (Leica DM 2500; Leica Microsystems GmbH, Wetzlar, Germany). Renal injury was assessed using a previously described 0-4 point scale (24) as follows: 0, none; 1, <10%; 2, 10-25%; 3, 25-75%; or 4, >75%.

Enzyme-linked immunosorbent assay (ELISA). The levels of inflammatory cytokines, TNF-α (cat. no. E-EL-M0049c), IL-1β (cat. no. E-EL-M0037c) and IL-6 (cat. no. E-EL-M0044c) were measured using mouse ELISA kits (Elabscience Biotechnology Co., Ltd., Wuhan, China) with a SpectraMax M5 ELISA plate reader (Molecular Devices, LLC., Sunnyvale, CA, USA), according to the manufacturer’s protocol.

Measurement of glomerular filtration rate (GFR). Serum cystatin C (CysC) and β2-microglobulin (β2-MG) are freely filtered by the glomerular membrane, which makes blood levels good indicators of GFR function. In the present study, CysC (cat. no. CSYS4004) and β2-MG (cat. no. RQ9114) were measured using a RANDOX enzymatic creatinine assay (Randox Laboratories Ltd., Antrim, UK). Blood urea nitrogen (BUN) was measured via an enzymatic kinetic method using a commercial kit (cat. no. C013-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum creatinine (Scr) levels were measured using an autoanalyzer according to the manufacturer’s protocol (cat. no. C011-1; Nanjing Jiancheng Bioengineering Institute).

Recombinant adenoviruses. Recombinant adenoviruses for the expression of miR-124 or control scrambled short hairpin RNA (miR-Con) were generated using the BLOCK-IT adenoviral RNAi expression system (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. High-titer stocks of amplified recombinant adenoviruses were purified as described previously (25). The viruses were diluted in PBS and administered at a dose of 10³ plaque-forming units per well in 12-well plates, 10³ plaque-forming units per were administered to mice via tail-vein injection every other day for 7 days.
The luciferase activity was measured using the dual Luciferase miR-124 mimics or miR-con were co-transfected at 50 nM containing the WT and MUT McP-1-3'-UTR (0.5 µg). The 293T cells were transfected with luciferase reporter vectors obtainted using online prediction software (miRanda-mirSVR; http://www.microrna.org), miR dB (http://www.mirdb.org/miRDB/) and TargetScan (http://www.targetscan.org/). The wild-type (WT, 5’-GAc UcG GAc UGU GUG ccU UA-3’) and mutant-type (MUT, 5’-GAc UcG GAc UGU GUA UUc cA-3’) 3’-UTR of McP-1 were synthesized by PCRI Corp., and the fragments were subcloned into the pmirGLO dual-Luciferase miRNA target expression vector (Promega corp., Madison, WI, USA). The potential binding site between miR-124 and McP-1 was determined using the pmirGLO Dual-Luciferase miRNA target expression vector (Promega Corp., Madison, WI, USA). The potential binding site between miR-124 and McP-1 was obtained using online prediction software (miRanda-mirSVR; http://www.microrna.org), miR dB (http://www.mirdb.org/miRDB/) and TargetScan (http://www.targetscan.org/). The 293T cells were transfected with luciferase reporter vectors containing the WT and MUT MCP-1-3’-UTR (0.5 µg). The miR-124 mimics or miR-Con were co-transfected at 50 nM. The luciferase activity was measured using the Dual Luciferase Reporter® assay system (cat. no. E1960; Promega Corp.) on a Luminoskan™ Ascent Microplate Luminometer (Thermo Fisher Scientific, Inc.). The dual-luciferase reporter gene assay was performed as previously described (22).

Results

*ITR improves survival outcome following candidiasis.* *C. albicans* injection induced animal death within 24 h, with
50% mortality by 70 h post-\textit{C. albicans} injection, and 25% of animals surviving at day 7. However, in the septic mice treated with ITR, no death occurred until 30 h post-\textit{C. albicans} injection, with 65% of the ITR-treated mice surviving for the duration of the investigation (Fig. 1).

\textbf{ITR inhibits systemic and local inflammatory responses in septic mice.} MCP-1 is a member of the CC chemokine family and it is an important molecule for monocyte recruitment under acute inflammatory conditions, regulating the progression of inflammation through the production of pro-inflammatory cytokines (27). A study by Labbe et al showed that antibody neutralization of MCP-1 prevented the endotoxin-induced upregulation of IL-1\textalpha, IL-1\beta, and IL-6 in the diaphragm (28). Therefore, the present study investigated the levels of MCP-1 in septic mice. The results showed that the mRNA (Fig. 2A) and protein (Fig. 2B) expression levels of MCP-1 in the kidneys from septic mice were significantly higher, compared with those in the control group, which were markedly decreased by ITR (Fig. 2A and B). The serum and renal levels of pro-inflammatory cytokines (TNF-\textalpha, IL-1\beta, and IL-6) were determined. As shown in Fig. 2C and D, compared with the control group, the serum and renal levels of pro-inflammatory cytokines were significantly increased in the septic mice. Treatment with ITR for 7 days resulted in a decrease of pro-inflammatory cytokines in septic mice, suggesting that ITR inhibited the excessive systemic and local inflammatory response in candidiasis-induced AKI mice.

\textbf{ITR alleviates candidiasis-induced AKI.} BUN is an indicator of kidney impairment, and SCR represents a good indicator of the severity of renal impairment, particularly of GFR (29). To evaluate renal damage, BUN and SCR were measured in healthy and septic mice with or without ITR treatment. As shown in Fig. 3A and B, candidiasis induced significant increases in BUN and SCR levels, which were significantly attenuated by treatment with ITR. In addition, the present study analyzed the GFR by measuring CysC and serum \(\beta\)-MG under different treatment conditions. As shown in Fig. 3C and D, CysC and \(\beta\)-MG levels were significantly higher in the septic mice, compared with those in the control group, however, ITR treatment significantly reduced the increased levels of CysC and \(\beta\)-MG in the septic mice. In accordance with these data, improved renal pathological changes were observed in the ITR-treated septic mice. As shown in Fig. 4A and B, the induction of candidiasis produced focal and segmental hyperplasia with diffuse mesangial proliferation and glomerular lesions. By contrast, the damage was markedly attenuated in the ITR-treated group.

\textbf{ITR regulates the expression of miR-124 in the kidneys of septic mice.} Based on the experiments, MCP-1 was closely associated with candidiasis-induced AKI. To investigate whether MCP-1 may be regulated by miRs, online prediction software
(miRanda-mirSVR, miRDB and TargetScan) was used for prediction. It was found that miR-124 was overlapping in the miRanda-mirSVR and TargetScan databases, and miR-290-5p and miR-292-5p were overlapping in the miRanda-mirSVR and miRDB databases. Therefore, the expression levels of miR-124, miR-290-5p and miR-292-5p were measured in the kidneys from septic mice. The results demonstrated that the levels of miR-124 were significantly decreased in the kidneys from the *C. albicans*-injected mice, compared with those in the healthy mice (Fig. 5). However, there was no significant difference in miR-290-5p or miR-292-5p between the cAN group and NC group (Fig. 6A and B). Of note, ITR-treated reversed the candidiasis-induced downregulation of miR-124 in the kidneys of the septic mice (Fig. 5), suggesting that the overexpression of miR-124 contributed to the improved candidiasis-induced AKI. To investigate whether McP-1 was a direct target of miR-124, the online prediction software was used for prediction. The results showed that the 3'-UTR of MCP-1 contained one conserved binding site of miR-124 (Fig. 7A). To confirm this, a luciferase activity assay was performed on the 293T cells. As shown in Fig. 7B, transfection with miR-124 mimics significantly reduced the luciferase activity elicited by the luciferase vector carrying the complementary sequence with the WT 3'-UTR of MCP-1. Following transfection with miR-124 mimics, no significant difference was observed in luciferase enzyme activity between the NC group and the reporter vector group containing the MUT 3'-UTR of MCP-1. These findings demonstrated that MCP-1 was a direct target gene of miR-124 by binding with its 3'-UTR.

Overexpression of miR-124 improves survival outcome following candidiasis. In septic mice, miR-Con transfection had no significant effect on survival outcome. However, the septic mice transfected with miR-124 mimics began to die at 35 h, with 60% of the septic mice surviving for the duration of the investigation (Fig. 8).

Intravenous injection of miR-124 inhibits the expression of MCP-1, inflammatory cytokines and AKI in septic mice. To investigate the effects of miR-124 on the inflammatory response and sepsis-induced AKI *in vivo*, male C57BL/6J
mice were infected with miR-124 mimics and miR-Con via tail-tail injection, and it was found that levels of miR-124 were significantly increased in the kidneys, compared with those in the NC group or miR-Con group (Fig. 9A). To determine the effects of miR-124 on inflammatory signaling, the effects of the overexpression of miR-124 on MCP-1 were examined in the kidneys of the mice. The mRNA and protein levels of MCP-1 were significantly inhibited in the kidneys of mice transfected with miR-124, compared with those in the miR-Con group (Fig. 9B-D). The effects of miR-124 mimics on the serum levels of inflammatory cytokines associated with MCP-1 were also examined in septic mice. It was found that the serum levels of TNF-α, IL-1β and IL-6 were markedly decreased by miR-124 mimics in septic mice (Fig. 9E). Furthermore, the miR-124 mimics led to significant decreases in the levels of BUN and Scr in serum of the septic mice (Fig. 9F and G), suggesting that miR-124 may improve the inflammatory response and AKI in septic mice through the inhibiting the expression of MCP-1.

**Discussion**

Experimental and clinical data support that candidiasis is a dominant factor for AKI (22,30). There are no effective therapies in candidiasis-induced AKI. In order to improve clinical outcomes, understanding the exact molecular mechanisms in candidiasis-induced AKI is considered essential. The present study used a mouse model of disseminated candidiasis, and the findings indicated that the upregulation of MCP-1 levels and inhibition of miR-124 were involved in candidiasis-induced AKI. In addition, a bioinformatics prediction approach showed that miR-124 was targeted to MCP-1 by binding with its 3′-UTR. The data revealed molecular insights into the involvement of miR-124 and its direct target MCP-1 in modulating sepsis-induced renal injury.

Previous studies have demonstrated that inflammation is pivotal in the pathogenesis of AKI, and that MCP-1, which is considered a key inducer of the proinflammatory response, has been shown to be linked with renal perfusion, glomerular filtration and glomerular lesions (31,32). Munshi et al showed that the pathologic increase in the expression of MCP-1 originates from the diseased kidney, and MCP-1 offers potential as a biomarker of AKI (31). MCP-1 is known to contribute to the toxic effect of LPS in vivo and in vitro (33,34); therefore, attenuating a pathologic increase in the expression of MCP-1 may be important in controlling the occurrence and development of AKI. The present study
provided evidence that ITR possessed a nephroprotective effect against candidiasis-induced toxicity via inhibiting the mRNA and protein expression of MCP-1 in the kidney. In candidiasis-exposed mice, significant increases in BUN, SCr and mortality rate were observed. By contrast, treatment with ITR resulted in a significant decrease of these parameters in septic mice. On histopathological examination, it was also observed that renal lesions were reduced significantly in septic mice receiving ITR treatment. Unexpectedly, the administration of exogenous miR-124 was able to efficiently restore and ameliorate the damaged kidney in candidemia mice, as ITR, through the suppression of MCP-1 levels. In addition, consistent with a previous study (35), miR-124 directly inhibited the serum levels of TNF-α, IL-1β and IL-6 in septic mice. Therefore, the evidence showed that miR-124 may be a potential therapeutic target for the treatment of inflammatory diseases.

In our previous study, it was demonstrated that the administration of exogenous miR-204 and miR-211 possessed the ability to efficiently ameliorate candidiasis-induced AKI (22), and is a type of gene therapy that has been well accepted in clinical practice (39). Zhou et al showed that the systemic administration of miR-155 mimics attenuated cardiac dysfunction and improved survival rates in late sepsis (40). The results of a study by Zheng et al showed that the silencing of miR-195 reduced multiple-organ injury and improved survival rates in sepsis (41). All the evidence indicates that exogenous miRs are involved in sepsis-induced inflammatory activity and tissues lesions. In the present study, adenovirus-delivered miR-124 increased by almost 2.5-fold in the kidneys from septic mice, and the overexpression of miR-124 may represent a novel therapeutic approach for sepsis-induced AKI.
Taken together, previous results and those of the present study provide evidence that MCP-1 is involved in the excessive inflammatory response induced by candidiasis in septic mice. However, ITR had a potent nephroprotective effect on the septic mouse model. In addition, an in vivo experiment demonstrated that adenovirus-delivered miR-124 can be introduced into the kidney to inhibit the expression of MCP-1 and the inflammatory response. These results provide a novel perspective on molecular-targeted therapy for candidiasis-induced AKI.

Competing interests

The authors declare that they have no competing interests.

References


