miR-3587 Targets HMOX1 to Attenuate Ferroptotic Renal Injury Following Ischemia-Reperfusion

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Research Article

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Abstract

Renal ischemia-reperfusion (IR) is frequently observed in patients who are critically ill, yet there are no reliable or effective approaches to treating this condition. This study aimed to investigate the miRNA-mRNA regulatory networks that are involved in IR-related ferroptotic renal injury. Bioinformatics method was used to screen critical hub gene and its upstream miRNA from Renal IR-related data sets in Gene Expression Omnibus, and further experiments were conducted to verify the regulatory effect of miRNA on critical hub gene. Heme oxygenase-1 (HMOX1) was identified as a critical hub gene that was found to be significantly upregulated during the early stages of renal IR injury. miR-3587 was identified as a putative regulator of HMOX1. When a miR-3587 inhibitor was applied in a hypoxia-reoxygenation model system using renal tubular epithelial cells, HMOX1 expression was significantly increased relative to that observed in the control hypoxia-reoxygenation group, with concomitant increases in glutathione peroxidase 4 (GPX4) protein levels, enhanced cell viability, a reduction in malondialdehyde content, and the restoration of normal mitochondrial membrane potential. Preliminary evidence suggests that utilizing miR-3587 inhibitors can further promote HMOX1 upregulation, thereby protecting renal tissues from IR-induced ferroptosis.

Introduction

Renal ischemia-reperfusion (IR) is a serious clinical condition that is often encountered in critically ill patients such as those that have undergone major surgery, resuscitation following cardiac arrest, or microcirculation recanalization after shock, potentially resulting in acute renal injury (AKI) [1]. However, the only treatment options available for patients suffering from renal IR at present is a combination of conservative treatment and renal replacement therapy owing to the heterogeneity of this condition and a lack of biomarkers that are reliably associated with its early stages. AKI can readily progress to chronic renal insufficiency or death [2], underscoring the critical importance of elucidating the pathophysiological mechanisms governing the onset and progression of AKI in order to treat this debilitating condition.

AKI is driven by a number of pathophysiological processes including oxidative stress, damage to endothelial cells, microvascular dysfunction, and renal tubular epithelial cell injury associated with ferroptotic, apoptotic, and pyroptotic cell death pathways [3]. Ferroptosis is a recently defined form of regulated cell death that is driven by the iron-dependent peroxidation of membrane lipids [4]. Renal tubular epithelial cell ferroptosis is reportedly involved in the incidence of AKI [5]. Indeed, the ferroptosis inhibitor XJB-5-131 is able to protect against renal tubular epithelial cell death and consequent kidney IR injury in a mouse model system [6].

MicroRNAs (miRNAs) are small RNAs which lack coding potential, yet can regulate the stability and translation of target mRNAs by binding to complementary sequences within the 3’-untranslated region (UTR) [7]. There is ample evidence that miRNAs can regulate key physiological processes including apoptosis, proliferation, and ferroptosis in a target-specific manner [8]. For example, in a murine traumatic brain injury model, Xiao et al. determined that miR-212-5p was able to target PTGS2 and to thereby
suppress ferroptosis and inhibit nerve injury [9]. Ding et al. further confirmed that hypoxia-reoxygenation was able to promote the upregulation of miR-182-5p and miR-378a-3p in renal tubular epithelial cells in vitro, resulting in glutathione peroxidase 4 (GPX4) and SLC7A11 downregulation and consequent ferroptotic injury. Consistently, silencing these two miRNAs in an in vivo rat renal IR model system was sufficient to suppress ferroptotic renal injury [10]. However, further work is required to fully elucidate the regulatory roles of individual miRNAs in the context of ferroptosis-induced renal IR injury. As such, the present study was designed to screen for potential genetic regulators of renal IR in an effort to define novel therapeutic targets. Through a series of bioinformatics analyses, heme oxygenase-1 (HMOX1) was ultimately identified as a key hub regulator of this disease state that was targeted by miR-3587. Preliminary in vitro analyses confirmed that miR-3587 inhibition was sufficient to suppress the ferroptotic death of renal tubular epithelial cells, attenuating renal injury by promoting HMOX1 upregulation.

Materials And Methods

Dataset selection

The GSE58438, GSE27274, GSE3219, and GSE9934 datasets were downloaded from the Gene Expression Omnibus (GEO) database. GSE58438 was utilized for hub gene screening, whereas GSE27274, GSE3219, and GSE9934 were used to confirm hub gene expression patterns. For further details regarding these datasets, see Table 1. The GSE58438 dataset was originally used to explore the protective effects of valproic acid and dexamethasone on acute renal ischemia-reperfusion injury, while the GSE3219 dataset was originally used to explore an early biomarker of ischemic acute renal failure and nephrotoxic acute renal failure. Therefore, samples with other intervention factors in the GSE58438 and GSE3219 datasets were not included in this study. The original purpose of the GSE27274 dataset was to explore the protective effect of upregulated fibrinogen expression on renal ischemia-reperfusion injury, and the renal cortex and medulla samples were sequenced separately. In order to assess genetic changes in the whole kidney, the cortical and medulla samples from the corresponding rats were combined and analyzed. The GSE9943 dataset was originally used to explore differential gene expression in the context of the renal ischemia-reperfusion response in BN and SD rats. In light of the possible heterogeneity among rat species, BN and SD rats in this dataset were analyzed separately in this study. For an overview of the study process, see Figure 1.

Common differentially expressed gene (cDEG) identification

The GSE58438 dataset was initially normalized using the R limma package, after which DEGs were identified using the following criteria: adjusted $P < 0.05$ and $|\logFC| \geq 1$. DEGs that were shared between comparisons of control (Ctr) and IR samples collected at the 3 h (IR_3h) and 24 h (IR_24h) time points, the RobustRankAggreg (RRA) R package was utilized. Volcano plots were constructed using the pheatmap package.

Pathway enrichment analyses
The DAVID online tool was used to conduct GO and KEGG pathway enrichment analyses, with resultant bubble charts being constructed using the R ggplot2 package.

**Protein-protein interaction (PPI) network construction, analysis, and hub gene validation**

The STRING database was used to construct a PPI network incorporating identified cDEGs using the default parameters, after which the network was imported into the Cytoscape program. The top 5 hub genes were identified using the Cytohubba plugin based on the Degree, Edge Percolated Component (EPC), Density of Maximum Neighborhood Component (DMNC), and Maximal Clique Centrality (MCC) topological measurements. HMOX1 was identified as the most critical hub gene based upon the weights of these top 5 hub genes under each screening method.

The R limma package was used to normalize and process the GSE27274, GSE3219, and GSE9934 datasets. HMOX1 expression was confirmed in all three datasets and for the Ctr vs IR_120h comparison in the GSE58438 dataset.

**Predictive miRNA identification**

The TargetScan, miRWalk, and miRDB databases were utilized to identify putative miRNA regulators of HMOX1. Venn diagrams were used to identify miRNAs that were predicted by all three of these databases.

**In vitro renal ischemia-reperfusion model establishment**

Rat renal tubular epithelial NRK-52E cells (Procell, CL-0174) were cultured in high-glucose DMEM containing 1% penicillin and streptomycin (Solarbio, 11965) and 10% fetal bovine serum (FBS; Gibco, 16000-044). Cells in the logarithmic phase of growth were added to culture plates. When cells were adherent after ~12 h, plates were transferred to a hypoxic incubator (1% O₂, 95% N₂, and 5% CO₂) for 24 h. Media was then changed, and cells were transferred to a normoxic incubator (21% O₂ and 5% CO₂) for 3, 6, or 9 h in order to establish a renal hypoxia-reoxygenation (HR) model of IR injury. Changes in cell morphology were assessed with an Axio Observer 3 microscope (Carl Zeiss).

**qPCR**

RNA was extracted from renal tubular epithelial cells following exposure to hypoxic conditions for 24 h and after 3, 6, and 9 h of reoxygenation at which time HMOX1 and miR-3587 expression levels were assessed. TriZol (RNAiso Plus, Takara, 108-95-2) was used to extract RNA from all samples, and the NanoDrop 2000 instrument was used to assess RNA concentrations and purity. The EasyScript One-Step gDNA Removal and cDNA synthesis supermix (TransS, AE311-02) were used to prepare cDNA using a StepOnePlus analyzer Real-Time PCR System. All qPCR analyses were performed using a TB Green PreMix Ex Taq TM kit (Takara, RR420A) and an Applied Biosystems 7500 Real-Time PCR instrument. Primer sequences were as follows: β-Actin, positive: 5'-CTA TGA GGG TTA CGC GCT CC-3' and reverse: 5'-ATG TCA CGC ACG ATT TCC CT-3'; HMOX1, positive: 5'-CAG AAG AGG CTA AGA CCG CC-3' and reverse: 5'-
TTG GTG AGG GAAA ATG TGC CA-3'. U6 and miR-3587 primers were synthesized by RiboBio (Guangzhou, China).

**Cellular treatment and transfection**

miR-inhibitor-nc, miR-3587-inhibitor, and a riboFECT™ CP Transfection Kit were purchased from RiboBi. NRK-52E cells in the logarithmic phase of growth were plated in 6-well plates and subjected to the following four treatment conditions: control, HR, HR + miRNA-inhibitor-nc transfection, and HR + miR-3587-inhibitor transfection. Cells were transfected with miRNA-inhibitor-nc and miR-3587-inhibitor constructs that were prepared based on provided kit directions. Briefly, after 12 h when cells were adherent and 50% confluent, the miRNA-inhibitor-nc or miR-3587-inhibitor transfection mixtures were applied and cells were incubated for 24 h. In this experiment, the HR model system was established as above, with the appropriate transfection mixture being added when the media was changed. For an overview of this experimental protocol, see Figure 7B.

**Western blotting**

RIPA buffer (Applygen, C1053) was used to harvest protein from appropriately treated cells, with protein levels in these extracts being quantified with a BCA kit (Applygen, P1511). Equal amounts of protein were then separated via 10% SDS-PAGE (20 μg/lane) and transferred to PVDF membranes (Millipore, IPVH00010 Immobilon-P Transfer Membrane) with a wet transfer system using 300 mA. Blots were blocked with 5% non-fat milk for 1 h at room temperature, followed by overnight incubation with primary antibodies (anti-HMOX1, Abcam, ab68477; anti-GPX4, Abcam, ab125066) at 4°C. Following three washes with TBST, membranes were incubated with HRP-conjugated secondary antibodies (ProteinTech, sa00001-2) for 1 h at room temperature. After three additional washes in TBST, Super ECL Prime (US EVERBRIGHT, S6008) was used to detect protein bands together with a BIO-RAD imaging system. ImageJ v1.8.0 (National Institutes of Health) was used for densitometric analyses of protein bands.

**Analyses of cell viability, malondialdehyde (MDA) levels, and mitochondrial membrane potential (MMP)**

Following successful cellular transfection and HR model establishment as detailed above, cell viability and MDA content were respectively analyzed using a cell counting kit-8 kit (CCK-8, Abmole, M4839) and an MDA determination kit (Nanjing Jiancheng, A003-1-2). Changes in MMP were assessed with an Olympus IX71 fluorescence microscope based on the directions provided with the JC-1 MMP assay kit (Abmole, ab113850).

**Statistical analysis**

GraphPad Prism 8.3.0 was used for all analyses, and experiments were repeated in triplicate. Differences between groups were compared via one-way ANOVAs, with P < 0.05 as the significance threshold.

**Results**
Dataset normalization

Samples included in the GSE58438 dataset were normalized with the R limma package (Figure 2).

Identification of ischemia-reperfusion-delayed DEGs

When analyzing the GSE58438 dataset, 638 DEGs were identified for the Ctr vs. IR_3h comparison, including 413 and 225 up- and down-regulated genes, respectively. Additionally, 1467 DEGs were identified for the Ctr vs. IR_24h comparison, including 671 and 796 up- and down-regulated genes, respectively. The resultant DEGs were arranged into heatmaps using the ggplot2 package (Figure 3A-B), while clustering heatmaps were drawn using the pheatmap package (Figure 3C-D). These heat maps revealed that one sample in the IR_24h dataset (Sample number: GSM1411067) clustered with the control group, so this sample was excluded from subsequent analyses. Following re-screening, 2746 total DEGs were identified for the Ctr vs IR_24h, of which 1304 and 1442 were up- and down-regulated, respectively.

A total of 80 cDEGs were identified using the RRA package when comparing the Ctr vs IR_3h and Ctr vs IR_24h datasets. Of these cDEGs, 55 and 25 were up- and down-regulated, respectively (Figure 3E).

Functional enrichment analyses of cDEGs

GO and KEGG functional enrichment analyses of these 80 cDEGs were next performed using the DAVID database. The top 10 biological process (BP), cellular component (CC), and molecular function (MF) GO terms are shown in Figure 4A-C. The top 5 cDEGs were the response to cytokine, positive regulation of cell proliferation, cellular response to interleukin-1, negative regulation of protein kinase activity, and positive regulation of gene expression categories, while the top 5 enriched CC terms were extracellular space, extracellular matrix, extracellular region, proteinaceous extracellular matrix, and cell surface, and the top 5 enriched MF terms were growth factor activity, cytokine activity, receptor binding, protein kinase inhibitor activity, and protease binding respectively.

KEGG pathway enrichment results are shown in Figure 4D, with the top 5 enriched pathways including the JAK-STAT, TNF, cytokine-cytokine receptor interaction, rheumatoid arthritis, and HIF-1 signaling pathways.

PPI network analysis and hub gene identification

Next, a PPI network was constructed for these cDEGs using the STRING database. The resultant network included 78 nodes and 124 edges, and was visualized using the Cytoscape program (Figure 5A).

This PPI network was analyzed with the MCODE plugin, leading to the identification of three functional clusters (module 1, MCODE score = 7.429; module 2, MCODE score = 3.6; and module 3, MCODE score = 3) (Figure 5B-D). Degree, EPC, DMNC, and MCC values were used to identify the top 5 hub genes in functional cluster 1 (Table 2). The top 5 hub genes identified based upon the Degree, MCC, and EPC
metric were identical, and scores for the top 5 hub genes as screened based upon Degree and MCC values were identical. Only DMNC values yielded distinct hub genes. HMOX1 was a hub gene that exhibited the highest EPC score, and it was thus chosen as a target for further study.

**Assessment of HMOX1 expression in different datasets**

Samples in the GSE58438 and GSE27274 datasets were from Wistar rats, while samples in the GSE3219 dataset were from SD rats, and samples from the GSE9934 dataset were from both SD and BN rats. HMOX1 expression in the GSE58438, GSE27274, GSE3219, and GSE9934 datasets is shown in Table 3 and Figure 6A-E. These analyses revealed that HMOX1 expression rose significantly from 3-8 h after reperfusion, before declining significantly at 24 h post-reperfusion.

**Predictive identification of miRNAs targeting HMOX1**

Using the TargetScan, miRWalk, and miRDB databases. 56, 48, and 11 miRNAs putatively targeting HMOX1 were identified, respectively. The only miRNA predicted by all three databases was miR-3587 (Figure 6F).

**Trends in HMOX1 and miR-3587 expression in renal tubular epithelial cells under hypoxia/reoxygenation conditions**

In order to confirm the above results, an *in vitro* renal IR model system was established by incubating renal tubular epithelial cells under hypoxic conditions followed by reoxygenation for 3, 6, or 9 h. When qPCR analyses were conducted at these time points, HMOX1 expression was gradually reduced as reoxygenation time increased, while miR-3587 was steadily upregulated over this timeframe, with significant differences among groups (Figure 6G-H). Light microscopic analyses for these cells are shown in Figure 6I-L. As reoxygenation time increased, the number of cells declined and they shrank.

**miRNA-3587 inhibition enhances HMOX1 expression and suppresses HR-induced ferroptosis in NRK-52E cells**

Sequence complementarity between HMOX1 and miR-3587 is shown in Figure 7A, while experimental conditions and sample collection are outlined in Figure 7B. Western blotting results indicated that there were significant increases in HMOX1 expression in the HR group, while miR-3587 inhibitor use further increased HMOX1 expression (Figure 7C). GPX4 is an important regulator of ferroptosis, and miR-3587 inhibitor transfection was associated with increased GPX4 expression (Figure 7D). The impact of miR-3587 on NRK-52E cell viability was assessed via CCK-8 assay, revealing that miR-3587 inhibition was associated with increased viability (Figure 7E. MDA is a lipid peroxidation byproduct that is reflective of the degree of lipid peroxidation in cells, thereby offering indirect insight regarding ferroptosis induction. MDA levels in NRK-52E cells declined significantly following miR-3587 inhibitor transfection (Fig. 7F). Ferroptosis is associated with significant reductions in MMP, and the normal control group exhibited red JC-1 fluorescence, whereas green fluorescence was observed following HR treatment, and miR-3587
inhibitor treatment normalized MMP in these HR-treated cells, which exhibited red fluorescent signal (Figure 7G-J).

**Discussion**

The kidneys are highly sensitive to insufficient perfusion, with renal IR often occurring in response to shock, trauma, transplantation, and other factors in critically ill individuals, resulting in AKI occurring in 5-20% of these patients [11]. Acute renal tubular epithelial cell necrosis is a primary pathological finding in AKI [12]. In the absence of timely intervention, small abnormalities in serum creatinine levels can result in severe complications or mortality [13]. Previous studies have shown that ferroptosis is one of the important mechanisms of renal tubular epithelial cell injury in IR [14]. As a unique regulatory mode of cell death, ferroptosis is essentially a metabolic disorder of lipid oxides in cells, that are produced in excess through the catalytic activity of iron ions [4]. When the antioxidant capacity of cells is weakened and lipid reactive oxygen species accumulate, this causes an imbalance of the intracellular redox state and thereby induces cell death. The biological characteristics of ferroptosis include increased reactive oxygen species production, iron ion aggregation, and ultrastructural changes including cell membrane rupture and blebbing, mitochondrial atrophy, decreased or absent mitochondrial cristae, increased membrane density, and normal nuclear morphology without chromatin agglutination [15]. The process of ferroptosis involves a variety of mechanisms and is precisely regulated by multiple signaling pathways [16]. Further study regarding the role of ferroptosis in different diseases is warranted in order to identify novel therapeutic targets and to guide drug development.

Recent advances in genetic sequencing technologies have highlighted a number of genetic changes associated with the progression of different diseases, enabling researchers to gain new insight into the etiological basis for early-stage renal IR injury and to thereby develop novel treatments for AKI. Herein, we utilized the GSE58438 dataset from the GEO database and employed a bioinformatics approach to identify cDEGs for the Ctr vs IR_3h and Ctr vs IR_24h comparisons. HMOX1 was identified as a key ferroptosis-related hub gene in this dataset, and after validating the differential expression of HMOX1 in additional datasets, miR-3587 was predicted to target HMOX1 by the TargetScan, miRDB, and miRWalk databases. Inhibiting miR-3587 was sufficient to decrease ferroptotic injury in renal tubular epithelial cells subjected to HR treatment by promoting HMOX1 upregulation. It is worth noting that, on the one hand, the present study only screened the cDEGs of IR_3h and IR_24h, in order to screen the early genetic changes of IR. On the other hand, with reference to other research methods [17, 18], the present study used external validation datasets which utilized different rat models, all of which exhibited comparable HMOX1 expression trends across these diverse sample types. In addition, this study was the first to report the use of miRNAs targeting HMOX1 to regulate ferroptotic injury in a renal IR model system.

When we analyzed DEGs in the Ctr vs IR_24h comparison for the GSE58438 dataset, clustering heat maps revealed that one IR_24h sample clustered with the control samples, suggesting that modeling was unsuccessful of that this sample was atypical. To improve the specificity of our overall results, this
sample was omitted from subsequent analyses as it had the potential to introduce false-negative experimental results.

Herein, GO analyses of 80 cDEGs indicated that BP terms were primarily associated with responses to cytokines and inflammatory factors, and the regulation of cell proliferation, while CC terms were mostly concentrated in the extracellular and cell surface compartments, and MF terms were primarily associated with cytokine and growth factor activity. These analyses suggested that IR-related genetic pathways were primarily associated with responses to the release of inflammatory mediators and the regulation of cell proliferation. KEGG pathway analyses revealed significant cDEG enrichment in the JAK-STAT and HIF-1 signaling pathways. JAK-STAT pathways play critical roles in diverse processes including proliferation and blood production [19]. HIF-1 signal pathway activation regulates cellular responses to hypoxia via engaging different compensatory pathways. Our enrichment analysis results were consistent with those published by Guo et al [20]. HMOX1 plays a critical role in the HIF-1 signaling pathway, providing a theoretical basis whereby HMOX1 may improve IR injury outcomes.

HMOX1 is an inducible enzyme that catalyzes heme conversion into biliverdin, carbon monoxide, and free ferrous during oxidative stress. From a mechanistic perspective, HMOX1 can also release carbon monoxide and free iron ions, thereby aggravating oxidative stress [21]. The upregulation of HMOX1 is evident in many different human malignancies, wherein it plays an important role in regulating the stability of the tumor microenvironment in a manner that promotes tumor cell proliferation, angiogenesis, and metastasis [21]. However, HMOX1 can also promote ferroptosis in cancer cells in therapeutic contexts [22]. There is also prior evidence that HMOX1 primarily plays a beneficial role in IR injury [23]. Pei et al. demonstrated that normobaric hyperoxia can promote HMOX1 upregulation, thereby protecting against renal IR injury [23]. Su et al. determined that Panx1 gene deletion can protect against IR-induced renal cell ferroptosis in a mouse model system by promoting HMOX1 upregulation [24]. In the present analysis, HMOX1 upregulation was observed in four datasets during the early stages of renal IR before decreasing significantly at 24 h, although no significant difference was observed in the Brown Norway rat model in the GSE9943 dataset at the 24 h timepoint. HMOX1 expression levels were also confirmed in vitro, with expression being significantly lower at 9 h post-HR relative to 6 h post-HR. Renal function changes continuously following reperfusion [25], such that animals ultimately develop secondary chronic kidney disease after reperfusion [26]. As such, approaches to regulating ferroptosis and other early stages of early renal IR injury are critical to improving outcomes.

miRNAs can serve as key regulators of gene expression [7], and both miRNA mimics and inhibitors have been highlighted as promising therapeutic tools in preclinical contexts [27]. Studies have shown that ischemia-reperfusion conditions result in the upregulation of miR-182-5p and miR-378a-3p, activating ferroptotic renal injury by downregulating GPX4 and SLC7A11 [10]. However, there have been relatively few prior analyses of how miRNAs modulate ferroptotic injury in the context of renal IR, and there have been no reports regarding miRNA-mediated HMOX1 regulation. We identified miR-3587 as a predicted regulator of HMOX1 through three online databases based upon shared sequence complementarity.
In prior studies, HMOX1 has been shown to be significantly upregulated under conditions of ischemia, with significant increases in Sprague-Dawley rats treated with cobalt protoporphyrin leading to reductions in oxidative stress, inflammation, and renal injury [28]. As such and in light of the observed HMOX1 upregulation in the context of renal IR, in the present study, a miR-3587 inhibitor was transfected into cells to promote further HMOX1 upregulation in order to explore its beneficial effects in this pathological context. MDA can be measured as a lipid peroxidation index, as it is a toxic aldehyde formed by the peroxidation of cell membrane polyunsaturated fatty acids. GPX4 is a key ferroptosis-related enzyme and the only enzyme capable of detoxifying lipid peroxides. The overexpression or knockout of GPX4 alters rates of lethality associated with different inducers of ferroptosis [29]. Herein, we established an in vitro HR model using renal tubular epithelial cells, and found that miR-3587 inhibitor transfection was sufficient to significantly increase HMOX1 expression, enhance cell viability, reduce MDA levels, and augment GPX4 expression. In the context of ferroptosis, mitochondria typically shrink, and cristae become less prominent or disappear entirely [4]. Herein, JC-1 was utilized to detect changes in MMP, indicating that miR-3587 inhibitor treatment attenuated mitochondrial membrane damage. In summary, in this study, based upon characteristic ferroptosis-related changes including the protein levels of HMOX1 and GPX4, cell viability, the degree of lipid peroxidation, and the mitochondrial membrane potential of our cells of interest, we preliminarily explored a mechanism whereby inhibiting miR-3587 can prevent IR-associated ferroptosis in renal tubular epithelial cells at least in part by promoting HMOX1 upregulation.

**Limitations**

There are certain limitations to the present study. For one, the sample size was relatively limited, as even though four datasets were included in the study, the sample size for each individual dataset was small. Further follow-up studies of murine and human samples are thus warranted to confirm and expand upon these results. Secondly, the in vitro validation approaches employed herein were not sufficiently comprehensive owing to technical limitations and time considerations. Further lentivirus-based approaches to modulating HMOX1 and miR-3587 were considered for the present study, and in the future additional immunohistochemical staining approaches, dual-luciferase reporter assays, and related techniques will be employed to further understand the regulatory roles of these genes in the context of renal IR injury.

**Conclusions**

Herein, a bioinformatics approach was employed leading to the identification of HMOX1 as a key regulator of renal IR injury targeted by miR-3587. By establishing an in vitro renal IR model, we were further able to confirm that this miR-3587 was able to regulate HMOX1 and to thereby attenuate renal IR-induced ferroptotic injury.

**Declarations**

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Conflicts of interest

The authors have no conflicts of interest.

Availability of data and material

Manuscript contains two parts of data. One part is the dataset downloaded from the GEO database for bioinformatic analysis, and the other part is the bioinformatic analysis and experimental research data uploaded in the form of supplementary materials.

Code availability

Not applicable.

Authors' contributions

TWQ designed the experiments. TWQ, FSM and ZH performed the experiments. TWQ, LF, and FSM analyzed the data. LF and QKJ participated in the coordination of study. ZJG and QKJ funded this project. TWQ, LF, and ZJG drafted the manuscript.

Compliance with ethical standards

Consent to participate

All authors have approved the final version of the manuscript and are accountable for all aspects of this study.

Consent for publication

All authors are consent for publication.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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None.

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### Tables

**Table 1 GSE58438, GSE27274, GSE3219, and GSE9934 dataset details**

| Aim         | Accession | Platform | Sample | Control, No. | Ischemia-reperfusion, No. (reperfusion time, h) | Exclusion, No. |
|-------------|-----------|----------|--------|--------------|-----------------------------------------------|---------------|
| Screening   | GSE58438  | GPL11534 | Kidney | 5            | 5 (3h), 4 (24h), 5 (120h)                      | 27            |
| Verification| GSE27274  | GPL6101  | Kidney | 6            | 6 (6h), 6 (24h), 6 (120h)                      | 0             |
| Verification| GSE3219   | GPL2774  | Kidney | 10           | 4 (2h), 5 (8h)                                | 17            |
| Verification| GSE9943   | GPL2996  | Kidney | 6            | 6 (24h)                                       | 0             |
Table 2 Scores for the top 5 hub gene in functional cluster 1

| Sorting | Gene   | Degree | EPC   | MCC   | DMNC | Gene   |
|---------|--------|--------|-------|-------|------|--------|
| 1       | HMOX1  | 7      | 4.351 | 840   | 0.713| TIMP1  |
| 2       | SERPINE1| 7      | 4.336 | 840   | 0.713| LCN2   |
| 3       | CXCL1  | 7      | 4.329 | 840   | 0.695| IL6    |
| 4       | MMP3   | 7      | 4.307 | 840   | 0.695| HMOX1  |
| 5       | IL6    | 7      | 4.256 | 840   | 0.695| SERPINE1|

Abbreviation: EPC, Edge Percolated Component. MCC, Maximal Clique Centrality. DMNC, Density of Maximum Neighborhood Component. HMOX1, Heme Oxygenase-1. TIMP1, Tissue Inhibitor of Metalloproteinase-1. SERPINE1, Serpin Family E Member 1. LCN2, Lipocalin 2. CXCL1, C-X-C Motif Chemokine Ligand 1. MMP3, Matrix Metallopeptidase 3.

Table 3 Differences in HMOX1 expression in different time points in different datasets

| Accession     | Ischemia time, min | Reperfusion time, h | adj. P-value   | LogFC |
|---------------|--------------------|---------------------|----------------|-------|
| GSE58438_Wistar| 45min              | 3h                  | 0.00003635     | 5.31  |
|               | 45min              | 24h                 | 0.00257        | 4.24  |
|               | 45min              | 120h                | 0.1052         | 1.56  |
| GSE27274_Wistar| 20min              | 6h                  | 0.00069931     | 2.17  |
|               | 20min              | 24h                 | 0.00841318     | 1.52  |
|               | 20min              | 120h                | 0.00947        | 0.70  |
| GSE3219_SD    | 40min              | 2h                  | 0.03042455     | 7.13  |
|               | 40min              | 8h                  | 0.02119530     | 29.07 |
| GSE9943_SD    | 45min              | 24h                 | 0.30506        | 2.29  |
| GSE9943_BN    | 45min              | 24h                 | 0.99986953     | 1.44  |

Figures
Figure 1

Study flow chart. Abbreviation: Ctr, control; IR, ischemia-reperfusion; cDEGs, common differentially expressed genes; RRA, RobustRankAggreg; PPI, protein-protein interaction; HMOX1, heme oxygenase 1; miR, microRNA; HR, hypoxia-reoxygenation; PCR, polymerase chain reaction; WB, western blotting; JC-1, a mitochondrial membrane potential indicator.
Figure 2

Normalization of data in the GSE58438 dataset. A, Before normalization; B, After Normalization.
**Figure 3**

DEG volcano plots and clustering heat maps. A. Volcano plot showing DEGs for the Ctr vs IR_3h comparison. B. Volcano plot showing DEGs for the Ctr vs IR_24h comparison. C. DEG clustering heat maps for the Ctr vs IR_3h comparison. D. DEG clustering heat maps for the Ctr vs IR_24h comparison. E, cDEGs clustering heat map.
Figure 4

cDEG functional enrichment analysis results. A. The top 10 GO BP terms. B. The top 10 GO CC terms. C. The top 10 GO MF terms. D. KEGG pathway enrichment results. GO, gene ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes.
Figure 5

A cDEG protein-protein interaction (PPI) network and three functional clusters. Green circles are used to denote downregulated hub genes. A. PPI network; B. functional cluster 1; C. functional cluster 2; D. functional cluster 3.
Figure 6

Analysis of HMOX1 expression patterns in different datasets and in vitro verification of the relationship between HMOX1 and miR-3587. A-E. Trends in HMOX1 expression in different datasets; F. Venn diagram plots for miRNAs targeting HMOX1; G-H. PCR-mediated detection of HMOX1 and miR-3587 expression at different time points following hypoxia-reoxygenation; I-L. Light microscopy analyses of cell morphology
In the Ctr, HR3h, HR6h, and HR9h groups. Scale bar = 4 µm. Magnification 200×. Abbreviation: SD, Sprague Dawley; BN, Brown Norway; HR, hypoxia-reoxygenation. ns, P > 0.05; *, P < 0.05; **, P < 0.01.

Figure 7

Inhibition of ferroptosis in NRK-52E cells during HR via the miR-3587-mediated regulation of HMOX1. A. Predicted sequence complementarity between miR-3587 and HMOX1; B. Experimental grouping and sample processing; C. HMOX1 protein band densitometric analyses; D. GPX4 protein band densitometric analyses; E. Cell viability (%); F. WST-8 absorbance; G. Confocal microscopy images.
analyses; E. Cell viability test results; F. MDA level measurements; G-J. Mitochondrial membrane potential was measured using JC-1 in different treatment groups. Abbreviation: HR, hyperoxia-reoxygenation; RO, reoxygenation; Ctr, control; MDA, malondialdehyde. *P < 0.05; **P < 0.01 vs. control group. ##P < 0.01; ns, P > 0.05 vs. HR group.