The Correlation between Ferroptosis and m6A Methylation in Patients with Acute Kidney Injury

Lihua Ni\textsuperscript{a}  Rui Bai\textsuperscript{b}  Qiuyuan Zhou\textsuperscript{a, c}  Cheng Yuan\textsuperscript{d}  Le-Ting Zhou\textsuperscript{e}  Xiaoyan Wu\textsuperscript{a}

\textsuperscript{a}Department of Nephrology, Zhongnan Hospital of Wuhan University, Wuhan, China; \textsuperscript{b}Department of Radiation and Medical Oncology, Zhongnan Hospital of Wuhan University, Wuhan, China; \textsuperscript{c}Department of Pathology, The Central Hospital of Enshi Autonomous Prefecture, Enshi, China; \textsuperscript{d}Department of Gynecological Oncology, Zhongnan Hospital of Wuhan University, Wuhan, China; \textsuperscript{e}Department of Nephrology, The Affiliated Wuxi People’s Hospital of Nanjing Medical University, Wuxi, China

Keywords
Acute kidney injury · Ferroptosis · N6-methyladenosine methylation

Abstract
Objective: The present research analyzed the correlation between N6-methyladenosine (m6A) methylation and ferroptosis associated genes (FAGs) in acute kidney injury (AKI) patients. Methods: Bioinformatics analysis of microarray profiles (GSE30718) was performed to select differential expression genes (DEGs). FAGs are derived from systematic analysis of the aberrances and functional implications. The m6A methylation related genes were derived from the molecular characterization and clinical significance of m6A modulators. The multi-gene correlation of ferroptosis and M6A methylation modification was displayed. Then, the CIBERSORT algorithm was used to analyze the proportions of 22 immune cell infiltration. Results: In total, 349 DEGs were extracted between the AKI and control samples, among which 172 genes were upregulated and 177 were downregulated. FAGs (SLC1A5, CARS, SAT1, ACSL4, NFE2L2, TFRC, and MT1G) and m6A methylation related genes (YTHDF3, WTAP, and IGF2BP3) were significantly increased in AKI patients (\(p < 0.05\)). FAGs (SAT1, ACSL4, and NFE2L2) were positively correlated with the expression level of m6A methylation genes (\(p < 0.05\)). NFE2L2 has high diagnostic value, and the level of NFE2L2 was negatively correlated with the degree of follicular helper T (T\textsubscript{FH}) cell infiltration. Conclusion: Our research could provide a new theoretical basis for the pathogenesis and immune mechanism of AKI.

Introduction

Acute kidney injury (AKI) is a common complication of sepsis with an incidence between 47 and 61\% [1, 2]. Specifically, 13 million people are affected by AKI each year, and 1.7 million died of AKI induced by ischemia reperfusion. Infection, trauma, renal insufficiency, and drug poisoning may all lead to the occurrence of AKI. The main pathological manifestations of AKI were death and abscission of renal tubular epithelial cells. In recent years, in addition to apoptosis, other regulatory forms of cell death, including ferroptosis, necroptosis, and pyrop-
Ferroptosis, have been gradually recognized [3]. The expression of ferroptosis related signal molecules is positively correlated with the incidence rate and mortality of AKI. On the other hand, iron chelators, and inhibitors of ferroptosis have renal protective effects in various AKI animal models, suggesting that ferroptosis plays an important role in the pathogenesis of AKI [4]. Therefore, exploring the mechanism of ferroptosis in renal tubular epithelial cells in AKI and clarifying the effect of ferroptosis on the process of AKI to chronic kidney disease is of great significance for developing effective treatment strategies for AKI.

N6-methyladenosine (m6A) modification is an important regulatory process of mRNA posttranscriptional modification. It plays an important role in regulating mRNA shear, localization, transcription, and stability [5, 6]. In the field of kidney diseases, it has been observed that Fto, the first-identified RNA demethylase, is abundant in the kidney and regulates the fibrogenic process through the TGF-β signaling pathway in obstructive nephropathy [7]. Li et al. [8] found a strong relationship between m6A methylation modification and the severity of renal interstitial fibrosis. Therefore, it is reasonable to believe that the m6A methylation modification might be involved in the process of kidney injury. In tumor models, it had been confirmed that m6A methylation modification can mediate ferroptosis. However, it is not clear whether m6A methylation is involved in ferroptosis in the AKI model.

The resulting activation of immune cells dysregulates the innate and adaptive immune system, which releases additional proinflammatory cytokines that are filtered in the glomerulus, thereby damaging the proximal tubules and promoting tubular epithelial cell loss and metabolic dysregulation [9, 10]. Therefore, targeted regulation of the degree of inflammation has been a potential strategy for AKI management [11]. However, there are few studies on the type of immune cell infiltration in AKI patients. Therefore, our research used the gene expression omnibus (GEO) database to analyze the relationship between m6A methylation and ferroptosis associated genes (FAGs) by the bioinformatics method, so as to provide a new theoretical basis for the pathogenesis and immune mechanism of AKI.
Method

Microarray Data
The gene expression dataset (GSE30718) were downloaded from the GEO (http://www.ncbi.nlm.nih.gov/geo/) and the platform used for both expression profiling arrays was the Affymetrix Human Genome U133 plus 2.0 Array. The GSE30718 dataset included 26 patients with AKI and 11 renal biopsy specimens from healthy controls.

Identification of Differential Expression Genes
The expression data was normalized by the transcript per million method. The Ensembl transcript ID was transformed into the
The mean value was regarded as the expression level of genes if diverse probes were annotated to the same genes. The Limma package of R software was used to study the differential expression genes (DEGs). \( p < 0.05 \) and \( \log(\text{fold change}) > 0.5 \) or \( \log(\text{fold change}) < -0.5 \) were defined as the thresholds for the screening of DEGs. To further confirm the underlying function of potential targets, the data were analyzed by functional enrichment. Gene Ontology (GO) is a widely-used tool for annotating genes with functions. The Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analysis was a practical resource for the analytical study of gene functions and associated high-level genome functional information.

**Ferroptosis and M6A Methylation Modification**

The FAGs were derived from the systematic analysis by Liu et al. [12] of the aberrances and functional implications. The m6A methylation related genes were derived from research by Li et al. [13] on the molecular characterization and clinical significance of m6A modulators. The multi-gene correlation of ferroptosis and M6A modification was displayed by the heatmap package. All the above analysis methods and the R package were implemented by the R Foundation for Statistical Computing (2020) version 4.0.3.

**Characteristics of Immune Cell Infiltration**

The CIBERSORT algorithm was used with the default parameter for estimating 22 immune cell fractions using FPKM values of each expressed gene. Permutations for significance analysis were 1,000 times; \( p < 0.05 \) was considered statistically significant. A heatmap was used to visualize the proportions of immune cells. The violin plot offers further visualization of the differences in the immune cell distribution between samples.

**Results**

**Identification of DEGs**

In total, 349 DEGs were extracted between the AKI and control samples, among which 172 genes were upregulated and 177 were downregulated. The volcano plots and heatmap were generated to show the correlation between DEGs (Fig. 1).

Given the ontology sources, including KEGG pathways and GO biological processes, we analyzed pathways and functional enrichment of the DEGs. All the results of enrich-
**Fig. 4.** The expression distribution of m6A methylation related genes in AKI and control samples. 

(a) The expression heatmap of m6A methylation related genes. 

(b) m6A methylation related genes analysis results. *p < 0.05, **p < 0.01, ***p < 0.001.
ment analysis were shown in online supplementary Tables S1 and S2 (see www.karger.com/doi/10.1159/000524900 for all online suppl. material). According to the calculated adjusted p values, the top 20 with statistically obvious KEGG pathways and GO biological processes associated with upregulated and downregulated DEGs were presented in Figure 2.

**FAGs**

It was known that ferroptosis was related to a variety of metabolic disorders such as iron metabolism, lipid metabolism, and antioxidant metabolism. In our study, we compared the expression levels of 24 FAGs in the kidney tissues of AKI patients and healthy controls. The significance of the samples between the two groups was tested by Wilcox, and it was found that FAGs (SLC1A5, CARS, SAT1, ACSL4, NFE2L2, TFRC, and MT1G) were upregulated in AKI (Fig. 3). It is worth noting that the FAGs were upregulated in the samples of AKI, which reflected that ferroptosis was likely to participate in the process of AKI.

**M6A Methylation Modification Related Genes**

RNA methylation is regulated by different types of regulators, including methyltransferases, RNA-binding proteins, and demethylases. We analyzed three types of regulators. The results showed that the expression levels of YTHDF3, WTAP, and IGF2BP3 were significantly upregulated in AKI patients (Fig. 4). This phenomenon also suggested that M6A methylation modification was activated in AKI.

The Correlation of Ferroptosis and m6A Methylation in AKI

The expression of the above genes is all activated in AKI patients. But the correlation between the ferroptosis and M6A methylation modification related genes has not been further studied. The results of Spearman correlation analysis found FAGs (SAT1, ACSL4, and NFE2L2) were significant positive correlated with the expression level of m6A methylation genes (Fig. 5).

In order to further explore the clinical diagnostic value of FAGs in AKI. A receiver operating characteristic curve is built on a univariate classification model. Although the FAGs have high diagnostic efficiency, only CARS and NFE2L2 have an area under the curve of more than 0.8 (Fig. 6). Therefore, we believe that NFE2L2 may be worthy of further study.

Identification of the Infiltrating Immune Cells

The microenvironment is composed of immune cells, inflammatory factors, extracellular matrix, and various growths, and it has an important impact on clinical treat-
Fig. 6. A ROC curve built on a univariate classification model to estimate the diagnostic efficiency. ROC, receiver operating characteristic.
ment sensitivity and disease diagnosis. We used the CIBERSORT algorithm to investigate the abundance of immune cells in individual samples (Fig. 7a). The median value of NFE2L2 expression level was used as the grouping standard; the heatmap suggested the abundance of 22 immune cells in low NFE2L2 samples and high NFE2L2 samples in AKI patients (Fig. 7b). Then the relationship results of various immune cells were shown in Figure 8a.
Fig. 8. a, b Correlation between 22 kinds of immune cell infiltration and NFE2L2 expression level.
Interestingly, in AKI kidney samples with high expression of NFE2L2, the degree of infiltration of follicular helper T (T\textsubscript{FH}) cells was significantly reduced ($p = 0.007$, Fig. 8b).

**Discussion**

The pathogenesis of AKI is very complicated. At present, a variety of molecular mechanisms that induce or aggravate AKI have been proposed. Among them, ROS-induced kidney injury is considered to be one of the key mechanisms of AKI. Previous studies have shown that ferroptosis is a promising therapeutic target, especially in diseases dominated by renal tubular necrosis [14]. Early animal studies have shown the role of non-heme iron in AKI [15], and the protection of iron chelators on renal function in AKI models directly proved that ferroptosis was involved in the occurrence of AKI [16]. In a mouse model of AKI, neither the apoptosis inhibitor vZAD nor the necroptosis inhibitor Nec-1 can reduce renal damage and renal tubular damage, while the specific inhibitor of ferroptosis (ferrostatin-1, Fer-1) and its derivatives have obvious protective effects [17]. The above results indicate that ferroptosis is an important form of renal tubular epithelial cell death in AKI. Our results also confirmed that the expression level of FAGs (SAT1, ACSL4 and NFE2L2) in the kidney tissue of AKI patients was significantly upregulated. Especially, NFE2L2 is not only related to m6A methylation modification but also has high diagnostic value. Therefore, we speculated that the NFE2L2 may have a potential correlation with m6A methylation modification in AKI.

Past research has confirmed understanding of the contribution of epigenetic mechanisms to AKI, especially histone modifications and DNA methylation [18–20]. However, the m6A mRNA methylation also affects the growth of variant cells in the development of organs [21]. In recent years, it has been discovered that m6A methylation also affects cell survival during AKI [22]. Herein, we studied this question in the correlation between AKI and levels of m6A methylation related gene. Notably, we found that the levels of SAT1, ACSL4, and NFE2L2 in the kidney biopsies of patients with AKI were significantly higher compared with healthy controls. Even so, there is not enough evidence to show whether ferroptosis is related to m6A modification in AKI model. This correlation has only been reported in a few researches [23, 24]. Therefore, our research also conducted a preliminary exploration of this correlation.

In recent years, ferroptosis has also been proven to be associated with immunotherapy. Wang et al. [25] found that ferroptosis was related to T-cell mediated immunity. Therefore, we further discussed the association with the FAG signature (NFE2L2) and immune cell infiltration. The results indicated the expression level of NFE2L2 was negatively correlated with the degree of T\textsubscript{FH} cell infiltration. T\textsubscript{FH} cells are a subset of CD4+ T cells specialized in supporting germinal center responses. Excessive T\textsubscript{FH} cell responses drive the production of pathogenic autoantibodies in autoimmunity. Previous research reported ferroptosis could regulate T\textsubscript{FH} cell homeostasis, which can be targeted to enhance T\textsubscript{FH} cell function [25]. It should be noted that there is not enough evidence to support the relationship between NFE2L2 and T\textsubscript{FH} cells.

However, our research still has some limitations. First, the method of bioinformatics is based on statistical results, which may have false negative or false positive errors. The GSE30718 dataset included 26 patients with AKI and 11 renal biopsy specimens from healthy controls, so the limited sample size may also lead to an increased false-positive rate. Second, although we discussed the correlation between m6A methylation and ferroptosis based on the expression level, we were unable to judge the causal relationship between m6A methylation and ferroptosis due to the lack of laboratory verification. Whether the correlation between NFE2L2 and T\textsubscript{FH} cell infiltration can reflect iron death and T\textsubscript{FH} cell infiltration in AKI patients is also debatable.

The lack of effective drug treatment is an urgent problem for AKI. Ferroptosis is involved in the occurrence and progression of AKI. Therefore, if the regulatory mechanism of m6A methylation and ferroptosis can be further clarified, our research could provide a new theoretical basis for the pathogenesis and immune mechanism of AKI.

**Statement of Ethics**

An ethics statement was not required for this study type, no human or animal subjects or materials were used.

**Conflict of Interest Statement**

The authors declare that they have no competing interests.
Ferroptosis and m6A Methylation in AKI

**References**

1. Platani M, Kashani K, Cabello-Garza J, Maladonado F, Kashyap R, Kor DJ, et al. Predictors of acute kidney injury in septic shock patients: an observational cohort study. *Clin J Am Soc Nephrol.* 2011;6(7):1744–51.
2. Uchino S, Kellum JA, Bellomo R, Doig GS, Morimatsu H, Morgera S, et al. Acute renal failure in critically ill patients: a multinational, multicenter study. *JAMA.* 2005;294(7):813–8.
3. Linkermann A, Chen G, Dong G, Kunendorf U, Krautwald S, Dong Z. Regulated cell death in AKI. *Clin J Am Soc Nephrol.* 2014;25(12):2689–701.
4. Martin-Sanchez D, Ruiz-Andres O, Poveda J, Carrasco S, Cannata-Ortiz P, Sanchez-Niño MD, et al. Ferroptosis, but not necroptosis, is important in nephrotic folic acid-induced AKI. *J Am Soc Nephrol.* 2017;28(1):218–29.
5. Lan Q, Liu PY, Haase J, Bell JL, Hüttelmaier S, Liu T. The critical role of RNA m6A methylation in cancer. *Cancer Res.* 2019;79(7):1285–92.
6. Jia G, Fu Y, Zhao X, Dai Q, Zheng G, Yang Y, et al. N6-methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. *Nat Chem Biol.* 2011;7(12):885–7.
7. Wang CY, Shie SS, Tsai ML, Yang CH, Hung KC, Wang CC, et al. FTO modulates fibrogenic responses in obstructive nephropathy. *Sci Rep.* 2016;6:18874.
8. Li X, Fan X, Yin X, Liu H, Yang Y. Alteration of N(6)-methyladenosine epitranscriptome profile in unilateral ureteral obstructive nephropathy. *Epigenomics.* 2020;12(14):1157–73.
9. Zarbock A, Gomez H, Kellum JA. Sepsis-induced acute kidney injury revisited: pathophysiology, prevention and future therapies. *Curr Opin Crit Care.* 2014;20(6):588–95.
10. de Pablo R, Monserrat J, Prieto A, Alvarez-Mon M. Role of circulating lymphocytes in patients with sepsis. *Biomed Res Int.* 2014;2014:671087.
11. Yao W, Chen Y, Li Z, Ji J, You A, Jin S, et al. Single cell RNA sequencing identifies a unique inflammatory macrophage subset as a druggable target for alleviating acute kidney injury. *Adv Sci.* 2022;e2103675.
12. Liu Z, Zhao Q, Zuo ZX, Yuan SQ, Yu K, Zhang Q, et al. Systematic analysis of the aberrations and functional implications of ferroptosis in cancer. *iScience.* 2020;23(7):101302.
13. Li Y, Xiao J, Bui J, Tian Y, Qu Y, Chen X, et al. Molecular characterization and clinical relevance of m(6)A regulators across 33 cancer types. *Mol Cancer.* 2019;18(1):137.
14. Linkermann A. Nonapoptotic cell death in acute kidney injury and transplantation. *Kidney Int.* 2016;89(1):46–57.
15. Baliga R, Ueda N, Shah SV. Increase in bleomycin-detectable iron in ischaemia/reperfusion injury to rat kidneys. *Biochem J.* 1993;291(Pt 3)(Pt 3):901–5.
16. Shah SV, Rajapurkar MM, Baliga R. The role of catalytic iron in acute kidney injury. *Clin J Am Soc Nephrol.* 2011;6(10):2329–31.
17. Guo C, Dong G, Liang X, Dong Z. Epigenetic regulation in AKI and kidney repair: mechanisms and therapeutic implications. *Nat Rev Nephrol.* 2019;15(4):220–39.
18. Chakraborty A, Viswanathan P. Methylation-demethylation dynamics: implications of changes in acute kidney injury. *Anal Cell Pathol.* 2018;2018:8764384.
19. Ingrosso D, Perna AF. DNA methylation dysfunction in chronic kidney disease. *Genes.* 2020;11(7):811.
20. Kagan VE, Mao G, Qu F, Angeli JP, Doll S, Croix CS, et al. Oxidized arachidonic and arachidonic acids induce cell ferroptosis. *Nat Chem Biol.* 2017;13(1):81–90.
21. Roundtree IA, Evans ME, Tan T, He C. Dynamic RNA modifications in gene expression regulation. *Cell.* 2017;169(7):1187–1197.
22. Xu Y, Yuan XD, Wu JJ, Chen RX, Xia L, Zhang M, et al. The N6-methyladenosine miRNA methylase METTL14 promotes renal ischemic reperfusion injury via suppressing YAP1. *J Cell Biochem.* 2020;121(1):524–33.
23. Ma L, Zhang X, Yu K, Xu X, Chen T, Shi Y, et al. Targeting SLCSA2 subunit of system Xc– is essential for m(6)A reader YTHDC2 to be an endogenous ferroptosis inducer in lung adenocarcinoma. *Free Radic Biol Med.* 2021;168:25–43.
24. Song Z, Jia G, Ma P, Cang S. Exosomal miR-4443 promotes cisplatin resistance in non-small cell lung carcinoma by regulating FSP1. *m6A modification-mediated ferroptosis. Life Sci.* 2021;276:119399.
25. Wang W, Green M, Choi JE, Gijon M, Kennedy PD, Johnson JK, et al. CD8(+) T cells regulate tumour ferroptosis during cancer immunotherapy. *Nature.* 2019;569(7755):270–4.