Identification of co-expression hub genes for ferroptosis in kidney renal clear cell carcinoma based on weighted gene co-expression network analysis and The Cancer Genome Atlas clinical data

Shengxian Li1,*, Ximei Xu1, Ruirui Zhang1 & Yong Huang1,2*

Renal clear cell carcinoma (KIRC) is one of the most common tumors worldwide and has a high mortality rate. Ferroptosis is a major mechanism of tumor occurrence and development, as well as important for prognosis and treatment of KIRC. Here, we conducted bioinformatics analysis to identify KIRC hub genes that target ferroptosis. By Weighted gene co-expression network analysis (WGCNA), 11 co-expression-related genes were screened out. According to Kaplan Meier’s survival analysis of the data from the gene expression profile interactive analysis database, it was identified that the expression levels of two genes, PROM2 and PLIN2, are respectively related to prognosis. In conclusion, our findings indicate that PROM2 and PLIN2 may be effective new targets for the treatment and prognosis of KIRC.

Abbreviations
DEGs  Differentially expressed genes
FDR  False discovery rate
GSEA  Gene set enrichment analysis
KIRC  Kidney renal clear cell carcinoma
OS  Overall survival
PCA  Principal component analysis
PI3K/AKT  Phosphatidylinositol 3-kinase/protein kinase B
PLIN2  Perilipin-2
PROM2  Prominin-2
RCC  Renal cell carcinoma
RNA-seq  RNA sequencing
TCGA  The Cancer Genome Atlas
TOM  Topological overlap matrix
WGCNA  Weighted gene co-expression network analysis

Renal cell carcinoma (RCC) is known to account for more than 90% of all adult kidney tumors. Its predominant histological subtype is renal clear cell carcinoma (also called kidney renal clear cell carcinoma; KIRC), which accounts for ~80% of RCC, the prognostic risk assessment of KIRC varies according to TNM staging, age, and

1National Center for International Research of Bio-Targeting Theranostics, Guangxi Key Laboratory of Bio-Targeting Theranostics, Collaborative Innovation Center for Targeting Tumor Diagnosis and Therapy, Guangxi Talent Highland of Bio-Targeting Theranostics, Guangxi Medical University, Nanning 530021, Guangxi, China. 2National Center for International Research of Biological Targeting Diagnosis and Therapy, Guangxi Key Laboratory of Biological Targeting Diagnosis and Therapy Research, Collaborative Innovation Center for Targeting Tumor Diagnosis and Therapy, Guangxi Medical University, Nanning 530021, Guangxi, China. *email: L914694127@126.com; huangyong503@126.com
gender. The five-year survival of patients is closely related to the pathological grade, depending on whether the
tumor is early, intermediate, or advanced. If the patient is in the early stage of the disease, i.e., a pathological grade
of I or II, the patient will have a better treatment effect and a longer survival period after surgical resection, with a
five-year survival rate as high as 80–90%. If the patient is in the middle and advanced stages of KIRC, with a
pathological grade of III or IV, the prognosis of the patient is relatively poor, tumor malignancy and the prob-
ability of metastasis and recurrence high, and the survival period shortened to five years. In these instances, the
survival rate is ~20%5–5. Therefore, it is recommended that patients must be detected and treated early. However,
only ~10% of patients with KIRC show characteristic clinical symptoms, and 20–30% of patients have metastases
already detected at the first visit. In ~30% of patients with localized KIRC, recurrence occurs twice after surgical
resection, whereas conventional radiotherapy and chemotherapy are furthermore largely ineffective in curing
KIRC4. Although the mechanism of its occurrence and development has been studied in-depth, its pathogenesis
and carcinogenesis are still unclear, and specific sensitive tumor markers are still lacking5. Therefore, it is imper-
tative to improve our understanding of the molecular mechanism of KIRC to identify prognostic biomarkers and
treatment targets that can guide the existing clinical phenotypic staging systems.

Modern high-throughput sequencing technologies include DNA sequencing, RNA sequencing (RNA-seq),
and epigenome research. Bioinformatics analysis of the results of these technologies, especially network analysis,
allows us to obtain genome assembly, genome annotation, and gene function annotation12–15. Bioinformatics
provides a new perspective by efficient integration of multiple large-scale datasets for various human diseases.
However, most bioinformatics research only focuses on identifying differentially expressed genes (DEGs)16, which
neglects the functional relationship and high correlation between genes with similar expression patterns17,18. Weighted
gene co-expression network analysis (WGCNA) explores the correlation between different genomes or
between samples and clinical features by constructing a free-scale gene co-expression network19,20. This method
is widely used to identify related clinical modules and hub genes of different types of cancer21. For example,
Yuan et al. used WGCNA as early as 2017 to discover that the expression of six pivotal genes is closely related
to the progression and prognosis of KIRC19. Lastly, Zhang et al. used WGCNA to verify the six hub genes of
colorectal cancer19.

Ferroptosis is a cell death pathway, first proposed by Professor Brent Stockwell of Columbia University in
201220, which is caused by an increase in iron ion load driving a large amount of lipid peroxidation. It primar-
ily involves three major metabolic mechanisms, including iron metabolism, lipid metabolism, and amino acid
metabolism21. Among these, lipid metabolism induces tumor development and treatment resistance by enhanc-
ing lipid synthesis, storage, and catabolism. In general, the membrane fatty acid composition, such as the ratio
of saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids, promotes cell growth.
However, tumor cells show plasticity in fatty acid metabolism which has resulted in a shift in research focus to
limit fat toxicity and ferroptosis as a means to improve overall survival (OS) in patients with cancer22,23.

Studies have shown that ferroptosis is closely related to the occurrence and development, as well as prognosis
treatment of tumors—with KIRC as a tumor type sensitive to ferroptosis24. In KIRC and normal tissues,
ferroptosis regulators are related to PD-L1 expression which affects the tumor immune microenvironment pro-
moting tumorigenesis25. Furthermore, Wu et al. showed that ferroptosis is related to the clinicopathological
characteristics of patients with KIRC and constructed five KIRC and ferroptosis-related prognostic models26.
Another study found that 11 ferroptosis genes (CARS, CD44, DPP4, GCLC, HMGCR, HSPB1, NCOA4, SAT1,
PHKG2, GOT1, HMBOX1) were significantly related to the OS of patients with KIRC27. Moreover, CHAC128, Acyl-CoA
Thioesterase 8 and 1129, and SUV39H130 may be effective prognostic indicators of KIRC. Ling et al. found
that the TAZ/WNT10B axis may serve as biomarkers and therapeutic targets for KIRC immunotherapy31.
Taken together, there are many studies on KIRC, but the current understanding of its pathogenesis, tumor
progression, and metastasis is still imperfect, with many of its characteristics differing from other cancers32–34.
Therefore, finding novel ferroptosis-related targets or pathways for the treatment of KIRC would two-fold curb
the high recurrence rate and growing drug resistance of KIRC.

In this study, we downloaded the RNA-seq data of KIRC samples from The Cancer Genome Atlas (TCGA),
used WGCNA to determine the genes and modules related to the clinical characteristics of patients with KIRC,
and aimed to identify the co-expression of genes related to clinical characteristics and ferroptosis. Then, through
survival analysis of the co-expressed genes, we determined hub genes with the highest prognostic potential.
Our study aims to find a link between KIRC-related genes with ferroptosis that may be used for the prognosis of
KIRC. This could be insightful for the development of potential biomarkers and treatment targets, providing a
new perspective for future research into KIRC.

Materials and methods
Data sources and study design. RNA-seq data and clinical information on patients were downloaded on
October 17th, 2021 from the ‘Colon and Rectal Cancer’ cohort of the TCGA database (https://www.cancer.gov/
about-nci/organization/ccg/research/structural genomics/tcga), hosted at the Xena website of the University of
California at Santa Cruz25 (http://xena.ucsc.edu/; Table 1 and Supplementary Tables S1–S2). The RNA-seq data
contained 538 tumor samples and 407 normal tissue samples from 945 patients with KIRC. Data on the clinical
information, including the OS of patients with KIRC, were also obtained from the TCGA database (http://xena.
ucsc.edu/; Supplementary Tables S3). In addition, data on genes related to induction and inhibition of ferroptosis
were downloaded from the FerrDB database (http://www.zhounan.org/ferrdb/; Supplementary Table S4). Using
principal component analysis (PCA), we excluded samples if the first two principal components identified were
unable to distinguish tumor tissue from normal tissue. The study process is shown in Fig. 1.
Principal Component Analysis (PCA) is a technique used for dimensionality reduction. It helps in visualizing high-dimensional data in a more comprehensible format. UMAP, tSNE, and PCA are among the commonly used dimensionality reduction methods.

For UMAP analysis, the R software package UMAP v0.2.7.0 was utilized. The Z-score was first applied to the expression spectrum, followed by the UMAP function for dimension reduction analysis to obtain the matrix. PCA analysis was performed using the R software package STATS v3.6.0. Similarly, for tSNE, the R package Rtsne v0.15 was employed. The Z-score was applied to the expression spectrum, followed by the Rtsne function for dimension reduction analysis to obtain the matrix.

Among these methods, UMAP demonstrated superior performance. UMAP possesses a highly efficient visualization and scalable dimensionality reduction algorithm. It offers competitive visualization quality compared to t-SNE, retains more global structure, presents superior running performance, and ensures better scalability. Moreover, UMAP allows for embedding dimensions without computational limits, making it a versatile tool for general-purpose dimension reduction in machine learning. UMAP also preserves more global structure, particularly in the continuity of cell subsets. Hence, UMAP was chosen for sample exclusion based on distinguishing tumors from normal tissue.

**Identification of KIRC DEGs**

To identify differentially expressed genes (DEGs) between distinct groups, the R software package Limma (Linear Models for Microarray Data) was utilized. This package performs multiple linear regression and computes moderated t-statistics, moderated F-statistic, and log-odds of DEGs by empirical Bayes moderation of the standard errors towards a common value.

The following table provides the clinical information and sample size for the TCGA KIRC dataset.

| Characteristics     | LIVING (N=605) | DECEASED (N=340) | Total (N=945) | FDR  |
|---------------------|----------------|------------------|---------------|------|
| Cancer type         |                |                  |               |      |
| Kidney clear cell renal carcinoma | 605 (64.02%)  | 340 (35.98%)     | 945 (100.00%) |      |
| Age                 |                |                  |               |      |
| Mean ± SD           | 58.74 ± 11.72  | 64.53 ± 11.82    | 60.82 ± 12.08 |      |
| Median [min, max]   | 59.00 [26.00, 86.00] | 64.00 [32.00, 90.00] | 61.00 [26.00, 90.00] |      |
| Gender              |                |                  |               | 0.36 |
| Female              | 203 (21.48%)   | 125 (13.23%)     | 328 (34.71%)  |      |
| Male                | 402 (42.54%)   | 215 (22.75%)     | 617 (65.29%)  |      |
| Stage               |                |                  |               | 9.20E-44 |
| Stage I             | 369 (39.17%)   | 87 (9.24%)       | 456 (48.41%)  |      |
| Stage II            | 72 (7.64%)     | 25 (2.65%)       | 97 (10.30%)   |      |
| Stage III           | 133 (14.12%)   | 98 (10.40%)      | 231 (24.52%)  |      |
| Stage IV            | 30 (3.18%)     | 128 (13.59%)     | 158 (16.77%)  |      |

This dataset was used to conduct a comprehensive analysis of the relationship between various clinical characteristics and patient outcomes. The analysis included the identification of key genes with significant expression differences, followed by validation of these genes in the expression level. The results were further analyzed for survival and OS predictions, leading to the identification of 11 hub genes, including PROM2 and PLIN2, which showed promising prognostic significance.
DEG significance of each gene. DEGs were defined as those showing $|\log2(\text{fold-change})|> 1$ and $P < 0.01$. Volcano plots of DEGs were plotted using the R function `ggplot2'.

**WGCNA.** At first, the Pearson's correlation matrices and average linkage method were both performed for all pair-wise genes, and then a weighted adjacency matrix was constructed using a power function $A_{nm} = |C_{nm}|^\beta$ ($C_{nm} = \text{Pearson's correlation between genes } m \text{ and } n$; $A_{nm} = \text{adjacency between genes } m \text{ and } n$)$^{39,40}$; and $\beta$ was a soft-thresholding parameter that could emphasize strong correlations between genes and penalize weak correlations. After choosing the power of 6, the adjacency was transformed into a topological overlap matrix (TOM)$^{39}$, which could measure the network connectivity of a gene defined as the sum of its adjacency with all other genes for network generation, and the corresponding dissimilarity (1-TOM) was calculated. To classify genes with similar expression profiles into gene modules, average linkage hierarchical clustering was conducted according to the TOM-based dissimilarity measure with a minimum size (gene group) of 100 for the genes dendrogram, with the sensitivity set to 4. To further analyze the module, we calculated the dissimilarity of module eigengenes, chose a cut line for module dendrogram and merged some modules$^{40}$. In addition, we also merged modules with a distance of less than 0.25, and finally obtained nine co-expression modules. It is worth noting that the grey module is considered to be a gene set that cannot be assigned to any module.

**Correlation analysis of clinical features and modules for identification of KIRC hub genes.** Using clinical features as input, we performed correlation analysis between the modules and clinical features, as well as between MM (module membership) and GS (gene significance). Based on the weighted correlation, a hierarchical clustering analysis was performed, and the clustering results were segmented according to set criteria to obtain different gene modules—represented by the branches and different colors of the clustering tree. The relationship between models was studied and an interaction network of different models was constructed on the system level. We used the “Venn” R package to draw a Venn map of ferroptosis-related DEGs and prognostic genes, while also preserving information related to the intersection genes.

**Functional enrichment analysis by gene set enrichment analysis (GSEA).** The GSEA v3.0 software was obtained from the GSEA (http://software.broadinstitute.org/gsea/index.jsp) website$^{36}$, whereas the c2.cp.kegg.v7.4.symbols.gmt subset was downloaded from the Molecular Signatures Database$^{40}$ (http://www.gsea-msigdb.org/gsea/downloads.jsp) to evaluate related pathways and molecular mechanisms based on gene expression profiles and phenotypes. The minimum gene set function was set to 5 and the maximum gene set was 5,000 resamplings ($5 \times 1,000$ resamplings). $P < 0.05$ and a false discovery rate (FDR) $< 0.25$ were considered statistically significant.

**Functional enrichment of DEGs.** We used the GO annotation of genes in the ‘org.hs.eg.db’ v3.1.0 R software package as the background, then mapped genes into the background set, and used ‘clusterProfiler’ v3.14.3 for enrichment analysis to obtain the result of gene accumulation. The minimum gene set function was 5 and the maximum gene set was 5,000. $P < 0.05$ and an FDR $< 0.25$ were considered statistically significant.

**Bioinformatics validation of hub genes.** The survival prediction of hub genes was assessed using Kaplan–Meier analysis with the ‘survival’ v3.2–7 R package. First, we obtained the DEG profile and prognostic data of 578 KIRC tumor samples from the TCGA and then determined the median expression value of each gene. Depending on whether the expression of a given gene was above or below the median, samples were assigned to either the "high expression" or "low expression" group. The log-rank test was used to evaluate the significance of the difference in survival between the high and low expression groups. If the test correlated with $P < 0.05$, we considered the gene to be a verified pivot gene.

Verifying that a gene is a DEG in most tumors is part of the broad-spectrum analysis. If gene is differentially expressed in most tumors, it means that it is broad-spectrum. Thereafter, a single gene is analyzed for its expression in tumors based on its differential expression in each tumor. We downloaded a unified standardized pan-cancer dataset from the UCSC (https://xenabrowser.net/) database: TCGA TARGET GEx (PANCAN, $N = 19,131$, $G = 60,499$), and further extracted ENSG00000155066 (prominin-2; PLIN2) gene expression data in each sample. We screened samples from the datasets for Solid Tissue Normal, Primary Solid Tumor, Primary Tumor, Normal Tissue, Primary Blood-Derived Cancer-Bone Marrow, and Primary Blood-Derived Cancer-Peripheral Blood. Thereafter, we performed $\log2(x + 0.001)$ transformation on each expression value. Lastly, we eliminated cancer types with less than 3 samples to obtain the expression data of 34 cancer types. We used R software (version 3.6.4) to calculate the expression difference between normal and tumor samples for each tumor and used the unpaired Wilcoxon Rank Sum and Signed Rank Tests to analyze the significance of the difference$^{36}$.41.

**Statistical analysis.** In this study, all statistical analyses were performed using R language (version 4.0.2). A two-sided $P$ value of $< 0.05$ was considered as statistically significant. The adjusted $P$ value was determined by the Benjamini–Hochberg method. All methods were carried out in accordance with relevant guidelines and regulations.

**Human and animal rights.** This article only collected data from existing databases without any direct involvement of human participants.
Results

Data pre-processing. The KIRC gene expression profile dataset downloaded from the TCGA database contains 72 normal samples and 534 tumor samples (Supplementary Table S1). Based on PCA, the first two principal components differentiated well between tumors and normal samples, forming two distinct clusters. In grouping tumor and normal samples, those with close distances had similar properties, and samples that deviated significantly from the population were removed. In total, 27 tumor tissues and 1 normal tissue were excluded (Fig. 2A,B). The gene expression profiles of the remaining 578 samples (Fig. 2C) and 259 ferroptosis-related genes were used for subsequent analysis.

Identification of DEGs. The data set after excluding outliers was analyzed by limma analysis. From the results of the volcano plot and heatmap (Fig. 3A,B), we found 5,151 DEGs with obvious differences after analysis, of which 2,522 genes were up-regulated and 2,629 genes down-regulated.
**GSEA.** GSEA is used to evaluate the distribution trend of genes in a predefined gene set ranked by phenotype correlation (based on its contribution to phenotype). As expected, several ferroptosis-related functions were involved, including pyruvate metabolism, propanoate metabolism, fatty acid metabolism, bladder cancer, butanoate metabolism, proximal tubule bicarbonate reclamation, steroid biosynthesis, arginine and proline metabolism, etc. (Fig. 4) (www.kegg.jp/kegg/kegg1.html) 42–44.

**WGCNA and identification of critical modules.** We performed a cluster analysis according to the expression matrix of 5,151 DEGs and the network of clinical data of 578 KIRC samples. It can be seen that all samples in the cluster were within the cut-off threshold (height < 200) which means that all values were normal (Fig. 5D). Four clinical variables were used in WGCNA (Fig. 5D): tumor-normal, gender, age, and stage. The 578 samples were divided into two clusters: tumor and normal. To build a scale-free network, we set the soft threshold power $\beta$ to 6, the independence to 0.83, and the average connectivity to 36.36 (Fig. 5A,B). The DEGs with similar expression patterns were gathered into the same module, and the modules showing the cutting height difference < 0.25 were merged. This process produced nine co-expression modules: blue, green, green-yellow, grey, magenta, purple, turquoise, black and yellow (Fig. 5C). The characteristic genes of the turquoise module ($r = 0.96$) and blue module ($r = 0.81$) are highly correlated with KIRC (Fig. 6C,D). These results were also confirmed by analysis of hierarchical clustering, heatmap, and adjacency relationship (Fig. 6A,B). Taken together,
these results indicate that turquoise and blue modules may be closely related to KIRC. Therefore, the central genes of the turquoise and blue modules were further analyzed.

**Identifying candidate hub genes from the most relevant modules.** As shown in Fig. 6C,D, the MM and GS scores were strongly correlated with each other in the turquoise and blue modules. The criteria for selecting pivot genes were relatively lower than the standard cut-off threshold (MM > 0.79). Under the unified
threshold of "cor. gene Module Membership" > 0.6 and "cor. gene Trait Significance" > 0.6, a total of 708 genes were determined to be satisfied in the turquoise module and 357 genes in the blue module.

**Screening of co-expressed genes of ferroptosis-related genes and KIRC DEGs.** By analyzing the ferroptosis genes contained in the DEGs, we found that there were a total of 11 KIRC DEGs related to ferroptosis (Fig. 7). As shown in Fig. 8, the GO enrichment analysis of these 11 genes was all related to lipid metabolism. It is well known that abnormal lipid metabolism is an important factor inducing ferroptosis. In other words, ferroptosis plays a vital role in KIRC.

**Hub gene expression and correlation with survival.** The Wald test method was used to analyze the hub genes with ACSF2, PROM2, and PLIN2 confirmed as possible prognostic-related genes (Fig. 9A). By examining the potential correlation between the expression of candidate central genes and patient survival, we found that PROM2 and PLIN2 were significantly related to prognosis (Fig. 9C,D), while no significance was found for ACSF2 (Fig. 9B). Therefore, we defined the PROM2 and PLIN2 genes as the "final" pivotal genes.

We also confirmed that the expression of PROM2 and PLIN2 were significantly different between the normal and KIRC tissues (Fig. 9E,F). PLIN2 was down-regulated in KIRC, while PROM2 was up-regulated.

As seen from Fig. 10A,B, the expression of PROM2 and PLIN2 were significantly different between multiple tumors. Furthermore, the protein–protein interaction network analysis chart is shown in Fig. 11A,B.
**Figure 9.** Survival analysis and validation of hub genes. (A) Wald Test. (B) Kaplan–Meier survival curves of patients with KIRC stratified by low or high expression of PLIN2. (C, D) Kaplan–Meier survival curves of patients with KIRC stratified by low or high expression of PLIN2 (C) and PROM2 (D). (E–F) Differences in expression of the PLIN2 (C) and PROM2 (D) genes between normal and tumor tissues. *P* < 0.01.

**Figure 10.** Validation of hub genes in various tumors. (A, B) Differences in expression of the PLIN2 (A) and PROM2 (B) genes in various tumors. *P* < 0.01.
Discussion

KIRC is one of the most serious malignant tumors worldwide. Various studies have used WGCNA to explore molecular markers for diagnosis and prognosis. Specifically, Zou et al. showed that KIRC has a unique metabolic state more prone to ferroptosis in response to the hypoxia-inducible factor pathway. On the therapeutic front, they indicated GPX4 as an effective therapeutic target for KIRC. However, their research only focused on specific genes in KIRC, and the mechanism of ferroptosis in KIRC remained uncertain.

In this study, we found 5,151 DEGs between normal and KIRC tissues using limma analysis, indicating that the occurrence and development of KIRC are regulated by a complex genetic network. By using WGCNA, we identified that genes of the turquoise and blue modules were most closely related to KIRC, with 1,066 relevant genes. By analyzing these 1,066 KIRC-related genes and 259 ferroptosis genes, a total of 11 ferroptosis co-expression hub genes were found. Then, GO function enrichment analysis showed these genes were mainly involved in abnormal fatty acid metabolism pathways which regulate ferroptosis in KIRC—consistent with previous related studies. These findings on the importance of fatty acid metabolism pathways in ferroptosis may be helpful to understand the tumorigenic mechanism and treatment options of KIRC. Finally, our survival analysis of these 11 hub genes showed that PROM2 and PLIN2 expression are closely related to the poor prognosis of patients with KIRC. Therefore, PROM2 and PLIN2 may be promising prognostic indicators for patients with KIRC.

PLIN2, also known as adipose differentiation-related protein, wraps lipid droplets and phospholipids, as well as participates in the storage of neutral lipids in lipid droplets. Ma et al. showed that the expression of PLIN2 decreased significantly in the iron overload group, iron overload caused ferroptosis in the liver of mice with a decrease in GPX4 expression and an increase in Ptg2 expression, resulting in a high level of lipid peroxidation. Another study showed that PLIN2 promotes the proliferation and apoptosis of gastric cancer cells by modifying the ferroptosis pathway, such as regulating various ferroptosis-related genes, including acyl-CoA synthase long-chain family member 3, etc. PLIN2 has also been shown to be an indispensable factor for inhibiting ferroptosis caused by abnormal fat metabolism in gastric cancer. However, no research has been done on PLIN2 in KIRC. In line with previous studies, our findings confirm the critical role of PLIN2 in ferroptosis inhibition caused by abnormal fat metabolism and further indicate PLIN2 as a potential prognostic risk factor of KIRC.

Studies have shown that PROM2 participates in the composition of the plasma membrane microstructure where it mainly participates in biological processes such as promoting cell growth, migration, and perception by interacting with membrane cholesterol. PROM2 expression may be closely related to both prostate and breast cancer. PROM2 is also a candidate gene marker for distinguishing renal chromophobe cell carcinoma and benign renal eosinophiloma. Nevertheless, no research had been done on the role of PROM2 in KIRC before our study.

A variety of signaling pathways are involved in the regulation of tumor proliferation, migration, and invasion, including the JAK-STAT, NF-kB, Ras-Raf-MAPK, and Notch signaling pathways amongst others. Specifically, the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway is most commonly dysregulated in human tumors, which plays an important role in a series of cell biological processes such as cell growth, proliferation, and angiogenesis. In this regard, studies have shown that PROM2 can promote the proliferation, migration, and invasion of breast cancer by activating the PI3K/AKT pathway. Furthermore, ferroptosis plays an important role in triggering inflammation by activating the PI3K/AKT signaling pathway. In our study, we found that PROM2 is an independent risk factor for KIRC and may have potential as a prognostic indicator. Moreover, we propose that PROM2 regulates ferroptosis via the PI3K/AKT signaling pathway in KIRC.
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Correspondence and requests for materials should be addressed to S.L. or Y.H.

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