STEAP3 Affects Ferroptosis and Progression of Renal Cell Carcinoma Through the p53/xCT Pathway

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Abstract
Renal cell carcinoma is particularly sensitive to ferroptosis, an iron-dependent non-apoptotic form of cell death. This mechanism does not require activation of caspase or the participation of other apoptotic effector molecules (such as BAX or BAK), nor is it accompanied by the morphological characteristics or biochemical processes of apoptosis. The STEAP3 gene was found because it promotes tumor apoptosis in prostate cancer, but its role in renal cell carcinoma has not been studied in depth. Through real-time quantitative polymerase chain reaction, we found that the expression of the STEAP3 gene was upregulated in renal cell carcinoma tissue samples and cell lines, and it was found to be highly expressed in renal cell carcinoma tissue through immunohistochemistry. This upregulation is related to poor survival and prognosis of patients. We used erastin, a ferroptosis inducer, found that renal cell carcinoma became more susceptible to ferroptosis after knocking down STEAP3. The results indicate that renal cell carcinoma cell lines with knocked down STEAP3 expression are more sensitive to ferroptosis, and this effect occurs through the p53/xCT pathway. In summary, our research helps to identify new biomarkers and provides new targets for the treatment of renal cell carcinoma.

Keywords
ferroptosis, STEAP3, renal cell carcinoma, p53, xCT

Abbreviations
CAT, catalase; FBS, fetal bovine serum; OS, overall survival; PPI, protein–protein interaction; qRT-PCR, real-time quantitative polymerase chain reaction; RCC, renal cell carcinoma; ROS, reactive oxygen species; siRNA, small interfering RNA; SOD, superoxide dismutase; T-AOC, total antioxidant capacity.

Received: June 29, 2021; Revised: January 14, 2022; Accepted: January 19, 2022.

Introduction
Renal cell carcinoma (RCC) is a malignant tumor originating in the urinary tubule epithelial system of the renal parenchyma. It is the most common renal malignant tumor, accounting for approximately 80% to 90% of renal malignant tumors and approximately 2% to 3% of systemic malignant tumors. In 2019, there were approximately 73 820 new RCC cases in the United States and 14 770 deaths.1 The incidence of RCC increases rapidly. The incidence of RCC is associated with obesity, and long-term intake of high-fat diets can induce RCC.2 Although most of the lesions found are small tumors, a considerable number of patients are still diagnosed with locally advanced disease, and as many as 17% of patients have distant metastases at the time of diagnosis.3 Smoking is also...
associated with the incidence of RCC, which includes a variety of carcinogens associated with the etiology of RCC. The incidence of RCC has been increasing, patients often diagnosed with advanced RCC. In response, researchers are seeking new therapeutic targets and molecular markers.

Since ferroptosis was first proposed in 2012, research on tumors, ischemia–reperfusion, neurodegeneration, and other fields has continued to deepen. Ferroptosis is iron-dependent non-apoptotic cell death. This cell death mechanism does not require activation of caspase or the participation of other apoptotic effector molecules (such as BAX or BAK), nor is it accompanied by the morphological characteristics or biochemical processes of apoptosis. The morphology mainly includes a decrease in mitochondria, volume decrease, density increase, mitochondrial cristae decrease or disappearance, and mitochondrial outer membrane rupture. Yang et al. found that RCC is particularly sensitive to ferroptosis regulated by GPX4. Lee et al. found that overexpression of AMPK in the Caki-1 cells can protect cells from erastin-induced ferroptosis. Research on ferroptosis in RCC, especially renal clear cell carcinoma, continues to deepen. Ferroptosis is being studied as a treatment strategy that may help resolve the drug resistance of RCC. Patients and Tissue Samples
From January 2019 to November 2019, 34 fresh RCC and its adjacent tissues were obtained from the Department of Urology, Renmin Hospital of Wuhan University. All specimens were collected from patients who had received adjuvant radical resection and had informed consent. The specimens were diagnosed by 2 pathologists. This study was conducted under the permission of Institutional Ethics Committee of the Renmin Hospital of Wuhan University and in accordance with the “Ethical Management Guidelines.” The prior written consent is fully informed and signed by all participants.

Cell Lines and Cell Culture
Human RCC cell lines 786-O, A498, and normal renal tubular epithelial cell line HK-2 were obtained from American Type Culture Collection. OSRC-2 was obtained from Central Laboratory of Wuhan Renmin Hospital. The identification of these cell lines was conducted at the China Centre for Type Culture Collection. All cell lines were cultured in RPMI-1640 medium (HyClone) and supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin G sodium/streptomycin sulfate. All cells were grown in an incubator with 5% CO₂ at 37 °C.

Materials and Methods
Bioinformatics Analysis
The original data of RNA-seq were obtained from 610 renal clear cell carcinoma samples by TCGA, including 72 normal tissue samples, 538 tumor tissue samples. Use DESeq2 for differential analysis, setting P<.000001, log2FoldChange > 1. Through the protein–protein interaction (PPI) analysis of 70 genes related to ferroptosis or iron metabolism through the string database and cytoHubba analysis, the top 10 Hub genes and their functional networks were obtained, and the differentially expressed mRNAs were selected for survival analysis. The ggsurv packets in R with P<.05 were used as the screening threshold in the survival analysis.

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Quantitative Real-Time PCR
RNA isolation was conducted using TRIzol reagent (Invitrogen). We used NovoStart® SYBR qPCR SuperMix Plus (Cat.E096-01A, Novoprotein, China) to perform real-time quantitative polymerase chain reaction (qRT-PCR). Fold increase was calculated with the 2^ΔΔCt method. The sequences of the primers are as follows: STEAP3: 5′-TGCAAAACTGCTCAACTGGAGG-3′, 5′-AGCCAGTAGGATTGTAGCGG-3′; GAPDH: 5′-GTCTCC TCTGACTTCAACAGCG-3′, 5′-ACCACCATGTGTAGGCA AA-3′. All samples were tested in triplicate and repeated 3 times.

Immunohistochemistry
Paraffin slices were sequentially dewaxed, hydrated, antigen retrieval, and antibody (anti-STEAP3, 1:50, 17186-1-AP, Proteintech) was used for 4 °C overnight. DAB was added to develop color, hematoxylin was used to redye, dehydrate, and
protein expression, which was normalized to the expression level of GAPDH.

Results

Bioinformatics Analysis

Through differential expression analysis of 538 RCC samples from TCGA and 72 normal samples, 7 genes with low expression and 18 genes with high expression were obtained from 70 ferroptosis- and iron metabolism-related genes (Figure 1A). The highly expressed genes were SLC11A1, CP, TF, HMOX1, SLC39A14, HAMP, TFR2, SFXN3, HPX, HAVCR1, STEAP3, ABCB6, ABCG2, STEAP4, SLC25A37, ALAS2, CD163, and TMPRSS6, and the poorly expressed genes were FECH, ACO1, SFXN5, BMP6, SFXN2, SCARA5, and SLC48A1. We performed a PPI analysis of 70 genes associated with ferroptosis or iron metabolism in the STRING database using cytoHubba.

Statistical Analysis

All the experimental data were expressed by mean value ± standard error of \( \geq 3 \) independent experiments. We used Kaplan-Meier method to estimate the OS rate. The significance of the difference was compared with \( t \) test and \( \chi^2 \) test. One-way analysis of variance was used to analyze the significant difference among groups. \( P < .05 \) was considered to be statistically significant.

Discussion

In this study, we investigated the correlation between ferroptosis and the prognosis of RCC patients. Our results showed that the expression of ferroptosis-related genes was significantly associated with the clinical outcome of RCC patients. These findings suggest that ferroptosis may play a role in the development and progression of RCC. Further studies are needed to clarify the underlying mechanisms and explore potential therapeutic targets for RCC.

Conclusion

In conclusion, our study suggests that ferroptosis is involved in the pathogenesis of RCC and may be a promising target for the development of new therapeutic strategies. Further studies are needed to confirm these findings and to explore the clinical relevance of ferroptosis in RCC.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81830071 and 81870086) and the National Key Research and Development Program of China (2016YFC1306600). We are grateful to all the participants and their families who contributed to this study.

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to identify the top 10 hub genes and their interaction networks (Figure 1B). HAMP, STEAP3, and TFR2 were differentially expressed in 10 hub genes. We analyzed the clinical information on RCC samples in the TCGA database and found that patients with higher expression of STEAP3 had poorer prognoses (Figure 1C).

**STEAP3 Is Highly Expressed in RCC Tissues and Cell Lines**

We tested the mRNA expression of STEAP3 in RCC and adjacent tissues. qRT-PCR showed that mRNA expression in RCC samples was significantly higher than that in adjacent tissues (Figure 2A). The qRT-PCR results of RCC cell lines were consistent with tissues detection (Figure 2B). Results from Western blot verified the tendency of STEAP3 higher expression in tissue samples (Figure 2C) and cell lines (Figure 2D). Immunohistochemistry staining results (Figure 2E) showed increased levels of STEAP3 expression in RCC tissue samples.

**Knockdown of STEAP3 Influences the Biological Behavior of RCC**

We examined the inhibitory effect of siRNA. After transfection, the expression of STEAP3 protein and mRNA level was effectively inhibited (Figure 3A-C). The CCK-8 showed that after treatment with erastin, cell viability of the STEAP3 knockdown group was significantly lower than that of the control group (Figure 3D). Through cell colony formation assays (Figure 3E), wound-healing assays (Figure 3F), and Transwell invasion assays (Figure 3G), and after erastin, the STEAP3 knockdown group differed significantly from the control group. Our results suggest that knocking down STEAP3 can inhibit the development of tumors.

**Knockdown of STEAP3 Increases the Sensitivity of RCC Cell Lines to Ferroptosis**

The ferroptosis-related protein (p53 and ACSL4) expression in the STEAP3 knockdown group was higher than that in the non-knockdown group, while expression of other ferroptosis-related
protein (xCT and GPX4) decreased remarkably (Figure 4A and B). However, apoptosis-related protein, such as BCL-2, BAX had no changes (Figure S1). By measuring the intensity of the MitoSox probe signal in the cells, we measured the level of ROS in the cells. It was found that the STEAP3 knockdown group had a significant increase in ROS levels compared with the control group (Figure 4C). In the knockdown group, the expression of GSH, CAT, T-AOC, and SOD was decreased more than that in the control group, and the MDA concentration, which can reflect the degree of cell ferroptosis, increased more than that in the control group (Figure 4D and E). It has been proven that knocking down STEAP3 can increase the sensitivity of RCC cell lines to ferroptosis.

**STEAP3 Affects Ferroptosis Sensitivity Through the p53/xCT Pathway**

We observed the expression and activity of p53 and XCT in STEAP3-knockdown RCC cell lines and NC groups. We found that after knockdown of STEAP3 in RCC cell lines, the expression of p53 was upregulated and the expression of xCT was decreased compared to the control group (Figure 5A and B). Then, we used pifithrin(PFT)-α, a p53 inhibitor to determine whether the effect of STEAP3 knockdown could be rescued. As the result showed, PFT-α reversed the protein expression caused by STEAP3 knockdown (Figure 5A and B). To study the role of the p53/xCT pathway in STEAP3-mediated cell migration, proliferation, and invasion, we conducted rescue experiments. The CCK-8 showed that after treatment with erastin, the cell viability of the STEAP3 knockdown group was significantly lower than that of the control group. In the presence of PFT-α, the changes in cell viability caused by the knockdown of STEAP3 can be rescued (Figure 5C). Transwell invasion showed that the invasion ability of cancer cells caused by the knockdown of STEAP3 can be rescued in the presence of PFT-α (Figure 5D). With the absence of erastin, GSH, CAT, T-AOC, SOD, and MDA which are affected by STEAP3 knockdown can be rescued by PFT-α (Figure S2).

**Discussion**

STEAP3 is a member of the 6-transmembrane epithelial antigen of prostate (STEAP) family, and they all have a common 6-transmembrane domain. STEAP3 was originally cloned from the mouse LTR6 cell line as a transcript induced by wild-type p53 activation. Steiner et al found that adenovirus-mediated STEAP3 gene expression can effectively inhibit the growth of prostate cancer *in vitro* and *in vivo* by inducing cell apoptosis. Studies by Ohgami and others have shown that STEAP3 can reduce Fe⁺⁺ in cells and can promote the absorption of ions by cells. Studies have shown that STEAP3 may be involved in cell apoptosis and cell cycle processes, especially the G2-M process. BCL2 interacting protein 3 like (BNIP3L also known as NIX) seems to enhance the apoptotic effect of STEAP3, and the interaction between STEAP3 and myelin transcription factor 1 (Myt1) implies the regulation of the phosphorylation state of Myt1. STEAP3 can be
regarded as a positive regulator of Myt1, and STEAP3 and Myt1 together have a significant impact on the cell cycle and delay the G2-M process. STEAP3 was first discovered in prostate cancer, and it can promote the apoptosis of prostate cancer. In colon cancer, IncRNA STEAP3-AS1 can modulate cell cycle progression via affecting CDKN1C expression through STEAP3. In glioblastoma, STEAP3 predicts poor prognosis and promotes tumor growth and invasion. STEAP3 is also associated with a transition from cirrhosis to hepatocellular carcinoma. In this experiment, we verified...
that STEAP3 is highly expressed in RCC tissues and cells, and through clinical information analysis, we found that its high expression is related to RCC. Our study provides genetic evidence that STEAP3 plays a key role in RCC. According to the analysis of clinical data, STEAP3 is an independent prognostic factor of RCC, and the expression of STEAP3 was negatively correlated with prognosis in RCC patients.

To further verify changes in the sensitivity of RCC cells to ferroptosis after knocking down STEAP3, we used erastin as an inducer and found that after knocking down STEAP3, erastin significantly reduced the antioxidant capacity of RCC cells, leading to increased lipid peroxidation and ROS levels. We can conclude that knocking down STEAP3 increases the sensitivity of RCC cells to ferroptosis. Then, we conducted a series of cell experiments and found that RCC cells after knocking down STEAP3 can significantly inhibit growth, migration, and invasion in vitro under the induction of erastin. We speculate that after knocking down STEAP3, the cystine/glutamate transport system becomes more sensitive to the effects of erastin.

Inactivation of the P53 tumor suppressor pathway is a key event in the formation of most human cancers.\textsuperscript{25–28} Le \textit{et al} inhibited SLC7A11 by transcription, a component of the cysteine/glutamate antiporter, and p53 inhibited the uptake of cystine and made cells sensitive to ferroptosis, finding that p53-mediated transcriptional inhibition of SLC7A11 plays a key role in ROS-induced ferroptosis.\textsuperscript{29} Brent \textit{et al} found that STEAP3 transcripts are upregulated by p53 and that STEAP3 antisense impaired p53-mediated apoptosis, hinting at the possibility that STEAP3 may participate in cell death-related activities.\textsuperscript{11} Our research found that STAP3 and p53 expression was complementary, and the Western blot results showed that after knocking down STEAP3, the expression of p53 increased, and the expression of xCT downstream decreased. This is a good explanation for the increased sensitivity to ferroptosis of RCC cells caused by knockdown of STEAP3. The rescue experiment saved the effect of knocking down STEAP3. Our study

Figure 4. Changes in ferroptosis-related proteins, antioxidant capacity, and lipid peroxidation in 786-O and A498 cell lines. (A, B) Ferroptosis-related proteins were significantly different between siNC group and siSTEAP3 group when treated with erastin. The concentration of erastin is 10 \(\mu\)M (786-O) and 15 \(\mu\)M (A498). (C) MitoSox analysis of reactive oxygen species (ROS) levels in A498 and 786-O cells. (D) Quantification analysis of antioxidant markers in 786-O and A498 cells. (E) Quantification analysis of lipid peroxidation levels in HK-2 cells. All the above data are the mean \(\pm\) SD from an average of 3 experiments.
demonstrated the important role of STEAP3 in RCC for the first time; STEAP 3 has the potential to be used as a biomarker to predict the survival of patients with RCC. STEAP3 may increase sensitivity to ferroptosis by regulating the p53/xCT pathway, thereby inhibiting tumor growth. Specifically, to determine whether knocking down the expression of STEAP3 can be used as a new strategy for the treatment of RCC, further study is warranted.

Authors’ Note
Cheng Lin Ye and Yang Du contributed equally to this work. The present study was approved by The Ethics Committee of Renmin Hospital of Wuhan University. All patients provided written informed consent prior to enrollment in the study.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This paper is funded by the National Natural Science Foundation of China (No. 81972408), the frontier project of Wuhan Applied Foundation (No. 2018060401011321).

Supplemental Material
Supplemental material for this article is available online.

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Figure 5. STEAP3 affects ferroptosis sensitivity through the p53/xCT pathway. (A, B) Knocking down of STEAP3 in 786-O and A498 cells, the expression of p53 was upregulated and the expression of xCT was decreased. PFT-α, an inhibitors of p53, could reverse it. (C, D) Cell counting kit-8 (CCK-8) assays and transwell invasion showed in the presence of PFT-α, the proliferation and invasion of siSTEAP3 kidney cancer cells(786-O, A498) were clearly elevated. 786-O and A498 cells in CCK-8 assays and transwell were treated with erastin. The concentration of erastin is 10 μM (786-O) and 15 μM (A498).
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