



Curcumin reverses the sunitinib resistance in clear cell renal cell carcinoma (ccRCC) through the induction of ferroptosis via the *ADAMTS18* gene

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Background: To explore the possible mechanism by which curcumin reverses the sunitinib resistance in clear cell renal cell carcinoma (ccRCC).

Methods: A sunitinib-resistant ccRCC cell model was established. The MTT assay was used to determine the half maximal inhibitory concentration (IC_{50}) and drug resistance (DR) index. The effects of curcumin plus sunitinib or sunitinib alone on drug-resistant cell lines were verified by the cell counting kit-8 (CCK-8) assay, colony formation assay, and apoptosis assay. The concentration of iron ions in the cell lines was analyzed using an Abcam Iron Assay Kit. The expressions of *ADAMTS18* gene and ferroptosis-related proteins (*NCOA4*, *FTH1* and *p53*) after curcumin plus sunitinib treatment were analyzed by reverse transcription polymerase chain reaction (RT-PCR) and Western blotting. After transfection of curcumin plus sunitinib/sunitinib alone-treated drug-resistant cell lines with si-*ADAMTS18*, cell proliferation activity was assessed by the CCK-8 assay, and the protein expression levels of *ADAMTS18*, *NCOA1*, *FTH1* and *p53* were analyzed by Western blotting. After treatment with ferroptosis-1 (Fer-1; a ferroptosis inhibitor), the cell proliferation activity of drug-resistant cell lines treated with curcumin plus sunitinib/sunitinib alone was reassessed using the CCK-8 assay.

Results: Curcumin plus sunitinib inhibited the proliferation of sunitinib-resistant ccRCC cells ($P < 0.05$). Curcumin significantly decreased the concentration of iron ions and increased the expression of *ADAMTS18* gene, while significantly inhibited ferroptosis-related protein expression ($P < 0.05$). After silencing the *ADAMTS18* gene, there was no significant difference in cell proliferation or ferroptosis-related protein expression between curcumin plus sunitinib and sunitinib-treated drug-resistant cell lines ($P > 0.05$). Ferroptosis inhibitors reversed the inhibitory effect of curcumin on sunitinib-resistant ccRCC cell lines.

Conclusions: Curcumin can reverse the sunitinib resistance in ccRCC, possibly by upregulating the expression of the *ADAMTS18* gene to induce ferroptosis.

Keywords: Curcumin; clear cell renal cell carcinoma (ccRCC); ferroptosis; *ADAMTS18*; sunitinib resistance

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Introduction

Clear cell renal cell carcinoma (ccRCC) is the most common renal malignant tumor and one of the most invasive urinary system cancers (1). Patients with ccRCC are often at an advanced stage when they are first diagnosed, and the prognosis is poor. The tyrosine kinase inhibitor of sunitinib inhibits the oxygen and nutrient supply of tumors by blocking angiogenesis. Although sunitinib can prolong the progression-free survival of patients, drug resistance (DR) easily develops during the treatment process, an issue that must be addressed for the successful treatment of advanced ccRCC (2).

Curcumin is the active ingredient of the perennial herb turmeric (*Curcuma longa*), which has long been used as a traditional medicine in China, India and other countries. Modern science and technology have deeply explored this ancient remedy, and it has been widely used as an antiinflammatory, antioxidative, and chemopreventative agent for tumor prevention and treatment (3).

Ferroptosis is iron-catalyzed programmed cell death, which is characterized by iron-catalyzed peroxidation of phospholipids and polyunsaturated fatty acids. This phospholipid peroxidation causes cell toxicity, which can be neutralized by iron chelators and lipophilic free radical scavengers, indicating that ferroptosis induces cytotoxicity via lipid peroxidation (4). Studies have shown that iron in cells is a central factor in cancer progression, which explain why antitumor drugs can specifically affect tumor cells instead of normal cells with low iron content (5). It has been proposed that the expression of iron-related regulatory genes in ccRCC cells is significantly increased (6). In addition, compared with that in healthy tissues, the expression of NCOA1 was significantly increased in tumor tissues, which was associated with poorer survival outcomes (7). Mancias *et al.* proposed that NCOA1 is closely related to ferroptosis mechanism in tumor cells (8).

In 2015, our team first identified that abnormal methylation of the *ADAMTS18* gene existed in ccRCC (9). In 2021, our team confirmed that the *ADAMTS18* gene could participate in the occurrence and development process of ccRCC and that curcumin, as a botanical drug, can inhibit this process by demethylating the *ADAMTS18* gene (10,11). Based on the above findings, this study aimed to explore whether curcumin could affect sunitinib resistance in ccRCC cells through the regulation of the *ADAMTS18* gene. We present the following article in accordance with the MDAR reporting checklist (available at <https://dx.doi.org/10.21037/tcr-21-227>).

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Methods

Drugs and reagents

Sunitinib and curcumin were purchased from Shanghai PureOne Biotechnology Co., Ltd. (purity $\geq 98\%$), and the stock solutions (150 mmol/L) were prepared using DMSO and stored at room temperature. DMEM, fetal bovine serum (FBS), CCK-8 reagent, and reverse transcription polymerase chain reaction (RT-PCR) kits were purchased from Thermo Fisher Scientific.

Cell culture

ccRCC cell lines (A498 and 786-O) were purchased from Medical Discovery Leader (Beijing, China) and cultured in medium containing 10% ultracentrifuged FBS (Invitrogen, CA, USA), 1 mM sodium pyruvate (Invitrogen), penicillin (100 U/mL; Invitrogen), and streptomycin (10 mg/mL; Invitrogen) at 37 °C in a humidified environment with 5% CO₂. The cell culture also contained 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (HyClone).

Establishment of sunitinib-resistant cell lines

A498 and 786-O cells were treated with sunitinib at an initial concentration of 0.5 μM , and the cell culture medium was replaced. After the cells were stabilized, the concentration of sunitinib was increased stepwise by 0.5 μM to gradually establish sunitinib-resistant (15 μM sunitinib) A498 and 786-O cells.

MTT assay

The cell concentration was adjusted to inoculate 100 μL per well into 96-well plates. After cells were adhered to the plates, serial dilution of sunitinib were added. Five replicate wells were used for each concentration as well as a blank control. After 48 h of culture, 20 μL of MTT solution (5 mg/mL) was added to each well. After 4 h of culture at 37 °C, 150 μL of DMSO was added to each well. Plates were shaken at low speed for 10 min to fully dissolve the purple crystals, and the absorbance was measured at 570 nm using a microplate reader. Cell inhibition rate (IC) = [1 – optical density (OD) value of the experimental group/OD value of the control group], and DR index = half maximal inhibitory

Table 1 The primers of *ADAMTS18*, *NCOA*, *FTH1*, *p53* and *GAPDH* for RT-PCR

Genes	F 5'-3'	R 5'-3'
<i>NCOA4</i>	GAGGTGTAGTGATGCACGGAG	GACGGCTTATGCAACTGTGAA
<i>FTH1</i>	GGTGGCCGAATCTTCCTCA	GCCAGTTTGTGCAGTCCAG
<i>ADAMTS18</i>	CGCCAGCGATTAAATGATGA	GAAAATCGGTAGTGCAGGGAG
<i>p53</i>	CAGCACATGACGGAGTTGT	TCATCCAAATACTCCACACGC
<i>GAPDH</i>	GGAGCGAGATCCCTCCAAAT	GGCTGTTGTCATACTTCTCATGG

RT-PCR, reverse transcription polymerase chain reaction.

concentration (IC_{50}) of drug-resistant cells/ IC_{50} of parental cells in the control group.

Cell counting kit-8 (CCK-8) assay

A498-DR and 786-O-DR cells were seeded into 24-well plates and divided into a sunitinib alone group and curcumin plus sunitinib group. After cells in each group were treated with corresponding drugs for 48 h, 30 μ L of CCK-8 reagent was added to each well, and the cells were incubated in a 5% CO_2 incubator at 37 °C for 4 h. The absorbance was measured at 450 nm using a microplate reader. The drug dose-response curves were plotted, and IC_{50} values were calculated.

Colony formation

Cells transfected with curcumin plus sunitinib or sunitinib alone were seeded into 6-well culture plates. Three replicate wells were set up in each group, and the culture medium was replaced once every 2–3 days. After a 2-week culture, the cells were fixed in 4% paraformaldehyde solution for 30 min and stained with 0.1% crystal violet solution for 15 min. The number of colonies with more than 50 cells was counted under an optical microscope.

Flow cytometry

Flow cytometry (BD FACSAria) was used to assess cell apoptosis. After the cell density was adjusted, the cells were cultured overnight. After the cells were adhered to the plates, drugs were added. After a 48-h culture, the cells were collected by centrifugation and washed twice with precooled phosphate-buffered saline (PBS). The cells were divided into a nonstaining group, annexin V-only staining group, propidium iodide (PI) staining group and PI and

annexin V double-staining group. A total of 100 μ L of binding buffer was added to each group to disperse cells, and the corresponding dyes were added in the dark. Cells were incubated in the dark at room temperature for 15 min, and then 300 μ L of binding buffer was added. Cells were transferred to the corresponding flow cytometry tubes for the assessment of apoptosis.

Detection of iron ion content

An Abcam Iron Assay Kit was used for detection in strict accordance with the instruction manual.

RT-PCR

RNA was extracted from target cells, and cDNA was synthesized by reverse transcription. After PCR amplification, agarose gel electrophoresis was performed. An ImageJ gel image analysis system was used to analyze the mRNA expression levels of *ADAMTS18*, *NCOA*, *FTH1* and *p53*. *GAPDH* was used as a reference gene. The primers of *ADAMTS18*, *NCOA*, *FTH1*, *p53* and *GAPDH* could be traced in *Table 1*.

Western blotting

Total protein in cells was extracted using a KeyGEN Bio TECH Protein Extraction Kit (KGP1100), separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. After blocking with skim milk powder, the blots were immunostained with primary and secondary antibodies, and the grayscale values for the bands were analyzed using a Bio-Rad imaging system. The following antibodies, with catalog numbers, were used: *NCOA4* (ab86707, phosphate), *FTH1* (ab65080, pH 7.4

PBS), *ADAMTS18* (orb226265, pH 7.4 PBS), and *p53* (ab26, Western primary antibody dilution).

Transfection with si-ADAMTS18

Cells were transfected with 25 μ L of si-*ADAMTS18* (orb226265, GeneChem, Shanghai, China), and si-NC was used as a control (Santa Cruz, CA, USA) at a final concentration of 40 nM. Lipofectamine 2000 (Invitrogen) was used in accordance with the manufacturer's instructions. The transfection efficiency was verified by RT-PCR.

Statistical analysis

Statistical analysis was performed using SPSS 22.0. The experimental results were analyzed using the rank sum test. Significant differences were based on a type I error $\alpha < 0.05$ and type II error $\beta < 0.01$.

Results

Successful establishment of sunitinib-resistant ccRCC cells

A498-DR and 786-O-DR exhibited similar changes, showing circular and dispersed growth, while A498 and 786-O cells were spindle-shaped and exhibited clustered growth (Figure 1A). Based on the MTT assay results, the IC_{50} values for A498 and 786-O cells were (4.68 ± 2.33) and (4.82 ± 1.62) μ M, respectively, and the IC_{50} values for A498-DR and 786-O-DR cells were (28.36 ± 5.81) and (29.42 ± 6.37) μ M, respectively; the difference was significant ($P < 0.05$) (Figure 1B). The DR indices for A498-DR and 786-O-DR cells were 6.06 and 6.10, respectively. A drug-resistant cell line model was successfully established. By applying the apoptosis assay, it was discovered that sunitinib could promote A498 and 786-O apoptosis in a dose-dependent manner, yet without influencing the apoptosis of A498-DR and 786-O-DR, which simultaneously verified the successfully established drug-resistant cell lines (Figure 1C).

Inhibition of A498-DR and 786-O-DR cell growth by curcumin

Curcumin plus sunitinib significantly inhibited the growth of 786-O-DR cells in a dose-dependent manner, as determined by the CCK-8 assay (Figure 2A). The IC_{50} values for 786-O-DR cells were significantly reduced in the curcumin plus sunitinib group (Figure 2B). As seen in

Figure 2C, curcumin significantly inhibited the colony formation ability of A498-DR and 786-O-DR cells. The differences between the above groups were significant ($P < 0.05$).

Reversal of the resistance of ccRCC to sunitinib by curcumin through the induction of ferroptosis

Analysis of the iron ion content in the above cell lines indicated that curcumin significantly reduced the iron content in the cell lines. The iron ion concentration in 786-O-DR treated with curcumin plus sunitinib was significantly decreased ($P < 0.05$, Figure 3A). The mRNA and protein expression levels of *ADAMTS18* and ferroptosis-related proteins were analyzed by RT-PCR and Western blotting (Figure 3B,C,D). The results showed that curcumin plus sunitinib treatment upregulated the expression of the *ADAMTS18* gene and significantly reduced *NCOA4*, *FTH1* and *p53* expression levels ($P < 0.05$). The results suggested that curcumin could reduce the mRNA and protein expression levels of *NCOA4*, *FTH1*, and *p53*, indicating that curcumin may induce ferroptosis through the upregulation of the *ADAMTS18* gene.

Effect of curcumin on 786-O-DR activity after ADAMTS18 gene silenced

After the transfection of 786-O-DR cells with si-*ADAMTS18*, the inhibitory effect of curcumin plus sunitinib on the proliferation of 786-O-DR was disappeared (Figure 4A). From Figure 4B,C,D, it could be found that by RT-PCR and WB assay, si-*ADAMTS18* was successfully transfected into 786-O-DR cells. After silencing the *ADAMTS18* gene, the difference in the expressions of *p53*, *NCOA4* and *FTH1* in each group was not significant (Figure 4B,C,D). The above results suggested that after silencing the *ADAMTS18* gene, the proliferation of drug-resistant cell lines was restored, and the inhibitory effect of curcumin on the growth of cell lines and ferroptosis was disappeared simultaneously, indicating that the *ADAMTS18* gene was critical in the process of curcumin-induced cell ferroptosis.

Reversal of curcumin-induced cell death by ferroptosis inhibitors

After incubation with 20 μ M ferroptosis-1 (Fer-1), a ferroptosis inhibitor, for 48 h, the proliferation of 786-O-

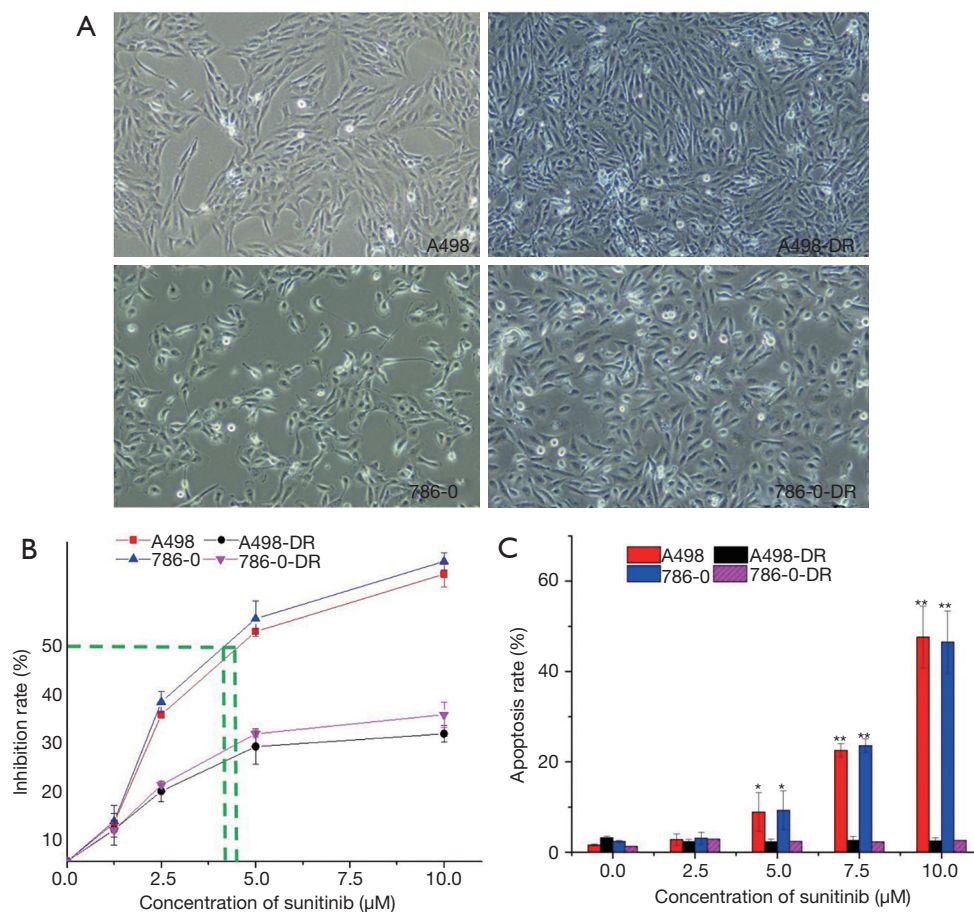


Figure 1 Successful establishment of drug-resistant ccRCC cell lines. (A) The cells of ccRCC and ccRCC-DR were observed under inverted microscope without staining, and the magnification was $10\times 10=100$; (B) inhibition rates and IC_{50} values were determined by the MTT assay; (C) the apoptosis assay revealed that sunitinib promoted A498 and 786-O apoptosis in a dose-dependent manner, yet without influencing the apoptosis of A498-DR and 786-O-DR. The rank sum test was used to compare the above groups, and the difference was significant (type I error $\alpha < 0.05$, and type II error $\beta < 0.01$). *, $P < 0.05$; **, $P < 0.01$. ccRCC, clear cell renal cell carcinoma; DR, drug resistance; IC_{50} , half maximal inhibitory concentration.

DR cells was analyzed using the CCK-8 assay. The results indicated that cell proliferation was significantly increased after Fer-1 treatment ($P < 0.05$). The Fer-1 reversed the inhibitory effect of curcumin on cells, resulting in increased cell proliferation. The results also suggested that the inhibitory effect of curcumin on cell proliferation might be related to the mechanism of ferroptosis (Figure 5).

Discussion

ccRCC is a group of heterogeneous diseases originating from renal tubular epithelial cells and is a malignant tumor of the urinary system (12). Currently, the global incidence

of ccRCC is relatively high. Sunitinib is a tumor-targeting drug mainly used to inhibit growth factor receptor tyrosine kinases. It is used to inhibit tumor angiogenesis. Studies have shown that sunitinib treatment can delay the progression of ccRCC by 6–7 months. However, most patients are in the stage of DR within 1 year of treatment (13), which is a clinical problem that urgently needs to be solved.

In 2015, ccRCC treatment entered the immunotherapy era. Many large-scale phase III clinical trials have been successful. Immunotherapy combined with targeted therapy or dual immunotherapy has become the first-line treatment standard for ccRCC. Based on the excellent performance of combination immunotherapy, the National Comprehensive

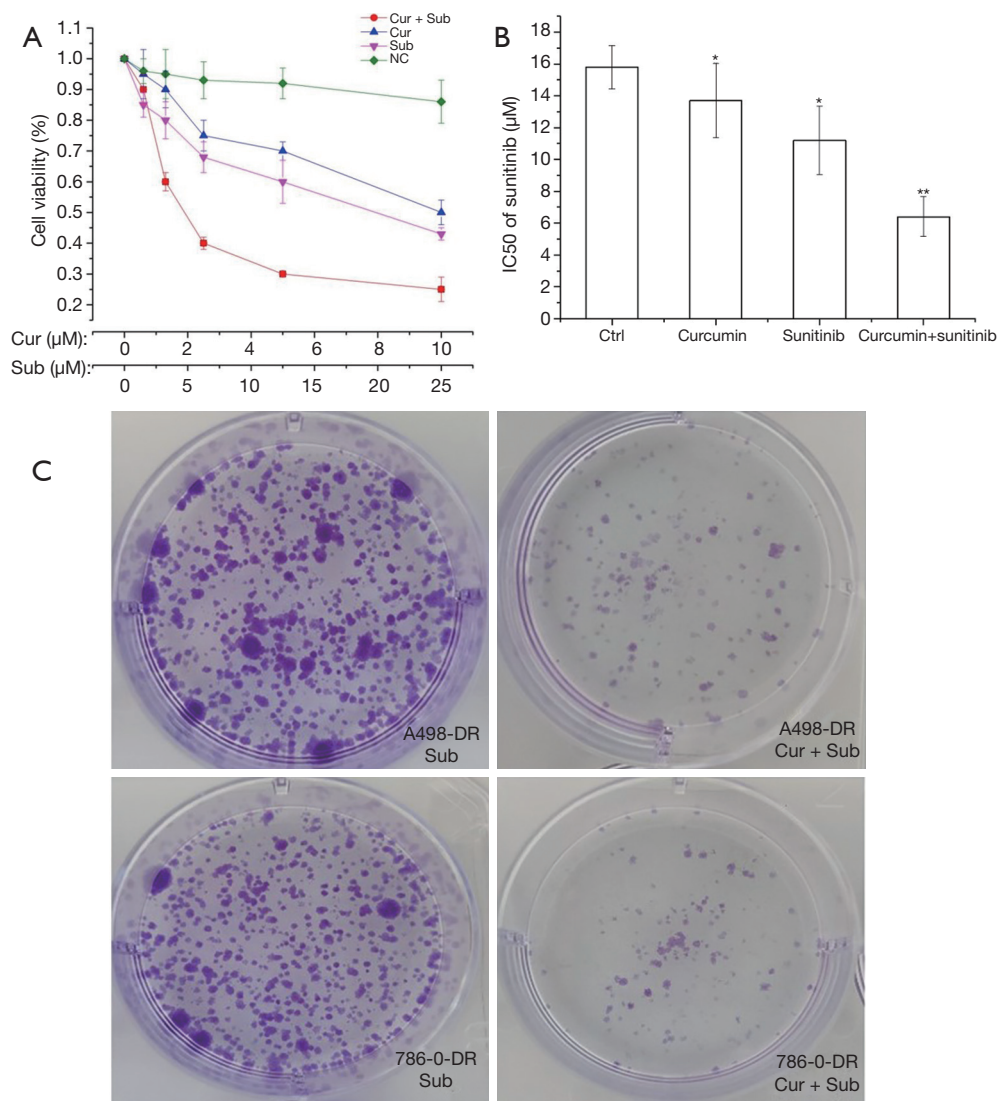


Figure 2 The inhibitory effect of curcumin on ccRCC-DR cell lines. (A) Curcumin plus sunitinib significantly inhibited the growth of 786-O-DR cells in a dose-dependent manner by the CCK-8 assay; (B) the effect of curcumin and sunitinib on the IC₅₀ value for 786-O-DR cells; (C) curcumin plus sunitinib significantly inhibited the colony forming ability of A498-DR and 786-O-DR cells. The immuno-histochemistry double staining method was used. The rank sum test was used to compare the above groups, and the difference was significant (type I error $\alpha < 0.05$, and type II error $\beta < 0.01$). NC = ddH₂O. *, $P < 0.05$; **, $P < 0.01$. ccRCC, clear cell renal cell carcinoma; DR, drug resistance; IC₅₀, half maximal inhibitory concentration; CCK-8, cell counting kit-8.

Cancer Network (NCCN) guidelines for kidney cancer version 1, 2021, recommend pembrolizumab combined with axitinib as the first-line preferred regimen for low-risk and intermediate-high-risk ccRCC patients and nivolumab combined with ipilimumab as the preferred first-line regimen for intermediate- and high-risk ccRCC patients. Although ccRCC treatment has entered the immunotherapy era, there are still many limitations of immunotherapy

in clinical practice due to the high costs and the risk of accelerated death caused by immune hyperprogression. In addition, some drugs have not yet been marketed in most developing countries, including China.

Curcumin is a natural polyphenol extracted from the turmeric rhizome. It has antiinflammatory and antioxidant properties, activates the immune system, and can be used as an antioxidant to capture free radicals and protect living

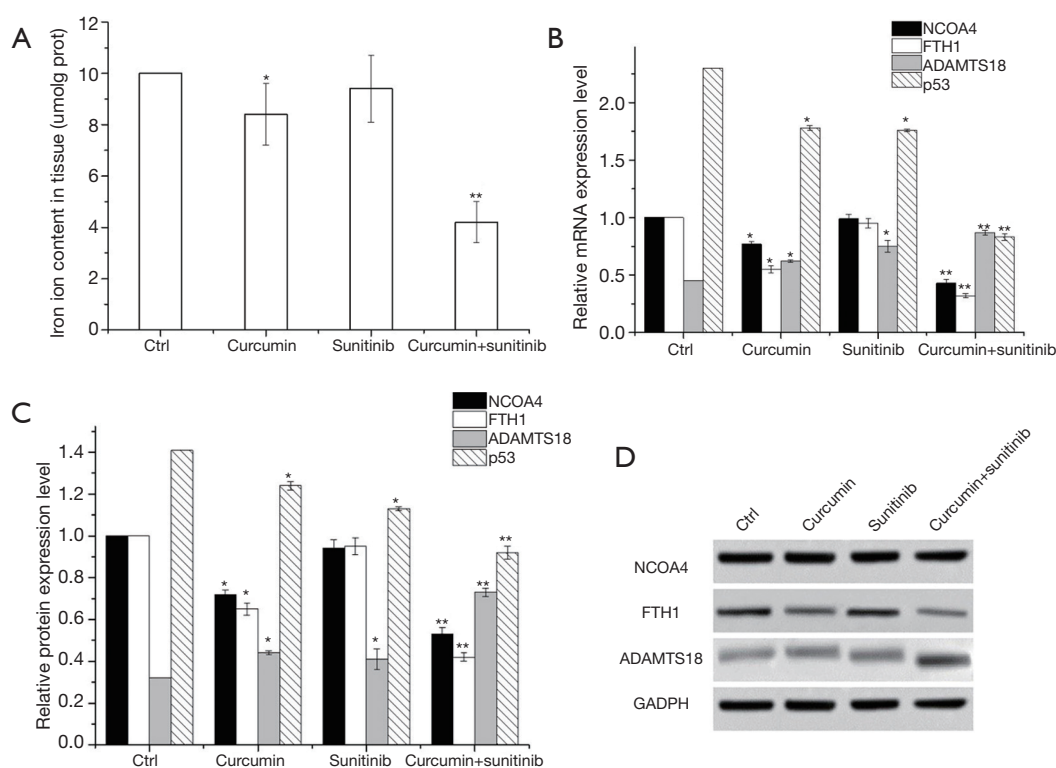


Figure 3 The ferroptosis was induced by curcumin. (A) The iron ion concentration in the different drug treatment groups; (B) RT-PCR analysis of *NCOA4*, *FTH1*, *ADAMTS18* and *p53* mRNA expression levels in each group; (C) Western blot analysis of *NCOA4*, *FTH1*, *ADAMTS18* and *p53* protein expression levels in each group; (D) The specific Western blot images in original format. The rank sum test was used to compare the above groups, and the difference was significant (type I error $\alpha < 0.05$, and type II error $\beta < 0.01$). *, $P < 0.05$; **, $P < 0.01$. RT-PCR, reverse transcription polymerase chain reaction.

cells (14). This study analyzed the effect of curcumin on the proliferation of sunitinib-resistant cells and found that the combination of curcumin and sunitinib reduced the proliferative activity of tumor cells. Curcumin can arrest cancer cells at different stages of the cell cycle and inhibit cancer cell proliferation by inducing apoptosis (15). Curcumin can also regulate different molecular targets and fight against human cancer. A large number of studies have also reported the inhibitory effect of curcumin on almost all types of tumor cells, such as reproductive cancers, digestive cancers, lymphoma, cancers of the immune system, urinary cancer, lung cancer, cancers of the nervous system, cancers of the skeletal system, and skin cancer. In these studies, the concentration of curcumin that yielded inhibitory activity ranged from 1 to 100 μM (16).

In the study of the mechanism of cellular ferroptosis, many studies have confirmed that the cystine/glutamate reverse transporter and the glutathione peroxidase 4

pathway are closely related to cellular ferroptosis. Recently, some studies have suggested that *p53* and *NRF2* are also involved in the mechanism of cellular ferroptosis (17,18). Current studies have proposed that these pathway proteins that may be involved in cellular ferroptosis will lead to the accumulation of high levels of iron ions and reactive oxygen species in cancer cells to promote their metabolic activity and growth (19). Metabolic remodeling of cancer cells is often associated with ferroptosis sensitivity. Researchers have proposed that this phenomenon may be an adaptive response to cellular metabolic imbalance (20). Researchers have also proposed that curcumin analogs can act as ferroptosis inducers to mediate cellular ferroptosis in a dose-dependent manner in the treatment of osteosarcoma (21). This study found that curcumin significantly reduced the iron ion content in drug-resistant tumor tissues, inhibited *NCOA4* and *FTH1* mRNA and protein expression, and enhanced sensitivity to sunitinib. Mancias *et al.* proposed

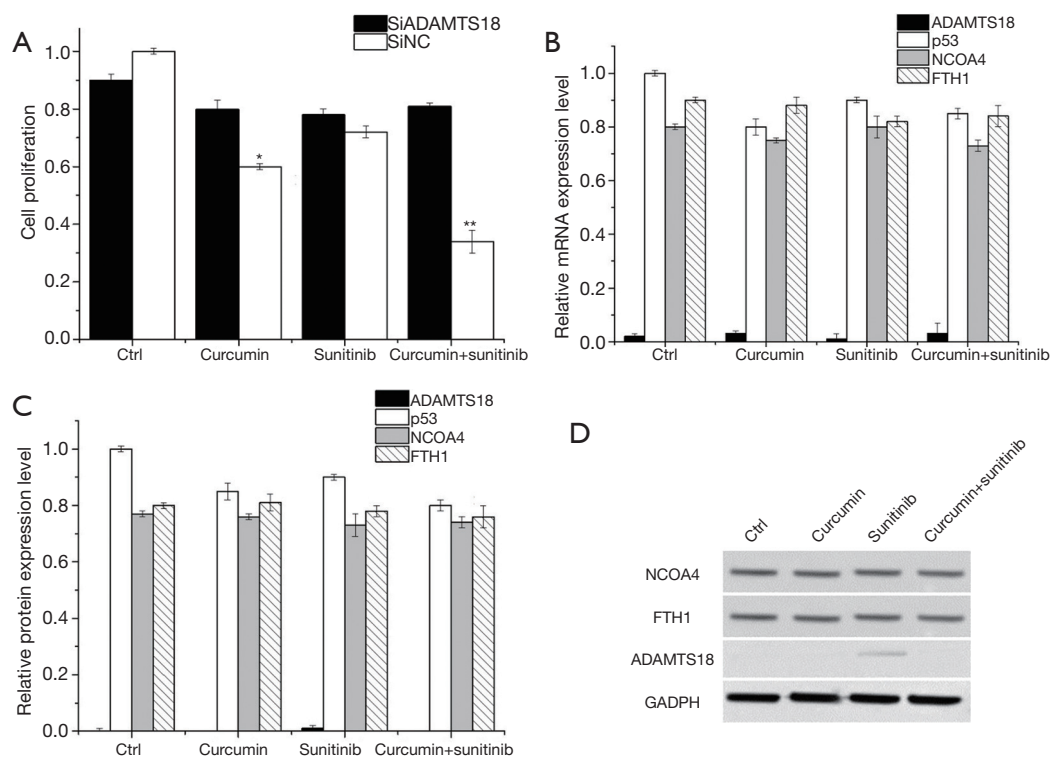


Figure 4 Effect of curcumin on the cell viability after silencing the *ADAMTS18* gene. (A) Analysis of cell proliferation activity in different groups of cells after *ADAMTS18* silenced; (B) analysis of *NCOA4*, *FTH1*, *ADAMTS18* and *p53* mRNA expression levels via RT-PCR; (C) analysis of the protein expression levels of *NCOA4*, *FTH1*, *ADAMTS18* and *p53* in each group via Western blotting; (D) the specific Western blot images in original format. The rank sum test was used to compare the above groups, and the difference was significant (type I error $\alpha < 0.05$, and type II error $\beta < 0.01$). NC = ddH₂O. *, $P < 0.05$; **, $P < 0.01$. RT-PCR, reverse transcription polymerase chain reaction.

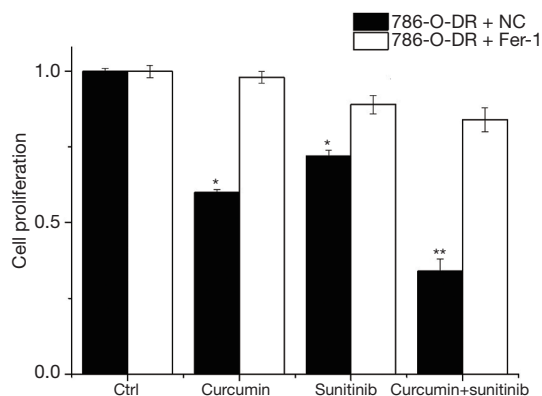


Figure 5 Reversal of curcumin-induced cell death by Fer-1. The rank sum test was used to compare the above groups, and the difference was significant (type I error $\alpha < 0.05$, and type II error $\beta < 0.01$). NC = ddH₂O. *, $P < 0.05$; **, $P < 0.01$. Fer-1, ferroptosis-1.

that *NCOA4* is highly aggregated in autophagosomes and found that the delivery of ferritin to lysosomes requires *NCOA4*. *NCOA4*-deficient cells cannot degrade ferritin, resulting in a decrease in bioavailable intracellular iron (8). Studies have shown that the regulation of ferritin and iron metabolism homeostasis may become an important regulatory point for the mechanism of ferroptosis (22). Curcumin can significantly inhibit *FTH1* and *FTL1* gene expression in tumor tissues of nude mice (23).

In this study, after 786-O-DR cells were transfected with si-*ADAMTS18*, curcumin-induced ferroptosis in ccRCC was disappeared. Therefore, we propose that curcumin induces ferroptosis in tumor cells by upregulating the expression of *ADAMTS18*, thereby enhancing the sensitivity of ccRCC to sunitinib. Currently, no relevant studies have confirmed that *ADAMTS18* is involved in the mechanism of cellular

ferroptosis. The results of this study indicate for the first time that curcumin could mediate ferroptosis in ccRCC through *ADAMTS18* gene. Unfortunately, data are limited to *in vitro* studies and the reported effects of curcumin still require confirmation in *in vivo* models in the long run.

Conclusions

In summary, this study established a sunitinib-resistant cell model and found that curcumin could reverse the sunitinib resistance in ccRCC and enhanced the sensitivity of ccRCC to sunitinib. This phenomenon may be induced through ferroptosis by upregulating *ADAMTS18* gene expression.

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Footnote

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Data Sharing Statement: Available at <https://dx.doi.org/10.21037/tcr-21-227>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/tcr-21-227>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived.

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