CSF2 Impairs Nrf2 Signaling Through Akt/Mtor Pathway in the Development of Bladder Cancer

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Abstract

Background

Cancer of the bladder (BCa) is one of the most common cancer of the urinary system. Colony-stimulating factor 2 (CSF2) was involved in lots of cancers, but BCa. We examined the effect of CSF2 on BCa in this study and the underlying molecular mechanisms.

Materials and methods

CSF2 mRNA levels in BCa were analyzed using the Cancer Genome Atlas (TCGA) database. Western blot was conducted to verify BCa tissue samples and cell lines expressing CSF2. The effect on growth of CSF2 was assessed by CCK8 and colony formation. To determine the migration and invasion capabilities of BCa cells, transwell analysis and wound healing assays were conducted. Next, western blot was used to explore the underlying mechanism. In the end, a xenografted BCa mouse model was established to examine CSF2 effects on tumorigenesis in vivo.

Results

These experiments showed CSF2 mRNA was upregulated in BCa samples. BCa proliferation and tumorigenesis were significantly inhibited in vitro and in vivo by CSF2 knockdown. Mechanism analysis revealed that BCa cells were inhibited from proliferating and invading when CSF2 was knocked down via AKT/mTOR signaling.

Conclusions

Based on these results, CSF2 promotes proliferation and tumorigenesis of BCa.

Introduction

There are estimated to be more than 17,000 deaths and 83,000 new cases of bladder cancer in the United States in 2021 as a result of bladder cancer (BCa)[1]. Cigarette smoking, occupational exposure, inflammation, and chemotherapy are thought to contribute to the high male-female ratio of new cases[2]. In the case of bladder cancer, there are a number of treatment options available. A number of treatment options are recommended in urology guidelines, including radical cystectomy, chemotherapy, and intravenous immunotherapy with bacteria Calmette Guerin (BCG)[3, 4]. After treatment, the quality of life of BCa patients decreases dramatically and most BCa patients are unable to avoid tumor progression and recurrence. Tumor recurrence and distant metastases are the leading causes of death in BCa patients, with over 90% of BCa patients dying from distant metastases BCa patients. In this regard, the development of improved treatments for BCa is a worthwhile endeavor.
In addition to stimulating stem cells to produce granulocytes and monocytes, colony-stimulating factor 2 (CSF2) is known as the macrophage colony-stimulating factor. [5]. Activated T lymphocytes, fibroblasts, endothelial cells, and dendritic cells (DC) secrete CSF2 to promote dendritic cell recruitment, maturation, and function to induce protective immunity. [6]. CSF2 requires the ligand-specific subunit (GM-CSFR) conductor to activate intracellular molecular signalling pathways and thus promote cell growth, proliferation and differentiation. Several recent studies have found that upregulation of CSF2 in the solid tumour microenvironment lead to poor prognosis which may result in the tumours when the immune system is suppressed [7]. Also in other studies CSF2 was found to be closely associated with poor prognosis in some tumours [8]. However, the relationship between CSF2 and bladder cancer is still unclear.

There is evidence that Nrf2 stimulates antioxidant and cytoprotective responses. A stable interaction between Nrf2 and Keap1 occurs in the cytoplasm under normal conditions. The Nrf2 protein translocates from the nucleus to the nucleus when cells undergo oxidative stress and binds to Maf to form a heterodimer that further activates ARE-mediated downstream gene expression, thereby inducing proliferation and other phenotypic changes in tumour cells [9]. Nrf2 has been shown to be highly activated in studies in bladder tumours, that upregulation of Nrf2 leads to poor prognosis in bladder cancer patients, and that overexpression in bladder cancer cells accelerates tumour cell progression [10, 11].

In this study, the function of CSF2 in the development of BCa and further research was conducted on the molecular mechanisms by which CSF2 regulates Nrf2 in BCa.

### Materials and Methods

**Bioinformatics analysis**

The public databases required in this paper are obtained through TCGA, GTEx, GEO and other public bioinformatics databases. The gene expression in TCGA and GTEx database comes from UCSC XENA, gene expression in GEO database, Methylation information comes from Smartapp database http://www.bioinfo-zs.com/smartapp/. All gene expressions in this study were standardized and log2 normalized. TCGA-BLCA contains 407 BCa samples and 19 BCa paracancerous tissues with paired adjacent samples; GTEx has 9 normal bladder mucosa tissues. GEO-GSE13507 contains 188 samples of BCa and 9 adjacent samples of BCa. The gene expression level of CSF2 in BCa and corresponding normal tissues was jointly mined by TCGA and GTEx, and then the ROC curve model of normal bladder tissue and bladder cancer tissue was constructed by R language.

**Patients and tissue samples**

We obtained a fresh BCa specimen as well as adjacent tissue specimens from Renmin Hospital of Wuhan University. After obtaining written consent from all patients, specimens were obtained by radical
resection from patients who had not undergone BCa or adjuvant chemotherapy. The study was approved by Ethics Committee of Renmin Hospital of Wuhan University.

**Antibodies**

Proteintech (Wuhan, China) provided all of the primary antibodies: Secondary antibodies against goat anti-rabbit IgG and goat anti-mouse IgG were provided by Proteintech (Wuhan, China).

**Cells and culture**

The human 5637 and T24 cell lines were purchased from ATCC. RPMI-1640 medium (HyClone, China) was used to maintain 5637 and T24 cells. They also received 1% penicillin G sodium/streptomycin sulfate along with 10% fetal bovine serum (FBS) (Gibco, Australia).

**Immunohistochemistry (IHC)**

Similar to HE staining, dewaxing and rehydration were performed. Then, the tissue sections were boiled at 100°C for 15 min. Blocking with 3.0% hydrogen peroxide (H₂O₂) at room temperature. The primary antibody was added to the tissue sections and incubated for one night at 4°C. After adding the secondary antibody, 30 minutes of incubation were performed at room temperature the next day. At last, Hematoxylin counterstained the sections after incubation with DAB chromogen.

**Western blot**

Using RIPA buffer solution, 5637, T24 cells were extracted of their total protein content. Discontinuous ultrasonic dispersion was used to split the samples. Lysates were centrifuged for 15 minutes at 4°C at 1*10⁵ g. The supernatant was detected with a bicinchoninic acid (BCA) assay. Protein samples were mixed with the loading buffer and denatured for 10 minutes at room temperature. Then, -20°C was used to store the protein samples. SDS-PAGE was used to separate 60 μg of protein from each sample and transfer it to NC membranes (Millipore, USA). The membranes were immersed in 5% nonfat milk for 1 hour at room temperature. Incubation with primary antibodies overnight was followed by 1 hour of incubation with secondary antibodies. As part of the protein development process, bands were detected using an appropriate development instrument.

**CCK-8 assay**

T24 and 5637 cells were seeded in 96-well plates at approximately 3×10³ cells/well and cultured for 24 h in different concentrations of brusatol. For each experiment, 10 μl of CCK-8 reagent (CK04, Dojindo, Japan) was added to each well and then cultured for 1 h. Absorption values were measured at 450 nm. The experiment was repeated three times.

**Colony formation assay**

T24 and 5637 cells were seeded in 6-well plates at approximately 300 cells/well and grown for approximately 14 days. The colonies were counted under the microscope after 4% paraformaldehyde (PFA) fixation and 0.1% crystal violet staining. The experiment was repeated three times. Transwell
migration assay. We used a transwell chamber system (Corning, USA). In the upper chamber, which was precoated with Matrigel (BD Biosciences, USA), $5 \times 10^4$ cells were suspended in serum-free medium. Medium containing 20% FBS was added to the lower chamber. After 48 h, cells on the other side of the membrane were fixed in 4% PFA (Paraformaldehyde) for 15 min and stained with 0.1% crystal violet for 1 h. The experiment was repeated three times.

**Wound healing assay**

A 6-well plate was seeded with cells and cultured so the bottom of the plate was completely covered with them. A sterile 200 mL plastic pipette was used to scratch the cell layer and washed with phosphate buffer solution (PBS) twice. Cells were cultured with medium containing 1% FBS for 48 hours. Photographs were taken at each time point. The experiment was repeated three times.

**Cell apoptosis**

Flow cytometry was used to analyze 5637 and T24 cells stained with Annexin-V FITC. Three repetitions of the experiment were conducted.

**TUNEL assay**

A kit for detecting apoptosis was used to perform the TUNEL assay. To observe the results, an optical fluorescence microscope (Olympus) was used.

**Reverse transcription (RT)-PCR and Quantitative (q)PCR**

Total RNA was extracted from T24 and 5637 cells. RNA was extracted with the TRI (Absin, China). Reverse transcriptase reactions were performed using a SuperScript First-strand Synthesis System (Thermo Scientific, the USA). The cDNA was stored in a -20 °C refrigerator for use. Real-time PCR reactions were performed with GAPDH as internal control and using a NovoStart were qPCR SuperMix Plus (Novoprotein). Pre-denaturation at 95°C for 30s; then enter the PCR reaction stage, the conditions are 95 °C 5s to 60 °C 30s, this stage cycle 40 times, and finally enter the lysis stage. Gene levels were shown as fold change relative to control calculated by the $2^{-\Delta\Delta CT}$ method. Primers used for qPCR were listed as follows:

CSF2: 5′-GGAGCATGTG$\triangleright$TG$\triangleright$CAT$\triangleright$CAG − 3, 5′ − CTGGAGGTC$\triangleright$ACA $\uparrow$ TCTGAGAT − 3;

NRF2: 5′-CACAT$\triangleright$CATCAG$\triangleright$ACAGTG$\triangleright$G − 3, 5′ − GG$\triangleright$TGTCCT$\triangleright$CGC$\triangleright$GGC$\triangleright$GCTG − 3;

GAPDH: 5′-GTCT$\triangleright$CTCTGAC $\uparrow$ C$\triangleright$CAGCG − 3, 5′ − ACACCTG $\uparrow$ GCTGTAGC $\triangleright$ − 3.

All experiments were conducted in triplicate and repeated three times.

**Cell transfection**
The small interfering RNAs (siRNAs) used to downregulate CSF2 expression were purchased from Genechem (Shanghai, China). The full sequence of NRF2 was inserted into a lentiviral vector (Sangon Biotech, China). For cell transfection, SiRNAs and cDNA were transfected to T24 and 5637 cells respectively according to the manufacturer's description. Infection efficiency was measured with Western blotting and real-time PCR.

**Xenograft mouse model**

Renmin Hospital Ethics Committee of Wuhan University approved the study. The animals used in the present study were performed in accordance with the Animal Welfare Act. A CSF2 knockdown lentivirus obtained from Genechem (Shanghai, China) was used to transfect T24 cells. Our xenograft mouse model was based on 4 week old male nude specific pathogen-free mice (SPF). They were subcutaneously injected with $1.5 \times 10^6$ CSF2 knockdown or negative control cells that were resuspended in PBS. Approximately four weeks later, the mice were sacrificed by sodium pentobarbital (100mg/kg) and the tumors were dissected and stained with IHC.

**Statistical analysis**

All values are presented as the mean ± Standard Deviation of the replicate samples. These experiments were repeated three times. The 22.0 SPSS software package was used for all statistical analyses.

**Results**

**CSF2 upregulation in Bca tissue specimens and cells**

The CSF2 expression level in 28 normal tissues and 407 Bca tissues included in TCGA and GTEx was different (Fig. 1A). The expression level of CSF2 in 19 cases of Bca tissues included in TCGA was different from that in paired adjacent tissues (Fig. 1B). The CSF2 expression level in 19 cases of Bca included in GEO was different from that in the paired adjacent tissues (Fig. 1C). The expression of CSF2 in Bca needs to be clarified, so we first examined it in Bca cell lines using WB and qRT-PCR. The results suggested that the protein expression (Fig. 1D) and RNA content (Fig. 1B) of CSF2 were significantly higher in RT4, 5637, UMUC3 and T24 relative to normal uroepithelial cells (SV-HUC-1). By extracting fresh tissue proteins for WB assay, we found that compared to paracancerous tissues, Bca tissues contained significantly more CSF2 protein (Fig. 1E). Using the TCGA database, we also obtained that compared with paraneoplastic tissues, the CSF2 RNA content of Bca tissue had a significantly higher level (Fig. 1F). CSF2 may be important in Bca, according to these results.

The previous results have shown that the CSF2 gene level in Bca is significantly lower. In order to further evaluate the accuracy of CSF2 in bladder cancer tissue and normal bladder tissue, TCGA and GTEx bladder cancer data were extracted and ROC model validation was carried out. The results suggest that CSF2 has a certain accuracy in distinguishing tumor tissue and normal tissue, with AUC reaching 0.787 (CI: 0.702–0.872) (Fig. 2A). According to Smartapp database analysis, multiple probes (cg17566874, cg13259290, cg08686879) in the CSF2 promoter region of bladder cancer tissue in prostate tumor
tissue β (Fig. 2B). The value is lower than that of normal tissue, and the expression of CSF2 follows β. The decrease of the value shows an upward trend (Fig. 2C). We believe that the demethylation of the promoter region is one of the reasons for the up regulation of CSF2 expression.

**Proliferation, migration, and invasion of BCa cells are triggered by CSF2**

In order to verify the role of CSF2 in the mid-stage of BCa, we first used shRNA to knock down CSF2 in BCa cell lines (T24 and 5637) using WB (Fig. 3A) and qRT-PCR (Fig. 3B) respectively. Next, we performed a clonogenic assay and found that the CSF2 knockdown group of T24 and 5637 cell lines showed a decrease in cell value added compared to the control group (Fig. 3C), and a significant decrease in cell viability of T24 and 5637 cell lines in the CCK-8 assay (Fig. 3D). The wound healing assay suggested the CSF2 knockdown group of T24 and 5637 cell lines showed a decrease in cell migration (Fig. 3E/3F). Additionally, the transwell assay showed a decrease in cell invasion ability (Fig. 3G). According to research, epithelial-mesenchymal transition (EMT) contributes to cancer metastasis. Therefore, we tested whether CSF2 induced EMT-associated markers by western blotting. The results showed that an increase in E-cadherin levels was observed, while Vimentin and N-cadherin expression levels decreased in CSF2 knockdown group (Fig. 3H). These results suggest that CSF2 knockdown inhibits the growth, migration and invasion ability of T24 and 5637 cells.

**CSF2 inhibits apoptosis in BCa cells**

Aiming to determine how CSF2 affects BCa cells to inhibit tumor growth, we use flow cytometry to determine whether it could induce apoptosis. In line with our expectations, compared to the control group, CSF2 knockdown group of T24 and 5637 cells exhibited a significantly higher rate of apoptosis (Fig. 4A). Western blotting was used to examine whether CSF2 suppresses apoptosis-associated markers in BCa cells. Flow cytometry showed an increase in Caspase3 cleavage and Bax expression, while Bcl-2 expression decreased (Fig. 4B). All these results suggested that BCa cells were more likely to undergo apoptosis when CSF2 was knocked out.

**CSF2 induces aggressive behavior in BCa cells by increasing Nrf2 expression**

Previous studies have shown that CSF2 regulates the biological functions of BCa cells. Increasing evidence shows that Nrf2 plays a key role in tumor cell proliferation by regulating key signaling pathways. Consequently, we investigated whether Nrf2 regulates cancer progression in 5637 and T24 cells. The results of WB showed that the CSF2 knockdown group showed greatly reduced expression levels of Nrf2 (Fig. 5A). A cell line overexpressing Nrf2 was constructed for further investigation (Fig. 5B). The CCK-8 assay (Fig. 5C, 4D), clone formation assay (Fig. 5E), transwell assays (Fig. 5F) revealed that boosting Nrf2 expression on BCa cells partially counteracted the inhibitory effects of CSF2 knockdown. These suggest that BCa cells' behavior can be mediated by CSF2 through regulating Nrf2...
CSF2 regulates Nrf2 via the Akt/Mtor pathway in BCa cells

The cBioPortal database was used to explore the specific mechanisms by which CSF2 influences bladder cancer progression using KEGG pathway analysis, and the results suggest that CSF2 may regulate bladder cancer progression through the signalling pathway in which AKT is located (Fig. 6A). The cBioPortal database also provides preliminary confirmation that there is no clear correlation between CSF2 and AKT1 expression (S1) and that AKT's function is generated post-transcriptionally through phosphorylation modifications. We then performed Western blotting experiments to verify changes in protein of p-Akt and Akt. Nrf2 is regulated by Akt/mTOR signaling in tumor cells[12, 13]. In order to determine whether Nrf2 is regulated by the Akt/mTOR pathway, we examined the activation of this pathway in T24 and 5637 cells. In the end, the levels of p-Akt and p-mTOR were significantly decreased in CSF2 knockdown T24 and 5637 cells (Fig. 6B). Subsequently, we use Akt agonist SC79. The cell biological activity assays were performed after the CSF2 knockdown T24 and 5637 cells were treated with SC79. Western blotting revealed an increase in Nrf2 protein levels following SC79 treatment in CSF2 knockdown T24 (Fig. 6C) and 5637 (Fig. 6D) cells. In other experiments, SC79 treatment significantly reversed the inhibition of CSF2 knockdown T24 and 5637 cell lines (Fig. 6E, 6F, 6G). Altogether, these results confirmed that CSF2 regulates Nrf2 in BCa cells by activating Akt/mTOR.

Knockdown of CSF2 inhibits the growth of BCa

BCa cells were inhibited when CSF2 was knocked down in vitro. With a xenograft mouse model, we tested whether knocking down CSF2 inhibited BCa cell growth in vivo. The mice were all randomly divided into two groups and received T24-NC or T24-shCSF2 cells injection. A tumor size measurement was performed after each injection. At last, T24-shCSF2 injection mice had smaller tumors than their control group (Fig. 7A). In T24-shCSF2 injection group, there was a noticeable difference between the tumor growth rate and the control group. (Fig. 7B). Consequently, TUNEL staining levels were increased (Fig. 7C), which indicated T24-shCSF2 induced apoptosis of tumor in vivo. Meanwhile, Ki-67 expression and Nrf2 levels were reduced by IHC analysis in the T24-shCSF2 injection group, as well as p-Akt and p-mTOR (Fig. 7D). These data confirmed that knockdown of CSF2 significantly inhibits the growth of BCa in vivo.

Discussion

The most common urogenital system tumor in adults is BCa [1]. Because of BCa's aggressive nature and proliferation rate, recurrence is common despite surgery, radiotherapy, and chemotherapy. [14]. Therefore, the search for new therapeutic targets is of great importance for treating bladder cancer. In this study, BCa tissues express more CSF2 than normal tissues, as opposed to normal tissues. As well, BCa with high CSF2 expression showed a high T stage and tumor grade. There was a superior survival rate in patients with low CSF2 expression than those with high CSF2 expression. BCa cells were inhibited in vitro and in vivo when CSF2 was knocked down. Consistently, we identified CSF2 could activate Nrf2
which played an important role in tumor biology. In addition, CSF2 activates the Akt/mTOR signaling pathway, up-regulating Nrf2 protein expression.

The upregulation of CSF2 in the solid tumour lead to poor prognosis in some tumours. Gu reported that Targeting CSF2/CSF2R for M2 macrophage reprogramming has been studied in clinical trials for cancer therapy. CSF2/CSF2R signaling plays an important role in macrophage polarization. [15]. Previous studies have demonstrated that The phosphorylation of STAT3/MYC by CSF2 regulates the phenotypic plasticity of small cell lung cancer [16]. Li et al performed a research about CSF2 in bladder cancer[17]. Patients with BCa and advanced disease status showed CSF2 was associated with poor clinical outcomes. The results suggested that prognosticators and potential therapeutic targets of BCa include CSF2. There is however a lack of knowledge about its role in BCa and the molecular mechanisms underlying it. Consistent with their findings, we found Among BCa patients, high levels of CSF2 were associated with a high T stage and tumor grade. In addition, we revealed knockdown of CSF2 inhibited BCa cells in vitro and in vivo.

Firstly, we found that BCa cells were less capable of proliferating and surviving when CSF2 was knocked down. With regard to the mechanism underline increasing tumour malignancy of CSF2, studies show that maybe connect to inducing apoptosis. Known as a programmed cell death, apoptosis occurs when cells die. A flow cytometry analysis confirmed the results in these reports that CSF2 knockdown induced apoptosis in BCa cells. BCa cells migrate and invade less when CSF2 is knocked down in another experiment. EMT of tumor cells may be the major cause of distant metastases from tumors, according to a growing number of studies [18–20]. The majority of BCa deaths are caused by distant metastases of tumors. The loss of cell-to-cell connection and apicobasal polarity occurs during EMT, and the cells assume a fibroblast-like appearance, allowing metastatic spread [21, 22]. E-cadherin is the key epithelial marker of EMT[23]. CSF2 knockdown cells showed a significant decrease in migration in our study. Additionally, knocking down CSF2 inhibited BCa cells' EMT, attenuating BCa's malignancy. It appears that CSF2 was an oncogene in BCa development, but further research is needed to confirm this.

An important transcription factor, Nrf2, plays a role in the survival of cancer cells. It has been shown that this protein can induce tumor-protective gene transcription, thereby promoting tumor growth and preventing cells from apoptosis.[24, 25]. As a result of the aryl hydrocarbon receptor (AhR), polycyclic aromatic hydrocarbons stimulate Nrf2.. An AhR-binding nuclear translocator (Arnt) transactivates Nfe2l2 by transactivating its promoter that contains xenobiotic response element-like sequences.. An activation of the Nrf2 pathway was caused by xenobiotic ligands induced by AhR [26]. Human MCF10A mammary cells activate NFE2L2 in response to breast cancer susceptibility 1 (BRCA1) [27]. A human mammary cell line, MCF10A, also activates NFE2L2 when breast cancer susceptibility 1 (BRCA1) is present. [28]. In addition to inhibiting EMT, Nrf2 also inhibited esophageal squamous cell carcinoma metastasis [29]. This is consistent with our findings. CSF2 knockdown cells showed a marked decrease in Nrf2 expression. A significant reduction of inhibition on BCa cells was observed with Nrf2 overexpression. Since Nrf2 is ubiquitinated by E3 ligases and degraded by 26S proteasomes under normal conditions, we hypothesized that CSF2 knockdown would also affect Nrf2. [30]. During the deeply experiment, Nrf2
overexpression improved BCa's biological behavior, suggesting Nrf2 has a role to play in CSF2 regulating BCA.

There is no doubt that the Akt/mTOR pathway plays a significant role in various types of cancer. Tumor cells are more likely to proliferate and metastasize when Akt is activated, since it inhibits its downstream molecules. Inhibition of downstream targets is accomplished by mTOR, a downstream marker [31]. The Akt/mTOR signaling regulates the expression of protein Nrf2 in cancer cells, which in turn regulates many biological behaviors[13, 32]. Lots of studies have shown that activated Akt could phosphorylate Nrf2, resulting in the release of Nrf2 from KEAP1-NRF2 complex[33]. And then Nrf2 transfers to the nucleus, ultimately leading to the transcriptional activation of phase II enzyme/antioxidant genes. Nrf2 is a well known transcription factor. When Nrf2 is blocked, the growth of cancer is inhibited. Cell proliferation and invasion were immediately affected by activating Akt phosphorylation in our research. In this study, Nrf2 was markedly down-regulated along with down-regulation of p-Akt and p-mTOR. The following, we used Akt agonist SC79 for the reverse experiment. As we expected, the expression of p-Akt and Nrf2 was increased in SC79 treatment group. And SC79 treatment reversed the inhibition of knockdown of CSF2 on the proliferation and invasion of cells. Our findings confirmed that CSF2 knockdown reduces Nrf2 expression and inhibits Akt/mTOR pathways. Hence, these results imply that the Akt pathway may be involved in the progression of BCA through CSF2.

In conclusion, we described the molecular mechanism of CSF2 in BCA process, in which CSF2 was high expression. We revealed the expression of CSF2 could attenuate BCA cell progression for the first time by regulating cell growth. Knockdown of it could obviously increase cancer cell apoptosis and inhibit cancer cell EMT process. Akt/mTOR signaling pathways also regulate the Nrf2 transcription factor via the underline mechanism. As a result of this mechanism, CSF2 may serve as a valuable diagnostic marker in BCA and as a therapeutic target.

Declarations

Acknowledgements

Not applicable.

Author contributions

Huaxin Wang and Lei Wang conceptualized the study. Shenglan Li contributed to the data curation. Qinghua Wang conducted the formal analysis. Xi Yu and Shuai Ke investigated the study. Chenglin Ye contributed to the methodology. Xi Yu and Shenglan Li wrote, reviewed, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Data availability

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

Ethics approval and consent to participate

The study was approved by Ethics Committee of Renmin Hospital of Wuhan University. The animal study was reviewed and approved by the Ethics Committee of Renmin Hospital of Wuhan University.

Consent for publication

All listed authors have actively participated in the study and have read and approved the submitted manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures
Figure 1

CSF2 is upregulated in bladder cancer. (A) CSF2 expression in 28 normal tissues and 407 BCa tissues included in TCGA and GTEx. (B) CSF2 expression in 19 cases of BCa tissues and adjacent tissues included in TCGA. (C) CSF2 expression in 19 cases of BCa and adjacent tissues included in GEO. (D) Western Blot showed that the protein level of CSF2 was upregulated in all of the four bladder cell lines compared to that in normal epithelial cells. (E) RT-qPCR analysis showed that the mRNA level of CSF2 was indeed upregulated in all of the four bladder cell lines compared to that in normal epithelial cells. (F) Western blot analysis revealed that the protein level of CSF2 were upregulated in malignant bladder cancer tissues compared to that in adjacent normal tissues. Values are expressed as the mean ±SDs. *P < 0.05.
(A) ROC curve of CSF2 in adjacent tissues of bladder cancer and normal tissues. (B) Using cg17566874, cg13259290 and cg08686879 as probes, the promoter methylation level of CSF2 gene in normal and tumor tissues was detected. (C) The probes of cg17566874, cg13259290, cg08686879 were negatively correlated with CSF2 gene expression.

**Figure 3**

CSF2 regulates the proliferation, migration and invasion of bladder cancer cells in vitro. We verified the knockdown efficiency of CSF2 in T24 and 5637 cells by WB (A) and qRT-PCR (B) respectively. (C) The representative images from the colony formation assay showed that knockdown of CSF2 significantly decreased the mean colony numbers. (D) CCK-8 assays showed that knockdown of CSF2 significantly decreased the growth rate of T24 and 5637 cells. The Wound healing assays showed that knockdown of CSF2 decreased the migration ability of T24 (E) and 5637 (F) cells. (G) The Transwell assay showed that knockdown of CSF2 decreased the invasion of T24 and 5637 cells(magnification ×200). (H) WB showed that EMT-associated markers such as E-cadherin was increased, whereas Vimentin and N-cadherin were decreased in CSF2 knockdown cells. Data are presented as the means ± SDs for 3 independent experiments.*P < 0.05 vs. the control group.
Figure 4

CSF2 inhibits apoptosis in BCa cells through activating caspase 3. (A) Flow cytometry assays showed that knockdown of CSF2 significantly decreased the apoptosis rate of T24 and 5637 cells. T24. Cell apoptosis was detected by flow cytometry using annexin V/PE staining. Representative flow cytometric images and statistical data are showed. (B) WB showed that Apoptosis-associated markers such as Bcl-2 was decreased, whereas Bax and cleaved-caspase3 were increased in CSF2 knockdown cells. The data are presented as means ± SDs for 3 independent experiments. *P<0.05 compared with the control group.
Figure 5

CSF2 prompts aggressive behaviors in BCa cells by increasing the expression of Nrf2. (A) The protein of Nrf2 was decreased in CSF2 knockdown T24 and 5637 cells. (B) The overexpression efficiency of Nrf2 (OE) in T24 and 5637 cells. The expression of Nrf2 were increased compared to negative control (NC). (C) CCK-8 assays showed that overexpression of Nrf2 reversed the inhibitory effect of CSF2 knockdown in T24 (C) and 5637 (D) cells. (E) Cell proliferation was assessed by the colony formation ability. The overexpression of Nrf2 reversed the inhibitory effect in CSF2 knockdown T24 and 5637 cells. (F) The Transwell assay showed that overexpression of Nrf2 reversed the ability of invasion in CSF2 knockdown T24 and 5637 cells(magnification ×200). Data are presented as the means ± SDs for 3 independent experiments. *, P<0.05; **, P<0.01 vs. the control group.
Akt/mTOR pathway is required for the regulation of Nrf2 by CSF2 in BCa cells. (A) There was a positive correlation between the expression of CSF2 and AKT pathway. Western blotting revealed the levels of p-Akt and p-mTOR were significantly decreased in CSF2 knockdown T24 and 5637 cells (B). Western blotting revealed that SC79 reversed the levels of Nrf2 and p-Akt in CSF2 knockdown T24 (C) and 5637 (D) cells. (E) CCK-8 assays showed that SC79 reversed the inhibitory effect of CSF2 knockdown in T24 and 5637 cells. (F) Cell proliferation was assessed by the colony formation ability. SC79 reversed the inhibitory effect in CSF2 knockdown T24 and 5637 cells. (G) The transwell assay showed that SC79 reversed the ability of invasion in CSF2 knockdown T24 and 5637 cells (magnification ×200). Data are presented as the means ± SDs for 3 independent experiments. *, P<0.05; **, P<0.01 vs. the control group.
**Figure 7**

Knockdown of CSF2 significantly inhibits the growth of BCa in vivo. (A) Morphology of the subcutaneous implanted tumor. (B) Mean tumor volume at each time point. (C) A TUNEL assay was performed to detect the apoptotic cells in the tumor tissue. (D) IHC was performed to detect the protein of Ki67, Nrf2, p-Akt, p-mTOR in the tumor tissue. *P < 0.05 vs. the control. All the above data are the mean ± SD from an average of three experiments.
Based on the above results, Csf2 may prompt BCa progression through by regulating Nrf2 via the Akt pathway (figure 8)