Experimental Research

ANNALS OF MEDICINE & SURGERY

OPEN

Inhibiting stanniocalcin 2 reduces sunitinib resistance of Caki-1 renal cancer cells under hypoxia condition

Hezhen Chu, MD[a], Wenchao Xie, MD[b], Chuanzhi Guo, MD[c], Haifeng Shi, PhD[c], Jie Gu, PhD[c], Zhenqian Qin, PhD[b], Yimin Xie, PhD[b,*]

Background: Our previous study has suggested that blocking stanniocalcin 2 (STC2) could reduce sunitinib resistance in clear cell renal cell carcinoma (ccRCC) under normoxia. The hypoxia is a particularly important environment for RCC occurrence and development, as well as sunitinib resistance. The authors proposed that STC2 also plays important roles in RCC sunitinib resistance under hypoxia conditions.

Methods: The ccRCC Caki-1 cells were treated within the hypoxia conditions. Real-time quantitative PCR and Western blotting were applied to detect the STC2 expression in ccRCC Caki-1 cells. STC2-neutralizing antibodies, STC2 siRNA, and the recombinant human STC2 (rhSTC2) were used to identify targeting regulation on STC2 in modulating sunitinib resistance, proliferation, epithelial-mesenchymal transition (EMT), migration, and invasion. In addition, autophagy flux and the lysosomal acidic environment were investigated by Western blotting and fluorescence staining, and the accumulation of sunitinib in cells was observed with the addition of STC2-neutralizing antibodies and autophagy modulators.

Results: Under hypoxia conditions, sunitinib disrupted the lysosomal acidic environment and accumulated in Caki-1 cells. Hypoxia-induced the STC2 mRNA and protein levels in Caki-1 cells. STC2-neutralizing antibodies and STC2 siRNA effectively aggravated sunitinib-reduced cell viability and proliferation, which were reversed by rhSTC2. In addition, sunitinib promoted EMT, migration, and invasion, which were reduced by STC2-neutralizing antibodies.

Conclusion: Inhibiting STC2 could reduce the sunitinib resistance of ccRCC cells under hypoxia conditions.

Keywords: clear cell renal cell carcinoma, drug resistance,, hypoxia, stanniocalcin 2, sunitinib

Introduction

Renal cell carcinoma (RCC) is the 14th most common cancers in the world, and 20–30% of patients with RCC have metastasis at the first diagnosis[1–3]. The clear cell RCC (ccRCC) is the most common type of metastatic RCC; however, several cases of collecting duct carcinoma were counted[4]. The drug resistance of RCC is still a problem for the survival of patients with RCC, and therefore, it needs to improve the drug sensitivity of the cancer cells. Sunitinib, one of the widely used multitargeted tyrosine kinase inhibitors, has been licensed for RCC treatment in clinical therapy[5,6]. However, the emerging sunitinib resistance overshadowed its application. Hence, it is urgent to clarify the underlying mechanisms and identify new treatments for sunitinib resistance.

HIGHLIGHTS

- Sunitinib was captured in Caki-1 cells under hypoxia.
- Sunitinib suppressed stanniocalcin 2 (STC2) expression under hypoxia conditions.
- Inhibiting STC2 reduces the sunitinib resistance of Caki-1 cells under hypoxia.
- Hypoxia-induced epithelial-mesenchymal transition and invasion of Caki-1 cells.
- STC2-neutralizing antibody reduced sunitinib-induced epithelial-mesenchymal transition, migration, and invasion under hypoxia.

The changes in the components of the cell membrane and extracellular matrix (ECM), the expressions of chemotaxis, and cell proliferation can promote drug resistance[7]. A xenograft study model revealed that epithelial-mesenchymal transition (EMT) is associated with acquired ccRCC resistance to sunitinib[8]. Increased EMT was also observed in sunitinib-resistant ccRCC cell lines, and ccRCC deteriorated in patients after TKI treatment[9]. Chronic sunitinib promotes EMT and increased cell migration and invasion of treatment of ccRCC cell line 786-O[10]. These *in vivo* and *in vitro* studies suggest that EMT is a mechanism of resistance to sunitinib in ccRCC.

[a]Department of Urology, Yixing Traditional Chinese Medicine Hospital, [b]Department of Urology, Affiliated Hospital of Jiangsu University-Yixing People's Hospital, Yixing and [c]School of Life Sciences, Jiangsu University, Zhenjiang, Jiangsu, People's Republic of China

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

*Corresponding author. Address: Department of Urology, Affiliated Hospital of Jiangsu University-Yixing People's Hospital, 75 Tongzhenguan Road, Yixing, Jiangsu, People's Republic of China. Tel.: +86 051087921000; fax: +86 051081550100. E-mail: 2221513084@stmail.ujs.edu.cn (Y. Xie).

Copyright © 2023 The Author(s). Published by Wolters Kluwer Health, Inc. This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.

Annals of Medicine & Surgery (2023) 85:5963–5971

Received 7 August 2023; Accepted 20 October 2023

Published online 1 November 2023

http://dx.doi.org/10.1097/MS9.0000000000001450

Previous studies have revealed that the lysosomes mediate the release of cathepsin B and matrix metalloproteinase-9 (MMP-9) into the ECM, a process named via exocytosis, which can degrade the ECM to promote cancer metastasis[11]. In 786-O cells, sunitinib accumulated in lysosomes and disrupted autophagic degradation by inhibiting the activity of the lysosomal protease cathepsin B, which promotes to sunitinib resistance of ccRCC[12,13]. In the case of metastasis RCC, the treatment with sunitinib for 3 months increased the lysosome exocytosis, which released cathepsin B into the ECM and degraded the ECM to induce RCC metastasis[14]. These studies indicate that sunitinib may change the ECM to regulate RCC metastasis, and its mechanism in sunitinib resistance of RCC cells still needs to be further investigated.

Hypoxia is a frequent occurrence in a wide range of human tumors, including urothelial and nonurothelial urinary tract tumors[15–17]. The majority of ccRCC cases have been linked to the inactivation of the von Hippel-Lindau tumor suppressor gene (pVHL) and the dysregulation of hypoxia-related pathways[18]. *In vivo* study of a sunitinib-resistant xenograft model of ccRCC, the bioinformatics analysis showed the differentially expressed genes were mainly involved in cell hypoxia response[19]. The hypoxia condition promotes tumor progression and increases adaptive responses and resistance to therapy through inhibition of proliferation and changes of ECM for cell invasion and migration[20]. Previous studies showed that induction of EMT and hypoxia resistance contributed to the sunitinib resistance of ccRCC[21]. In addition, sunitinib targeting inhibits vascular endothelial growth factor receptor and platelet-derived growth factor receptor, which plays critical roles in response to hypoxia stress[22]. These studies indicate that tumor hypoxia may play a crucial factor in the sunitinib resistance of ccRCC by hypoxia-induced EMT, cell invasion, and migration.

Stanniocalcins (STC1 and STC2) were originally identified as hormones to regulate the metabolism and homeostasis of calcium and phosphorus, recently were also reported to regulate various cell processes in tumor development[23–25]. In ovarian cancer cell SKOV3, STC2 induced the production of reactive oxygen species (ROS) under hypoxia and the activation of extracellular signal-regulated kinase1/2 (ERK1/2), thereby improving the ability of tumor cells to resist apoptosis under hypoxic stress[26]. The study of lung cancer cell H460 showed that STC2 silencing could significantly increase the intracellular ROS level and inhibit cell activity, indicating that STC2 may play dual roles in different cancers[27]. Under hypoxic conditions, stable STC2 expression can promote EMT of ovarian cancer SKOV3 cells[26]. It found that high expression of STC2 was associated with tumor invasiveness and shortened overall survival in patients with RCC[28,29]. Our previous study showed that blocking STC2 could reduce sunitinib resistance in ccRCC cells[30]. However, the effect of STC2 in ccRCC under hypoxia and its molecular mechanism is still unclear. Therefore, in the present study, the effect of hypoxia on sunitinib resistance associated with EMT and cell migration of ccRCC cells was investigated. In addition, the efficacy of STC2-neutralizing antibodies on the sunitinib resistance of ccRCC cells was detected so as to provide the experimental basis to overcome the sunitinib resistance of RCC.

Materials and methods

Reagents

Sunitinib was purchased from Cell Signaling Technology (Shanghai, China). MTT (3- (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) was purchased from Solarbio (M1020, Beijing, China). Acridine Orange (AO) was purchased from Leagene (DM0630, Beijing, China). Antihuman Ki-67 was purchased from Cell Signaling Technology (9449S, Shanghai, China). LysoTracker TM Green DND-26 (L7526), Alexa Fluor 488 goat antimouse IgG (A-11001), DAPI (D1306), and antihuman STC2 antibodies (PA5-34841) were purchased from Thermo fisher Scientific (Shanghai, China). Formaldehyde and Triton X-100 were purchased from Sangon Biotech (Shanghai, China). Human STC2-neutralization antibodies (AF2830) and recombinant human STC2 (O76061) were purchased from R&D System (USA). Autophagy inducer Rapamycin (Rap) and lysosome inhibitor Chloroquine (CQ) were purchased from Sigma (USA).

Cell culture

Caki-1 cells obtained from American Tissue Culture Collection (ATCC, Manassas, VA, USA) were cultured in McCoy's 5A medium (pH 7.4) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (pH 7.4) in CO2 (5%) incubator setting 37°C. The culture medium and supplements were purchased from Gibco (Thermo Fisher Scientific, USA). For the hypoxia treatment, the cells were cultured in low O_2 (5%) in the hypoxia incubator (Thermo Fisher Scientific, USA).

MTT assays

Caki-1 cells were collected and seeded in 96-well with a density of 2×10^3 cells/well. After being treated with sunitinib under a hypoxia incubator for 24 h, cell growth was detected by the MTT assay. In brief, cells were incubated in 100 μl MTT solution (0.5 mg/ml in culture medium) in a 96-well plate for 4 h. Then, the discarded medium and the colored product (formazan) were dissolved in 100 μl DMSO. The absorbance was measured by a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, USA) at 540 nm (690 nm as a reference). In some experiments, STC2-neutralizing antibodies (0.2 μg/ml) were added into the culture medium with sunitinib (5 μM).

Real-time quantitative PCR (RT-qPCR) analysis

The total RNA was isolated by using Trizol (Vazyme, Nanjing, China). The purity and concentration of RNA were detected by NanoDrop 2000. The cDNA was synthesized by using 10 μg total RNA according to the protocol of the RNA-to-cDNA kit (Vazyme, Nanjing, China). RT-qPCRs were carried out by Quantstudio 3 (Thermo Fisher Scientific, USA) using the SYBR Green I kit (Vazyme, Nanjing, China), using the primers STC2-F: GGTGGACAGAAC-CAAGCTCTC, STC2-R: CGTTTGGGTGGCTCTTGCTA, and β-Actin-F: GACTACCTCATGAAGATCCTC ACC, β-Actin-R: TCTCCTTAATGTCACGCACGATT. The relative expression levels of the target gene were calculated by the ΔΔCt method.

Western blotting analysis

The radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) was used to lysis the cells on ice for 30 min. Then, the samples were centrifuged at 13 000 g for 15 min at 4°C, the total protein in the supernatant was collected, and the concentration was detected by a BCA kit (Thermo Fisher Scientific, USA). The 30 μg total cellular protein was used to electrophoresis on 10% polyacrylamide gels, the transfer onto PVDF membranes (Merck, Shanghai, China). Western blotting was conducted using primary polyclonal antibodies (1:1000). Anti-STC2 was purchased from Thermo fisher Scientific (PA5-34841, Shanghai, China), Anti-E-cadherin, Anti-N-cadherin, Anti-vimentin, Anti-LC3B, Anti-P62 were purchased from Cell Signaling Technology (49398, Shanghai, China) and β-Actin was purchased from Boster (BM3873, Wuhan, China), and horseradish peroxidase-conjugated goat antirabbit antibodies were purchased from Cell Signaling Technology (7074S, Shanghai, China) (1:4000). Specific bands were visualized by chemiluminescence with the Westar Supernova kit (Vazyme, Nanjing, China).

Cell migration and invasion assay

The cell migration assay was performed by the wound healing assay. Cells were seeded in a 6-wells plate (2×10^5 cells/well) within a complete medium. Then, the cells (90–95% confluence) were manually scratched through the confluent monolayer by the tips. After being washed with PBS to remove cellular debris, the cells were incubated in a fresh medium (no FBS) with or without sunitinib (5 μM). In some experiments, STC2-neutralizing antibodies (0.2 μg/ml) were added into the culture medium with sunitinib (5 μM). Images were taken at 0 h and 24 h by Olympus IX73 microscopy (Japan). The cell migration distance was measured in μm and calculated to indicate relative wound healing rates.

Cell invasion of Caki-1 cells was detected by Transwell 8 μm pore size (BD Biosciences). The cells were seeded at 2×10^5 cells in Matrigel-coated inserts in the up compartment. After incubation for 24 h, the upper-side cells (noninvaded) on the insert membrane were removed. The invaded cells were fixed in ice-cold methanol for 10 min and stained with 0.1% crystal violet for 20 min, then the images were taken.

Knockdown of STC2

Knockdown of the expression of STC2 in Caki-1 cells was using siRNA: sense 5´GCGUGUUUGAAUGUUUCGATT-3´; and antisense 5´-UCGAAACAUUCAAAC ACGCTT-3´, which were purchased from GenePharm (Shanghai, China). The siRNAs were pack-aged by Lipo2000 reagent (Thermo Fisher Scientific, W I, USA) and transfected into cells (6-well, 2×10^5 cells/well). After being transfected within 24 h, the cells were harvested for further experiments.

Lysosomal acidity detection

AO staining was performed to detect the lysosomal acidity, which was indicated by the ratio between the red and green signal of AO. After treatment, Caki-1 cells were washed stained by AO (1 mM) for 15 min at room temperature and replaced with medium. Then, the fluorescence images were taken by microscope (Olympus BX53, Japan). Quantification of the red and green signal was performed by using the software Image J.

Immunofluorescence

After treatments, the Caki-1 cells were prepared for proliferation detection. The cells were fixed in 4% formaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 20 min. The cells were blocked with 3% normal goat serum for 1 h, then incubated with mouse anti-Ki-67 antibody or rabbit anti-E-cadherin antibody overnight at 4°C. After washing twice with PBS, incubated with Alexa Fluor 488 goat antimouse IgG or Alexa Fluor 488 goat antirabbit IgG 1–2 h. After washing twice with PBS, the cell nucleus was stained by the DAPI for several minutes, then washed with PBS. The fluorescence images were taken by a microscope (Olympus BX53, Japan).

Statistical analysis

Drug treatments were performed in triplicate in each experiment and every experiment was repeated at least three times. The results are expressed as means ± SD. Statistical significance was assessed with a Student's *t*-test or one-way analysis of variance followed by Duncan's multiple comparison tests. The significant differences between the indicated groups were considered if $P < 0.05$ or $P < 0.01$.

Ethics committee approval

This article does not contain any studies with human participants or animals performed by any of the authors. All experimental protocols used cell culture *in vitro*.

Results

Sunitinib was captured in Caki-1 cells under hypoxia conditions

In our previous study, the sunitinib resistance of ccRCC cells in normoxia condition had been detected[30]. In the present study, we aimed to identify the efficacy of sunitinib on Caki-1 cells and the underlying mechanism under hypoxia conditions. The cell viability was detected by the MTT assay. It showed that Caki-1 cells were resistant to 5 μM sunitinib (Fig. 1A), the most efficient dose in the normoxia condition. To identify whether lysosomal sequestration and inhibition of the autophagic flux are involved in the sunitinib resistance of ccRCC, we detected the accumulation of sunitinib in Caki-1 cell pretreated with the lysosome inhibitor CQ and the autophagy inducer Rapamycin. It appeared as yellow color particles after being treated with sunitinib (Fig. 1B). It is interesting that there are clustered punctate in the pretreatment of lysosome inhibitor CQ, but there is no significant change in the pretreatment of autophagy inducer Rapamycin (Fig. 1B). The results of Western blotting showed that the sunitinib-increased expressions of the autophagy flux biomarkers LC3B and P62 (Fig. 1C, D). AO staining was performed to further detect the effect of sunitinib treatment on the acidic environment of lysosomes. Compared with the control, sunitinib decreased the ratio of red/green fluorescence (Fig. 1E, F). These results suggested that sunitinib disrupted the acidic environment of lysosomes and increased sunitinib accumulation and resistance in Caki-1 cells under hypoxia conditions.

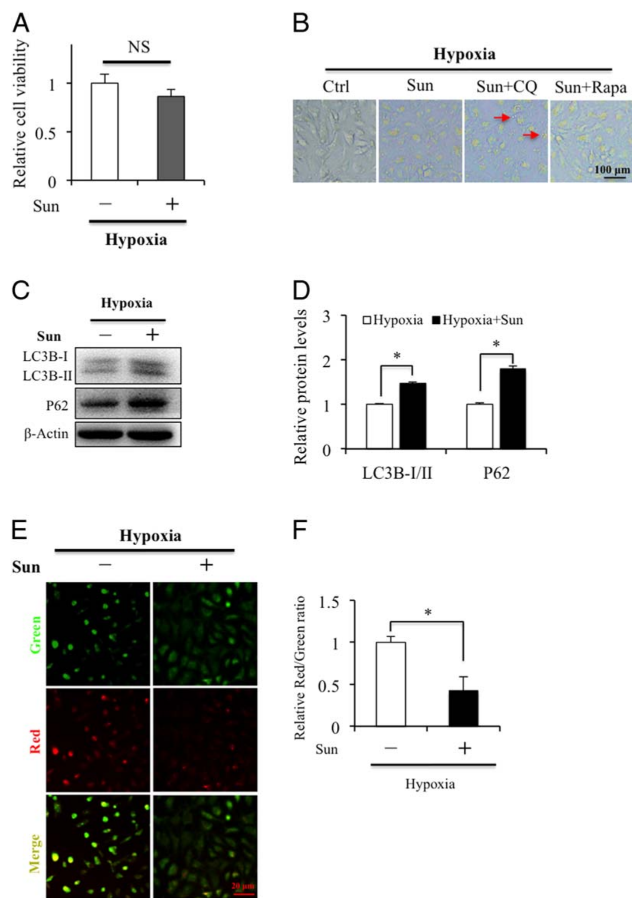

Figure 1. Effects of sunitinib on the cell viability, sunitinib accumulation, autophagy and lysosomal pH in Caki-1 cells under hypoxia condition. After treated with or without Sun (5 μM) for 24 h, the relative cell viability of Caki-1 cells was evaluated by MTT assay (A). Or pretreated with autophagy inducer Rapamycin (Rap) or lysosome inhibitor Chloroquine (CQ) for 1 h, followed by Sun (5 μM) for 24 h, accumulation of yellow granules of Sun in Caki-1 cells was investigated by phase contrast microscopy (B). Relative protein levels were evaluated by Western blotting and quantified by Image J 1.54c. (C, D). AO staining of lysosome in Caki-1 cells (E). Quantification of the ratio between the red and green signal of AO was performed by the software Image J 1.54c (F). *, $P < 0.05$, indicates significant difference. NS, no significant difference; Sun, sunitinib.

Sunitinib suppressed STC2 expression under hypoxia conditions

To identify the effect of hypoxia on STC2 expression, we treated the cells with cobalt chloride ($CoCl_2$), a mimic of a hypoxic state or cultured in hypoxic condition for 24 h. The results of RT-PCR and Western blotting showed that the expression of STC2 was upregulated after $CoCl_2$ treatment, as well as after being cultured under the hypoxic condition (Fig.2A-C). Moreover, the results of Western blotting revealed that sunitinib could inhibit the expression of STC2 in Caki-1 cells under hypoxia conditions (Fig.2D, E).

Inhibiting STC2 reduces the sunitinib resistance of Caki-1 cells under hypoxia conditions

To further verify the role of STC2 on the sunitinib resistance of Caki-1 cells under hypoxia condition, STC2 siRNA was

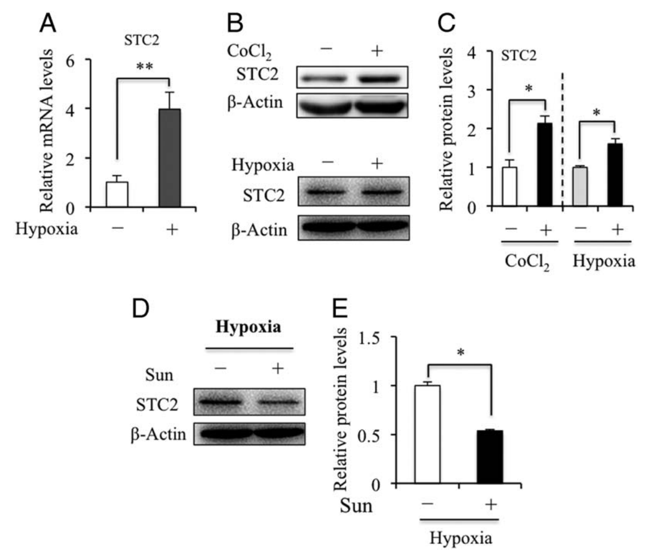

Figure 2. Effect of sunitinib on STC2 expression in Caki-1 cells under hypoxia condition. (A) The mRNA level of STC2 in Caki-1 cells was evaluated by RT-qPCR. (B, C) The protein levels of STC2 in Caki-1 cell lines were evaluated by Western blotting after treated with hypoxia mimic $CoCl_2$ (100 μM) or treated in hypoxic incubator for 24 h. (D, E) The protein levels of STC2 in Caki-1 cells were evaluated by Western blotting after treated with or without Sun (5 μM). Relative protein levels were normalized by β-Actin and analyzed by Image J 1.54c. *, $P < 0.05$ indicates significant difference. Sun, sunitinib.

transfected into the cells, and STC2-neutralizing antibodies and human recombinant STC2 (hSTC2) were applied to add into the culture medium. The results of the MTT assay showed that STC2-neutralizing antibodies and knockdown of STC2 by siRNA could promote the sensitivity of Caki-1 cells to sunitinib (Fig. 3A), while addition of hSTC2 increased sunitinib resistance of Caki-1 cells (Fig. 3A). In addition, the anti-Ki-67 staining showed that sunitinib inhibited cell proliferation, which was aggravated by STC2-neutralizing antibodies and STC2 siRNA, but abated by the addition hSTC2 (Fig. 3B, C). Phase contrast microscopy observed more yellow color particles (more sunitinib were captured and accumulated in the cells) in addition to hSTC2. Still, lesser in co-treatment of STC2-neutralizing antibodies (Fig. 3D). These results indicated that inhibiting STC2 could reduce the sunitinib resistance of ccRCC cells under hypoxia conditions.

Hypoxia-induced EMT and invasion of Caki-1 cells

The EMT-associated with the invasion of Caki-1 cells was determined under hypoxia conditions, which might contribute to sunitinib resistance in ccRCC. Western blotting analysis revealed that N-cadherin and vimentin protein levels were upregulated, whereas E-cadherin was reduced (Fig. 4A, B). Transwell assays revealed that hypoxia promoted the invasion of Caki-1 cells (Fig. 4C, D).

STC2-neutralizing antibodies reduced sunitinib-induced EMT, migration, and invasion under hypoxia conditions

Next, we detected the effect of sunitinib on EMT, migration, and invasion of ccRCC under hypoxia conditions. The results of Western blotting analysis showed that E-cadherin was

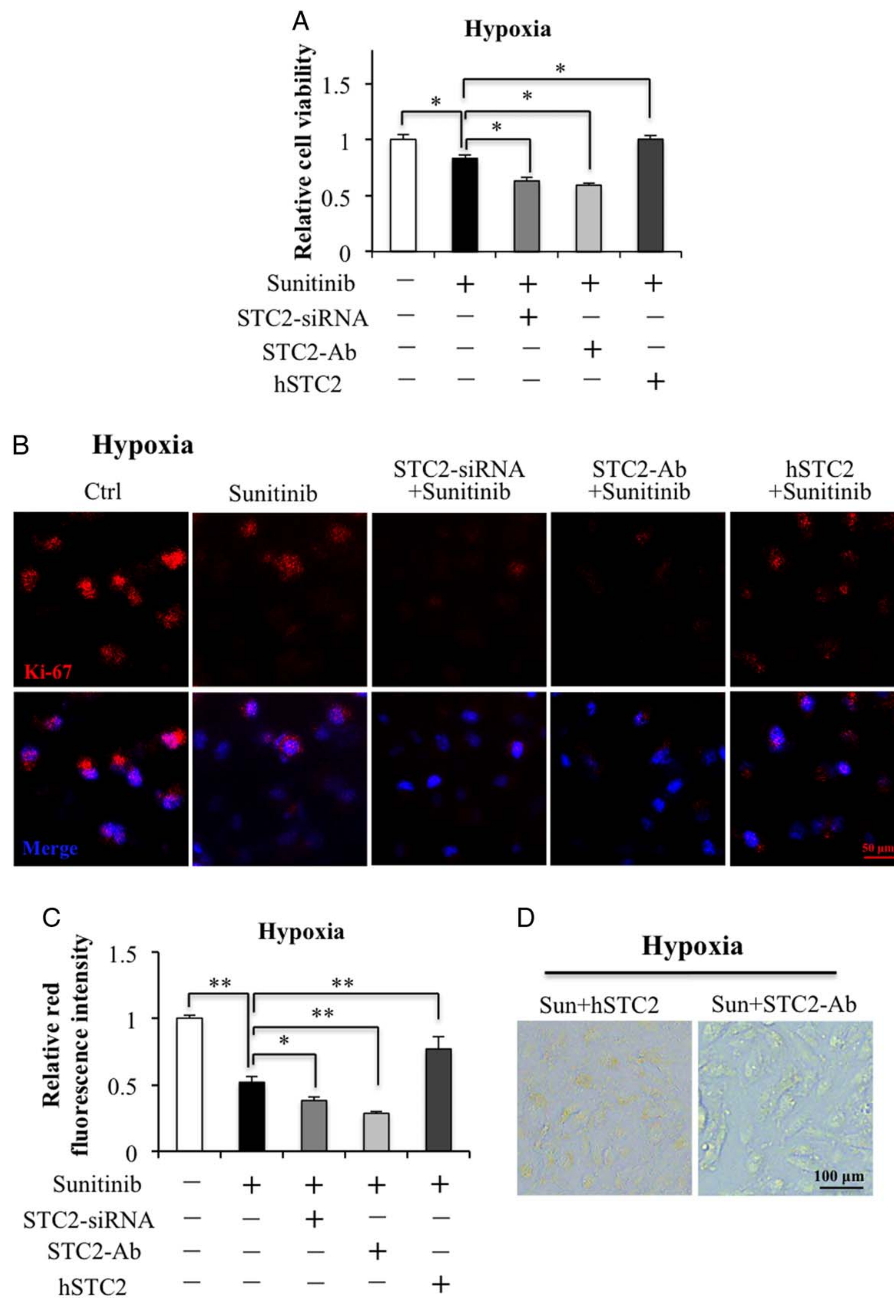

Figure 3. Regulation of STC2 on sunitinib efficacy in Caki-1 cells under hypoxia condition. Determination of cell viability of Caki-1 cells treated with or without 5 μM Sun, 5 μM sunitinib + 0.2 μg/ml STC2-neutralizing antibodies siRNA-STC2 knockdown + 5 μM Sun or 5 μM Sun + 500 ng/ml hSTC2 by MTT (A). The cell proliferation of Caki-1 cells was determined by immunofluorescence staining of proliferation maker Ki-67 and quantified by the software Image J 1.54c (B, C). The cells were incubated with 5 μM Sun + 0.2 μg/ml STC2-neutralizing antibodies, or 5 μM Sun + 500 ng/ml hSTC2 for 24 h, the accumulation of s Sun in Caki-1 cells were observed by phase contrast microscopy (D). * $P < 0.05$, ** $P < 0.01$, indicate significant difference. Sun, sunitinib.

significantly reduced and N-cadherin protein levels were significantly upregulated in Caki-1 cells with sunitinib treatment, which were reversed by STC2-neutralizing antibodies (Fig. 5A, B). In addition, sunitinib treatment increased the migration and invasion of Caki-1 cells, which were suppressed by STC2-neutralizing antibodies (Fig. 5C-F). These results suggested that inhibiting STC2 could reduce sunitinib-induced EMT, migration, and invasion of Caki-1 cells under hypoxia conditions.

Discussion

Like most other solid tumors, RCC also has tissue hypoxia caused by the imbalance of oxygen supply or consumption, which are very important for the occurrence and development of RCC[2,3]. Stanniocalcins (STCs) are hypoxia-inducible endocrine factors and potentially serve as universal tumor biomarkers and therapeutic targets[31,32]. In triple negative breast cancer cells, hypoxia-induced overexpression of STC1 to promote tumor

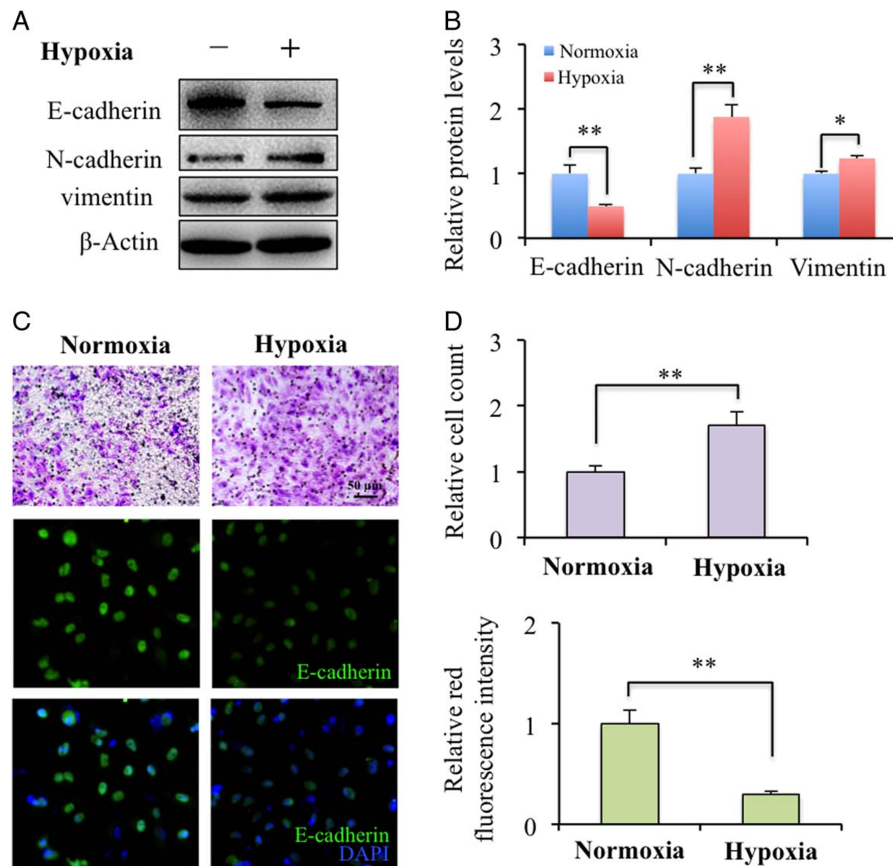

Figure 4. Effect of hypoxia on Caki-1 cells EMT and invasion. (A, B) The protein levels of EMT biomarkers in Caki-1 cells were evaluated by Western blotting. (C, D) The cell invasion of Caki-1 cells was evaluated by transwell. The expressions of E-cadherin in Caki-1 cells were detected by immunofluorescence staining. Green: E-cadherin, Blue: DAPI. Relative protein levels were normalized by β-Actin and relative immunofluorescence intensities were quantified by Image J 1.54c. *, $P < 0.05$; **, $P < 0.01$ indicate significant difference.

progression and metastasis[33]. The STC2 is one of the hypoxic characteristic genes in the immunosuppressive microenvironment of hepatocellular carcinoma to promote tumor evasion[34]. In RCC tissues, high mRNA and protein levels of STC2 were detected, which is associated with the aggressiveness of RCC and shortened overall survival[28,35], and hypoxia mimics $CoCl_2$ treatments induced the expression of STC1 to promote the metastasis of early-stage ccRCC[36]. However, the role and mechanism of hypoxia on the expression of STC2 in RCC remain unclear. In addition, as there are some differences between $CoCl_2$-induced chemical hypoxia and low oxygen-induced hypoxia model, we applied both hypoxia mimics $CoCl_2$ and hypoxia CO_2 incubator to identify the effects of hypoxia on the expression of STC2 in ccRCC cells. Our present study identified that hypoxia-induced STC2 expression could promote RCC cell proliferation, migration and invasion, as well as sunitinib resistance.

STC2 is regulated by hypoxia to promote the adaptation of tumor cells to a hypoxic environment. It reported that STC2 was upregulated to promote cell proliferation of breast cancer cells MCF-7 in hypoxia[37]. In ovarian cancer cell SKOV3, STC2 could induce the production of more ROS under hypoxia and activate the ERK1/2 signaling pathway, thereby improving the ability of tumor cells to resist apoptosis under hypoxic stress[26]. These studies indicated that hypoxia-induced STC2 expression could promote cell proliferation or antiapoptosis. Our results here confirmed that the expression of STC2 was upregulated in Caki-1 under hypoxia conditions, and inhibition of STC2 could reduce cell proliferation of Caki-1 cells.

The elevated expression of STC2 in cervical cancer cells has been reported to significantly increase cisplatin resistance[38]. Both *in vivo* and *in vitro* results indicated that STC1 mediated gemcitabine resistance in pancreatic ductal adenocarcinoma[39]. Our previous study showed that sunitinib treatments reduced the expression of STC2 both mRNA and protein levels, which could reduce sunitinib resistance of Caki-1 cells[30]. All these studies detected the roles of STCs in drug resistance under normoxia but not hypoxia, and the latter one is a real pathophysiological environment of tumors and important for the cancer incidence and development. The present study confirmed that under hypoxia conditions, sunitinib suppressed the expression of STC2. Moreover, the sunitinib efficacy was also improved by inhibiting STC2 using STC2-neutralizing antibodies and STC2-siRNAs, which is meaningful for ccRCC targeting therapy or combination treatment with sunitinib.

In the past decade, available systemic therapies have dramatically increased to treatment of metastatic renal cell carcinoma (mRCC)[40]. The efficacy of sunitinib has been studied in both the nonclear cell renal cell carcinoma (nccRCC) (e.g. papillary renal cell carcinoma, PRCC, and chromophobe renal cell carcinoma, chRCC) and ccRCC[41,42]. The hepatocyte growth factor

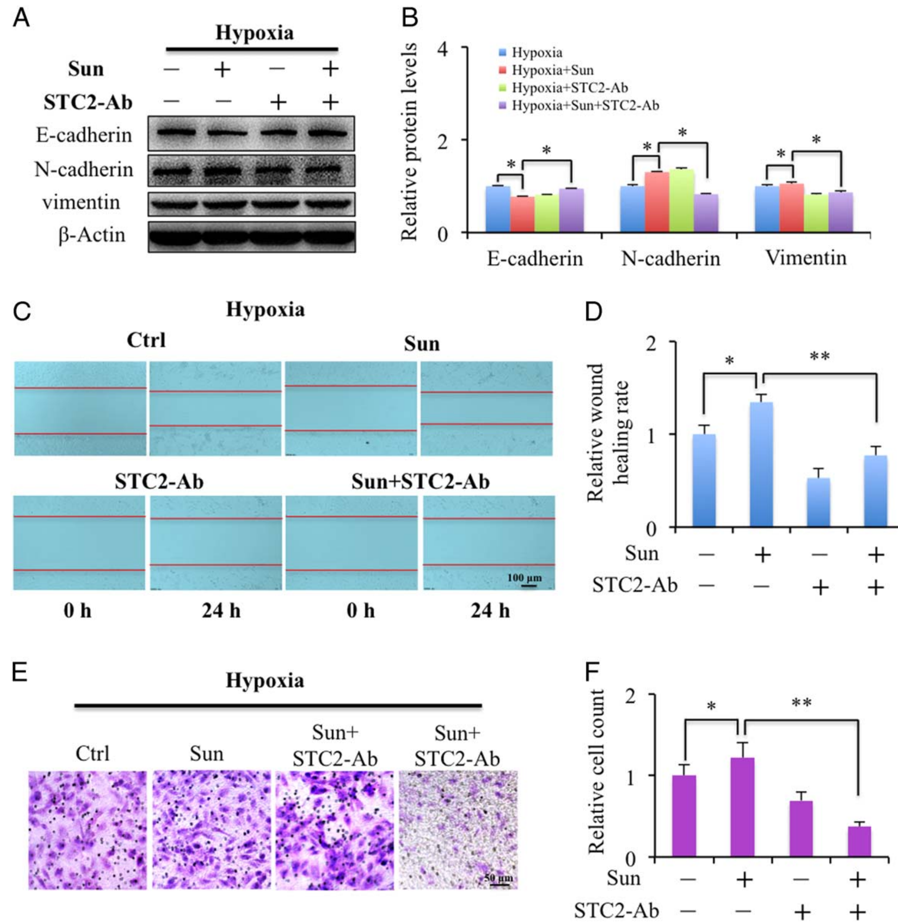

Figure 5. Effects of STC2-neutralizing antibodies on EMT, migration and invasion of Caki-1 cells treated with sunitinib under hypoxia condition. After treated with or without 5 μM Sun, 5 μM Sun + 0.2 μg/ml STC2-neutralizing antibodies for 24 h, the protein levels of EMT biomarkers in Caki-1 cells were evaluated by Western blotting (A), and quantified by Image J (B). The cell migration and invasion of Caki-1 cells were evaluated by wound healing assay and transwell (C-F). *, $P < 0.05$; **, $P < 0.01$ indicate significant difference. Sun, sunitinib.

receptor (MET) kinase inhibitor Cabozantinib treatment could suppress the metastasis of PRCC and resulted in longer progression-free survival than sunitinib in patients with metastatic PRCC[43]. Functions of fatty acid in the regulation of migration and invasion have been studied in PRCC and could be an attractive target for studies of PRCC therapy[44,45]. EMT is a morphologic transformation process of epithelial cells to a mesenchymal phenotype, which promotes tumor cell migration, invasion, and metastasis. Both *in vivo* and *in vitro* studies showed that EMT of ccRCC is acquired to enhance sunitinib resistance, and sunitinib treatment alters the expression of EMT-associated gene or protein and increases migration and invasion of ccRCC cells[8–10]. Our result showed that sunitinib treatment promoted EMT of Caki-1 cells by reducing E-cadherin and increasing N-cadherin and vimentin expression under hypoxia, which might contribute to sunitinib resistance. In ovarian cancer SKOV3 cells, it had suggested that overexpression of STC2 promotes cell EMT under hypoxia by upregulating the expression of N-cadherin, vimentin, and Snail and reducing the E-cadherin expression[26]. In ccRCC cell A498, it proved that hypoxia could increase the expression of STC1 and knockdown STC1 by siRNA reduced EMT by down-regulating the N-cadherin and vimentin, upregulating E-cadherin expression and inhibited the cell migration and invasion[33]. However, the roles of STC2 on the EMT of ccRCC cells under hypoxia conditions have not been identified before. Here, our results firstly showed that STC2-neutralizing antibodies could reduce sunitinib-induced EMT, migration, and invasion under hypoxia.

The role of autophagy in cancer is a hot topic and contention, and the effect of hypoxia on autophagy in cancers is also dependent on cancer types. In cervical cancer cell Hela, hypoxia-activated autophagy accelerates the degradation of SQSTM1/p62[46], while hypoxia inhibits the autophagy and reduces the viability of hepatocellular carcinoma cells[47]. Low loss and mutation of autophagy-related genes (e.g. ATG7) are highly correlated with the ccRCC progression[48]. Our results demonstrated that hypoxia-induced the expression of LC3B and accumulation of P62 in Caki-1 cells, indicating that hypoxia-induced autophagy initiation but inhibited autophagy flux.

The efficiency of sunitinib was enhanced by inhibiting autophagy in clear-cell ovarian carcinoma[49], but sunitinib resistance in ccRCC is induced by promoting autophagy initiation[50]. The inhibition of autophagy flux indicates the loss function of lysosomal degradation, which depends on the lysosomal acidic

environment. Studies have shown that resistance to sunitinib in ccRCC is attributed to lysosomal sequestration and inhibition of autophagy flux[11,12]. Our previous study reported that sunitinib accumulated in the cells and destroyed the lysosome pH[30]. However, STC2-neutralizing antibodies suppressed sunitinib accumulation was independent of restoring the lysosome pH[30]. In the present study, hypoxia promoted the disruption of lysosomal acidity by sunitinib. Moreover, sunitinib accumulation was aggravated by hSTC2 but alleviated by STC2-neutralizing antibodies.

There are still some limitations in the present study, such as no clinic investigation and samples, and the mechanisms of sunitinib-reduced expression of STC2 under hypoxia conditions are still unclear, and the therapeutic effect targeting on STC2 downregulation in the human renal carcinoma mouse xenograft model have not been further verified. Combine the clinical data and mechanism study in cells and animals can provide more information for RCC treatment. Future *in vivo* studies of the biological roles of STC2 and synergistic effect with autophagy regulators will be helpful for potential target STC2 immunotherapy for RCC.

Conclusion

In this study, we present data to identify the roles of STC2 in ccRCC cell sunitinib resistance under hypoxia conditions. Hypoxia-induced expression of STC2 in Caki-1 could promote the process of sunitinib resistance, and inhibiting STC2 may enhance sunitinib efficacy. Furthermore, sunitinib treatment induced EMT, migration, and invasion. This study reveals that sunitinib disrupts lysosomal acidity, which contributes to the increased sunitinib accumulation in ccRCC cells. STC2-neutralizing antibodies suppressed sunitinib accumulation in the cells and aggravated the inhibitory of sunitinib on cell proliferation.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors. All experimental protocols used cell culture in vitro.

Consent

This article does not contain any studies with human participants or animals performed by any of the authors. All experimental protocols used cell culture in vitro.

Sources of funding

This research was funded by the Foundation of Health and Family Planning Com-mission of Wuxi City (Q201807 and MS201812), the Foundation of Clinical Medical Science and Technology Development project of Jiangsu University (JLY20180005 and JLY20180070), the Foundation of Science and Technology Development Medical and Health Guidance Plan Project of Wuxi City (2020061), and Innovation Foundation of Science and Technology Bureau of Yixing City (2020SF08).

Author contribution

J.G., Z.Q., and Y.X.: conceptualization; J.G., Z.Q., and H.C.: methodology; H.C.: software; H.C., W.X., and C.G.: validation; H.C. and C.G.: formal analysis; H.C. and C.G.: investigation; J.G., Z.Q., and Y.X.: resources; H.C.: data curation; Y.X.: original draft preparation; J.G., H.S., Z.Q., and Y.X.: review and editing; Z.Q., and Y.X.: visualization; Z.Q. and Y.X.: supervision; J.G., Z.Q., and Y.X.: project administration; Z.Q. and Y. X.: funding acquisition. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest disclosure

The authors declare no conflict of interest.

Research registration unique identifying number (UIN)

JGu-683.

References

[1] Cohen HT, McGovern FJ. Renal-cell carcinoma. N Engl J Med 2005;353: 2477–90.
[2] Hsieh JJ, Purdue MP, Signoretti S, *et al*. Renal cell carcinoma. Nat Rev Dis Primers 2017;3:17009.
[3] Rini BI, Campbell SC, Escudier B. Renal cell carcinoma. Lancet 2009; 373:1119–32.
[4] Hasan HAA, Youssef A, Fayad S, *et al*. A rare case of collecting duct carcinoma with first presentation of respiratory symptoms. Urol Case Rep 2020;33:101367.
[5] Ornstein MC, Wood LS, Elson P, *et al*. A phase II study of intermittent sunitinib in previously untreated patients with metastatic renal cell carcinoma. J Clin Oncol 2017;35:1764–9.
[6] Venkatesan P. Intermittent sunitinib for metastatic renal cell carcinoma. Lancet Oncol 2017;18:e139.
[7] Rausch M, Rutz A, Allard PM, *et al*. Molecular and functional analysis of sunitinib-resistance induction in human renal cell carcinoma cells. Int J Mol Sci 2021;22:6467.
[8] Hammers HJ, Verheul HM, Salumbides B, *et al*. Reversible epithelial to mesenchymal transition and acquired resistance to sunitinib in patients with renal cell carcinoma: evidence from a xenograft study. Mol Cancer Ther 2010;9:1525–35.
[9] Hwang HS, Go H, Park JM, *et al*. Epithelial-mesenchymal transition as a mechanism of resistance to tyrosine kinase inhibitors in clear cell renal cell carcinoma. Lab Invest 2019;99:659–70.
[10] Zhou L, Liu XD, Sun M, *et al*. Targeting MET and AXL overcomes resistance to sunitinib therapy in renal cell carcinoma. Oncogene 2016; 35:2687–97.
[11] Olson OC, Joyce JA. Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. Nat Rev Cancer 2015;15:712–29.
[12] Gotink KJ, Broxterman HJ, Labots M, *et al*. Lysosomal sequestration of sunitinib: a novel mechanism of drug resistance. Clin Cancer Res 2011; 17:7337–46.
[13] Giuliano S, Cormerais Y, Dufies M, *et al*. Resistance to sunitinib in renal clear cell carcinoma results from sequestration in lysosomes and inhibition of the autophagic flux. Autophagy 2015;11:1891–904.
[14] Li L, Zhao S, Liu Z, *et al*. Sunitinib treatment promotes metastasis of drug-resistant renal cell carcinoma via TFE3 signaling pathway. Cell Death Dis 2021;12:220.
[15] Lodhi T, Song YP, West C, *et al*. Hypoxia and its modification in bladder cancer: current and future perspectives. Clin Oncol 2021;33:376–90.
[16] Hasan YMA, Basiony M, Hanbazazh M, *et al*. Clinico-pathological features and immunohistochemical comparison of p16, p53, and Ki-67 expression in muscle-invasive and non-muscle-invasive conventional urothelial bladder carcinoma. Clin Pract 2023;13:806–19.

[17] Myszczyszyn AMCA, Matak D, Szymanski L, *et al*. The role of hypoxia and cancer stem cells in renal cell carcinoma pathogenesis. Stem Cell Rev Rep 2015;11:919–43.

[18] Schodel J, Grampp S, Maher ER, *et al*. Hypoxia, hypoxia-inducible transcription factors, and renal cancer. Eur Urol 2016;69:646–57.

[19] Xie Y, Shangguan W, Chen Z, *et al*. Establishment of sunitinib-resistant xenograft model of renal cell carcinoma and the identification of drug-resistant hub genes and pathways. Drug Des Devel Ther 2021;15: 5061–74.

[20] Gilkes DM, Semenza GL, Wirtz D. Hypoxia and the extracellular matrix: drivers of tumour metastasis. Nat Rev Cancer 2014;14:430–9.

[21] Berkers J, Govaere O, Wolter P, *et al*. A possible role for microRNA-141 down-regulation in sunitinib resistant metastatic clear cell renal cell carcinoma through induction of epithelial-to-mesenchymal transition and hypoxia resistance. J Urol 2013;189:1930–8.

[22] Garcia-Donas J, Leandro-Garcia LJ, Alba AGD, *et al*. Prospective study assessing hypoxia-related proteins as markers for the outcome of treatment with sunitinib in advanced clear-cell renal cell carcinoma. Ann Oncol 2013;24:2409–14.

[23] Kanellis J, Bick R, Garcia G, *et al*. Stanniocalcin-1, an inhibitor of macrophage chemotaxis and chemokinesis. Am J Physiol Renal Physiol 2004; 286:F356–62.

[24] Yeung BH, Law AY, Wong CK. Evolution and roles of stanniocalcin. Mol Cell Endocrinol 2012;349:272–80.

[25] Chu SJ, Zhang J, Zhang R, *et al*. Evolution and functions of stanniocalcins in cancer. Int J Immunopathol Pharmacol 2015;28:14–20.

[26] Law AY, Wong CK. Stanniocalcin-2 promotes epithelial-mesenchymal transition and invasiveness in hypoxic human ovarian cancer cells. Exp Cell Res 2010;316:3425–34.

[27] Na SS, Aldonza MB, Sung HJ, *et al*. Stanniocalcin-2 (STC2): a potential lung cancer biomarker promotes lung cancer metastasis and progression. Biochim Biophys Acta 2015;1854:668–76.

[28] Meyer HA, Tolle A, Jung M, *et al*. Identification of stanniocalcin 2 as prognostic marker in renal cell carcinoma. Eur Urol 2009;55:669–78.

[29] Girgis AH, Iakovlev VV, Beheshti B, *et al*. Multilevel whole-genome analysis reveals candidate biomarkers in clear cell renal cell carcinoma. Cancer Res 2012;72:5273–84.

[30] Qin ZQ, Li KD, Chu HZ, *et al*. Blocking stanniocalcin 2 reduces sunitinib resistance in clear cell renal cell carcinoma. Neoplasma 2022;69:145–54.

[31] Yeung HY, Lai KP, Chan HY, *et al*. Hypoxia-inducible factor-1-mediated activation of stanniocalcin-1 in human cancer cells. Endocrinology 2005; 146:4951–60.

[32] Qie S, Sang N. Stanniocalcin 2 (STC2): a universal tumour biomarker and a potential therapeutical target. J Exp Clin Cancer Res 2022;41:161.

[33] Zandberga E, Zayakin P, Ābols A, *et al*. Depletion of carbonic anhydrase IX abrogates hypoxia-induced overexpression of stanniocalcin-1 in triple negative breast cancer cells. Cancer Biol Ther 2017;18:596–605.

[34] Mo ZM, Liu DY, Rong DD, *et al*. Hypoxic characteristic in the immunosuppressive microenvironment of hepatocellular carcinoma. Front Immunol 2021;12:611058.

[35] Dondeti VR, Wubbenhorst B, Lal P, *et al*. Integrative genomic analyses of sporadic clear cell renal cell carcinoma define disease subtypes and potential new therapeutic targets. Cancer Res 2012;72: 112–21.

[36] Ma X, Gu LY, Li HZ, *et al*. Hypoxia-induced overexpression of stanniocalcin-1 is associated with the metastasis of early stage clear cell renal cell carcinoma. J Transl Med 2015;13:56.

[37] Low AYS, Wong CKC. Stanniocalcin-2 is a HIF-1 target gene that promotes cell proliferation in hypoxia. Experimental Cell Research 2010; 316:466–76.

[38] Wang YX, Gao Y, Cheng HR, *et al*. Stanniocalcin 2 promotes cell proliferation and cisplatin resistance in cervical cancer. Biochem Bioph Res Co 2015;466:362–8.

[39] Zhao FY, Yang G, Qiu JD, *et al*. HIF-1α-regulated stanniocalcin-1 mediates gemcitabine resistance in pancreatic ductal adenocarcinoma via PI3K/AKT signaling pathway. Mol Carcinog 2022;61:839–50.

[40] Choueiri TK, Motzer RJ. Systemic therapy for metastatic renal-cell carcinoma. N Engl J Med 2017;376:354–66.

[41] Keizman SDD, Lee JL, Sella A, *et al*. Outcome of patients with metastatic chromophobe renal cell carcinoma treated with sunitinib. Oncologist 2016;21:1212–7.

[42] Shi TJHZ, Li CL. Safety and efficacy of sunitinib for advanced non-clear cell renal cell carcinoma. Asia Pac J Clin Oncol 2015;11:328–33.

[43] Pal TCSK, Thompson IMJ, Balzer-Haas N, *et al*. A comparison of sunitinib with cabozantinib, crizotinib, and savolitinib for treatment of advanced papillary renal cell carcinoma: a randomised, open-label, phase 2 trial. Lancet 2021;397:695–703.

[44] Karmokar MNPF. Free-Fatty Acid Receptor-4 (FFA4/GPR120) differentially regulates migration, invasion, proliferation and tumor growth of papillary renal cell carcinoma cells. Biochem Pharmacol 2023;213: 115590.

[45] Karmokar MNPF. Free-fatty acid receptor-1 (FFA1/GPR40) promotes papillary RCC proliferation and tumor growth via Src/PI3K/AKT/NF-κB but suppresses migration by inhibition of EGFR, ERK1/2, STAT3 and EMT. Cancer Cell Int 2023;23:126.

[46] Pursiheimo JP, Rantanen K, Heikkinen PT, *et al*. Hypoxia-activated autophagy accelerates degradation of SQSTM1/p62. Oncogene 2009;28: 334–44.

[47] Chang Y, Yan W, He XX, *et al*. miR-375 inhibits autophagy and reduces viability of hepatocellular carcinoma cells under hypoxic conditions. Gastroenterology 2012;143:177–U357.

[48] Liu XD, Tripathi DN, Ding Z, *et al*. Autophagy mediates HIF2α degradation and suppresses renal tumorigenesis. Oncogene 2015;34:2450–60.

[49] DeVorkin L, Hattersley M, Kim P, *et al*. Autophagy inhibition enhances sunitinib efficacy in clear cell ovarian carcinoma. Mol Cancer Res 2017; 15:250–8.

[50] Zhu LS, Ding R, Yan H, *et al*. ZHX2 drives cell growth and migration via activating MEK/ERK signal and induces Sunitinib resistance by regulating the autophagy in clear cell renal cell carcinoma. Cell Death Dis 2021; 12:399.