doi:10.4149/neo_2021_210823N1206

Blocking stanniocalcin 2 reduces sunitinib resistance in clear cell renal cell carcinoma

Zhen-Qian QIN[1], Kong-Dong LI[2], He-Zhen CHU[3], Xue-Feng YUAN[1], Hai-Feng SHI[2], Jie GU[2,*], Yi-Min XIE[1,*]

[1]Affiliated Hospital of Jiangsu University-Yixing Hospital, Yixing, Jiangsu, China; [2]School of Life Sciences, Jiangsu University, Zhenjiang, Jiangsu, China; [3]Affiliated Hospital of Yangzhou University-Yixing Traditional Chinese Medicine Hospital, Jiangsu, China

**Correspondence: 1000004531@ujs.edu.cn; 308947685@qq.com*

Received August 23, 2021 / Accepted November 8, 2021

Stanniocalcin 2 (STC2) has been identified as a prognostic marker in renal cell carcinoma. However, the role of STC2 in renal cell carcinoma is still unclear. In this study, we investigated the relationship between high expression of STC2 and sunitinib resistance in cells and the underlying mechanism. Through GEPIA platform analysis based on TCGA database, it showed that the expression of STC2 in kidney renal clear cell carcinoma (KIRC) was significantly higher than that in the normal population. Real-time quantitative PCR and western blotting detected significantly higher expression levels of STC2 in clear cell renal cell carcinoma (ccRCC) cells than that in normal renal cells. Enzyme-linked immunosorbent assay (ELISA) determined whether there is a high secretion of STC2 in ccRCC cells. The sunitinib resistance could be significantly reduced by STC2 neutralizing antibody but aggravated by the addition of recombinant human STC2 in ccRCC cells. Sunitinib suppressed STC2 expression and secretion, destroyed lysosomal acidic pH, and accumulated in the cells. However, STC2 neutralizing antibody can reduce the accumulation of sunitinib in cells to improve the inhibitory efficiency of sunitinib on cell proliferation. This study suggested STC2 could serve as a potential novel target for the treatment of ccRCC, anti-STC2 antibody might be an option of immunotherapy in the future.

Key words: stanniocalcin 2, clear cell renal cell carcinoma, sunitinib, drug resistance

Renal cell carcinoma (RCC) accounts for about 85% of the total renal cancer, originates from renal tubular cell carcinogenesis, and ranks the second in urinary malignancy [1–3]. Clear cell renal cell carcinoma (ccRCC) is the most common pathological type of RCC, accounting for 75% of all cases, and is the leading cause of cancer-related death [1–3]. However, its molecular pathogenesis is incompletely understood. To identify the underlying mechanism, some genetic studies such as whole-genome and/or whole-exome and RNA sequencing as well as array-based gene expression, copy number, and/or methylation analyses integrated the bioinformatics analysis such as gene set enrichment analysis (GSEA) and weighted gene co-expression network analysis (WGCNA) have been performed. Through these studies, some key pathways i.e., PI3K-AKT-mTOR signaling and p53-related pathways, and some candidate genes were screened [4]. However, their molecular mechanism is still unclear.

By multilevel whole-genome analysis and a meta-analysis, stanniocalcin 2 (STC2) was identified as a biomarker of ccRCC [5, 6]. STCs (STC1 and STC2) are involved in various physiological functions of the body in paracrine and autocrine ways. They not only regulate calcium and phosphorus metabolism, but also play an important role in cardiovascular diseases, inflammatory cell migration, embryo implantation, and decidualization [7–11]. In recent years, the role of STCs in tumor development has attracted increasing attention [12]. Among them, STC2 is overexpressed in various tumor tissues, such as renal cell carcinoma [5, 13], breast cancer [14, 15], liver cancer [16–18], gastric cancer [19], colorectal cancer [20], and prostate cancer [21]. However, the role of STC2 on the occurrence, development, and treatment of renal carcinoma is still unclear.

The causes of ccRCC are not fully understood, so the treatment options available are limited. Overall response rates remain unsatisfactory due to the drug resistance of tumors to chemotherapy and radiotherapy and the limitations of targeted therapy provided. In addition, some patients did not respond to targeted treatment or recurred after initial improvement. Therefore, revealing the resistance mechanism is needed, especially those widely used multi-targeted tyrosine kinase inhibitors (TKI). Furthermore, the advances

in mechanisms of resistance provided the basis for combining targeted therapies with newer generation immunotherapies to gain better therapeutic outcomes [22, 23].

Sunitinib (SUTENT), one of the TKIs, has been licensed for ccRCC treatment because of its good target therapeutic effect [24, 25]. However, its application has been overshadowed by the emergence of drug resistance. Previous studies have revealed that sunitinib is sequestered in lysosomes as a novel mechanism of drug resistance of ccRCC [26, 27]. Therefore, in this study, the relationship between STC2 expression and sunitinib resistance in ccRCC cells was investigated. STC2 neutralizing antibody was used to explore the roles of blocking STC2 in sunitinib resistance of ccRCC cells, so as to provide an experimental basis for comprehensive treatment 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). TUNEL kit was purchased from KeyGEN (KGA7073, Jiangsu, China). Anti-human Ki-67 was purchased from Cell Signaling Technology (9449S, Shanghai, China), LysoTracker™ Green DND-26 (L7526), and anti-human STC2 antibodies (PA5-34841) were purchased from Thermo Fisher Scientific (Shanghai, China). Human STC2 neutralization antibody (AF2830), recombinant human STC2 (O76061), and STC2 ELISA Kit (CSB-EL022822HU) were purchased from R&D system (USA).

Cell culture. HK-2, Caki-1, 786-O, 769-P, and ACHN cells obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) were grown at 37 °C in McCoy's 5A (for Caki-1) and RPMI 1640 (for HK-2, 786-O, 769-P, and ACHN) medium, respectively (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 a humidified atmosphere containing 5% CO_2. RPMI 1640 medium, FBS, penicillin, and streptomycin were purchased from Gibco (Thermo Fisher Scientific, USA).

MTT assays. DU145 and PC-3 cells were seeded in 6-well and transfected with vector control, FAM66C siRNA-2, and siRNA-3 for 24 h. Cells were then collected and seeded in 96-well with a density of 2 x 10^3 cells/well. After culturing for 1, 2, 3, or 4 d, cell growth was measured by the MTT assay. In brief, cells were incubated in 100 µl MTT solution (0.5 mg/ml in MEM medium) in a 96-well plate for 4 h before the end of incubation. The supernatant was then discarded, and 100 µl DMSO was added to dissolve the colored product (formazan). The absorbance was measured at 540 nm (690 nm as reference) using a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, USA). In some experiments, STC2 neutralizing antibody (0.2 µg/ml) and human recombinant STC2 (500 ng/ml) were added into the culture medium with sunitinib (5 µM).

Real-time quantitative PCR (RT-qPCR) analysis. The cell total RNA was isolated by using TRIzol (Vazyme, Nanjing, China). All RNA isolations were performed as directed by the manufacturer. Gel electrophoresis and staining confirmed the purity and integrity of the samples. Quantification of RNA was based on spectrophotometric analysis at 260/280 nm. cDNA was made from 10 µg total RNA using an RNA-to-cDNA kit (Vazyme, Nanjing, China). Real-time PCRs were carried out by Quantstudio 3 (Thermo Fisher Scientific, USA) using the SYBR Green I kit (Vazyme, Nanjing, China). Gene-specific primers for STC2-F: GGTGGACAGAACCAAGCTCTC, STC2-R: CGTTTGGGTGGCTCTTGCTA, and β-Actin-F: GACTACCTCATGAAGATCCTCACC, β-Actin-R: TCTCCTTAATGTCACGCACGATT were used. The occurrence of primer-dimers and secondary products was inspected using melting curve analysis and agarose gel electrophoresis. Control amplification was done either without reverse transcriptase or without RNA. The relative expression ratio of the target gene was calculated according to their threshold cycle Ct values.

Western blotting analysis. The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). After centrifugation at 13,000×g for 15 min at 4 °C, the supernatant was collected and the total protein concentration was determined by BCA (Thermo Fisher Scientific, USA). The protein lysates containing 30 µg total cellular protein in RIPA buffer were subjected to electrophoresis on 10% polyacrylamide gels. The gels were then blotted onto PVDF membranes (Merck, Shanghai, China). Western blotting was conducted using rabbit polyclonal antibodies against STC2 (PA5-34841, Thermo Fisher Scientific Shanghai, China) and β-Actin (BM3883, Boster, Wuhan China), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:4000) (7074S, Cell Signaling Technology, Shanghai, China). Specific bands were visualized using a chemiluminescent reagent (Vazyme, Nanjing, China).

Measurement of STC2 content in culture medium. Caki-1 cells were treated with sunitinib (0, 1, 5, 10 µM) for 24 h, and then the culture mediums were collected for STC2 content detection. The STC2 levels were measured using an STC2 ELISA Kit according to the manufacturer's instructions (R&D system, USA). The OD was measured with Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, USA) at 450 nm.

Knockdown of STC2. Caki-1 cells were seeded in a 6-well plate with a density of 2×10^5 cells/well and cultured overnight. Then the cells were transfected with STC2 siRNAs packaged by Lipo2000 reagent according to manufacturer instructions (Thermo Fisher Scientific, USA). At 24 hours after transfection, the cells were harvested for further evaluation. The siRNAs used in this study were STC2 siRNA-1: sense 5′-GUGGAGAUGAUCCAUUUCATT-3′ and

antisense 5′-UGAAAUGGAUCAUCUCCACTT-3′; STC2 siRNA-2: sense 5′-CAGGGCAAGUCAUUCAUCATT-3′ and antisense 5′-UGAUGAAUGACUUGCCCUGTT-3′; STC2 siRNA-3: sense 5′-GCGUGUUUGAAUGUUUCGATT-3′; and antisense 5′-UCGAAACAUUCAAACACGCTT-3′; from GenePharm (Shanghai, China).

Lysosomal acidity detection. Acridine Orange (AO), a fluorochrome, emits red fluorescence in cell acidic compartments such as lysosomes or autophagolysosomes and green fluorescence in the cytosol and the nucleus. The ratio between the red and green signal of AO indicates the acidic characteristics of lysosomes. Caki-1 cells were treated with sunitinib (0, 1, 5, 10 μM) for 24 h. After washing with PBS twice and AO stain buffer once, the cells were stained by AO (1 mM) for 15 min at room temperature, and then the fluorescence micrographs were obtained by Olympus BX53 (Japan). Quantification of the ratio between the red and green signal of AO was performed by using the software Image J.

LysoTracker™ Green DND-26 is a green fluorescent probe, which can indicate the acid environment in the lysosome. Caki-1 cells were treated with sunitinib (0, 1, 5, 10 μM) for 24 h. After washing with PBS twice, the cells were stained by LysoTracker™ Green DND-26 (50 nM) in a CO_2 incubator for 15 min at 37 °C. Fluorescent intensity was recorded by excitation at 488 nm and emission at 525 nm using a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, USA).

Immunofluorescence and fluorometric cell death detection. After treating with sunitinib (10 μM) for 24 h, the Caki-1 cells were prepared for proliferation and apoptosis detection. The cells were fixed for 30 min in 4% formaldehyde (Sangon Biotech, Shanghai, China) and permeabilized with 0.1% Triton X-100 (Sangon Biotech, Shanghai, China) in PBS for 20 min. After blocking with 3% normal goat serum, the cells were incubated with mouse anti-Ki-67 (9449S, proliferation marker, 1:100, Cell Signaling Technology) antibody overnight at 4 °C, followed by 1 h incubation with Alexa Fluor 488 goat anti-mouse IgG (1:200, A-11001, Thermo Fisher, Shanghai, China). After washing twice with PBS, the cell nuclei were stained by the DAPI (D1306, Thermo Fisher Scientific, USA) for several minutes. The cells were washed in PBS for 10 min and investigated by Olympus IX73 microscope (Japan). Fluorometric cell death was detected by terminal deoxynucleotidyl transferase (dUTP) nick end labeling (TUNEL) assay according to the manufacturer's instructions and counterstained with DAPI as previously described. Then the images were taken by an Olympus IX73 microscope (Japan).

Statistical analysis. Drug treatments were performed in triplicate in each experiment and every experiment was repeated at least three times. All data are represented as means ± S.D. Statistical significance was assessed with Student's t-test or one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Groups were considered significantly different if $p<0.05$ and extremely significantly different if $p<0.01$.

Results

High expression of STC2 in clear cell renal cell carcinoma cells. Differential analysis was performed on TCGA database for kidney renal clear cell carcinoma (TCGA-KIRC) using the GEPIA analysis platform (http://gepia.cancer-pku.cn/). Log2 (TPM+1) was used as the logarithmic range. Jitter Size: 0.4; matching with TCGA GTEx and normal data, |Log2FC| Cutoff = 1, p-value Cutoff = 0.01. The results showed that the expression of STC2 in the tumor tissues of patients with human renal clear cell carcinoma was significantly higher than that of the normal population (Figure 1A). The results of RT-PCR and western blotting showed that the expression levels of STC2 in Caki-1, 769-P, 786-O, and ACHN ccRCC cells were significantly higher than that in human renal tubular epithelial cells HK-2 (Figures 1B, 1C). In addition, it shows that there is no significant difference in STC1 expression between tumor patients vs. normal and HK-2 cells vs. Caki-1, 769-P, 786-O, and ACHN ccRCC cells (Supplementary Figures S1A, S1B). As a secreted protein, the result of ELISA showed that the STC2 contents in the culture medium of Caki-1, 769-P, 786-O, and ACHN ccRCC cells were significantly higher than that in HK-2 cells (Figure 1D).

Sunitinib suppressed the growth of human renal clear cell cancer cells. MTT assay showed that sunitinib treatment could significantly inhibit the cell viability of human renal clear cell carcinoma cells Caki-1, 786-O, and 769-P after 2 days. CaKi-1 cells were most sensitive to sunitinib at 5 μM, while 786-O and 796-P cells showed a dose-dependent sensitivity (Figure 2A). After 2 and 3 days of continuous treatment of sunitinib (5 μM), the cell viabilities of the treatment groups were significantly lower than that of the control group (Figures 2B–2D). Compared with 786-O and 796-P cells, Caki-1 cells showed more resistance to sunitinib (Figures 2A–2D). Moreover, Caki-1 cells showed more resistance to high dose sunitinib treatment (10 μM) (Figure 2A).

STCs regulate various biological functions as paracrine/autocrine factors. To determine whether is secreted STC2 involved in sunitinib resistance of ccRCC cells, STC2 neutralizing antibody and human recombinant STC2 (hSTC2) were applied to the culture medium. The results of the MTT assay showed that neutralizing antibody significantly increased the sensitivity of Caki-1, 786-O, and 769-P cells to sunitinib treatment (Figures 3A–3C). Knockdown of STC-2 by siRNA could promote the sensitivity of Caki-1 cells to sunitinib (Supplementary Figures S2A, S2B). The addition of hSTC2 could significantly increase sunitinib resistance of Caki-1, 786-O, and 769-P cells (Figures 3D–3F). The results suggested that sunitinib resistance of ccRCC cells could be increased by secreting STC2 but reduced by blocking STC2.

Effects of sunitinib on STC2 expression. To clarify whether sunitinib affects the expression of STC2 in ccRCC cells, the expression and secretion of STC2 were examined after treatment with sunitinib. The results and western blotting showed that sunitinib treatment could induce the

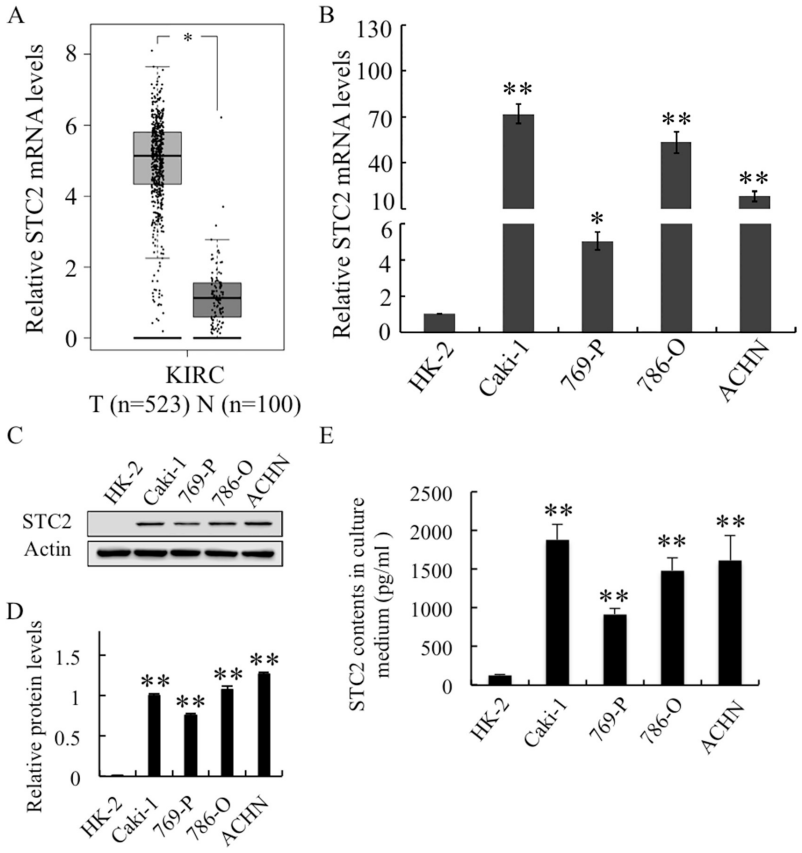

Figure 1. Expression pattern of STC2 in KIRC specimens and ccRCC cell lines. A) GEPIA analysis shows the mRNA expression levels of STC2 in 523 KIRCs and 100 non-KIRCs. B, C) The relative expression levels of STC2 in HK-2 cells and ccRCC cell lines were evaluated by RT-qPCR and western blotting. β-actin was used as a loading reference. D) The STC2 contents in the culture medium of HK-2 cells and ccRCC cell lines were detected by ELISA. *p<0.05, **p<0.01

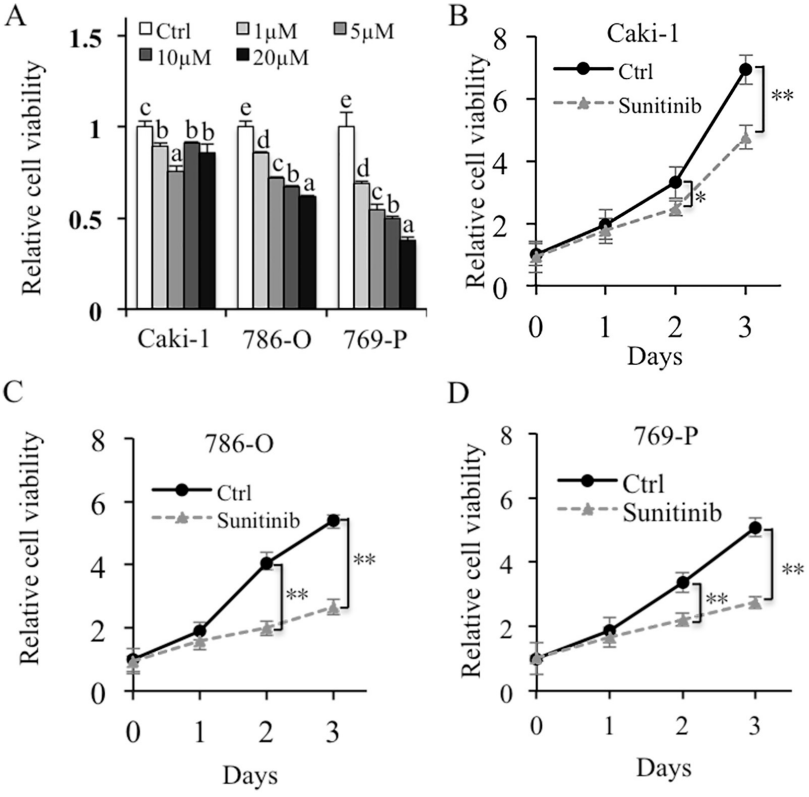

Figure 2. Effect of sunitinib on ccRCC cells growth. A) The relative cell viability of Caki-1, 786-O, and 769-P cells in the absence (Ctrl) or presence of increasing concentrations of sunitinib (1, 5, 10, 20 μM) was evaluated by the MTT assay after treatment for 2 d. Different letters indicate a significant difference between treatments. B–D) The relative cell viability of Caki-1, 786-O, and 769-P cells in the absence (Ctrl) or presence of sunitinib (5 μM) was evaluated by the MTT assay after treated for 1, 2, and 3 d. *p<0.05; **p<0.01

STC2 expression (Figures 4A–4C). However, sunitinib treatment did not affect the expression of STC1 (Supplementary Figure S1C). Therefore, STC2 but not STC1 contributed to the sunitinib treatment. The result of ELISA showed that STC2 secretion in the medium was reduced by sunitinib treatments. However, the STC2 content in the culture medium of high dose of sunitinib group (10 μM) was higher than low dose group (5 μM) (Figure 4D). This result indicated that Caki-1 cells were more resistant to high dose sunitinib might due to the high content of STC2 in the culture medium.

Sunitinib disrupted the lysosomal acidic environment. Lysosomal sequestrations of sunitinib acted as a mechanism of drug resistance of ccRCC. Phase-contrast microscopy revealed more yellow color particles after treatment with a higher dose of sunitinib for 2 d. It suggested that sunitinib was captured and accumulated in the cells (Figure 5A). To detect whether sunitinib treatment disrupts the acidic environment of the lysosome, AO and LysoTracker™ Green DND-26 stainings were performed. AO staining was used as an indicator of lysosomal pH. Compared with control, sunitinib treatments reduced the red fluorescence and increased the ratio of green/red fluorescence (Figures 5B, 5C). In addition, LysoTracker™ Green DND-26 staining showed that sunitinib treatments significantly increased the green fluorescence (Figure 5D). These results indicated that sunitinib accumulated in the cells and disrupted the acidic environment of lysosomes.

STC2 neutralizing antibody alleviates sunitinib resistance by improving the inhibitory efficiency of sunitinib on cell proliferation. To investigate the mechanism of STC2 neutralizing antibody alleviating sunitinib resistance, the accumulation of sunitinib in Caki-1 cells was observed. Phase-contrast microscopy showed fewer yellow color particles in co-treatment sunitinib+STC2 neutralizing antibody compared with sunitinib treatment alone (Figure 6A). However, the AO and LysoTracker™ Green DND-26 staining assay showed that the sunitinib-disrupted acidic environment of the lysosomes could not be restored by STC2 neutralizing antibody (Figures 6B-6D). These results suggested that STC2 neutralizing antibody alleviated sunitinib accumulation independent of restoring lysosomal acidity.

To further identify the underlying mechanism, cell apoptosis and proliferation were detected. The Ki-67 staining showed that sunitinib inhibited cell proliferation, which was aggravated by the STC2 neutralizing antibody. The results of TUNEL staining showed that neither sunitinib nor STC2 neutralizing antibody induced cell apoptosis (Figures 6E, 6F). These results indicated that STC2 neutralizing antibody could reduce the accumulation of sunitinib in cells to improve the inhibitory efficiency of sunitinib on cell proliferation.

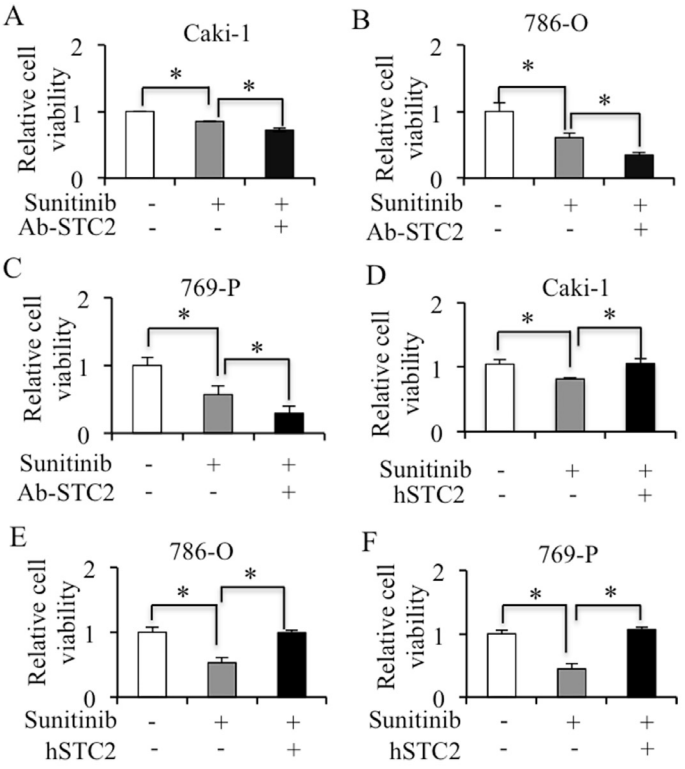

Figure 3. Effect of STC2 on sunitinib resistance in ccRCC cells. A–C) Determination of cell viability of Caki-1, 786-O, and 769-P cells in the absence (Ctrl) or presence of 5 μM sunitinib or 5 μM sunitinib+0.2 μg/ml STC2 neutralizing antibody. D–F) Determination of cell viability of Caki-1, 786-O, and 769-P cells in the absence (Ctrl) or presence of 5 μM sunitinib or 5 μM sunitinib+500 ng/ml hSTC2. *p<0.05

Discussion

Effective treatment of renal clear cell carcinoma has been a major issue. As a multi-target inhibitor of receptor tyrosine kinase, sunitinib has both anti-tumor and anti-angiogenic effects and is more effective than conventional therapies (radiotherapy or chemotherapy). It has been approved and marketed in many countries, and has been widely used in the treatment of renal cell carcinoma, and has entered clinical stage II and III [24, 25]. However, there is also the phenomenon of drug resistance, and its specific influencing factors and mechanisms are receiving close attention.

The elevated expression of STC2 has been reported in a variety of cancers, in which it promotes tumor cell growth, invasion, and migration, as well as drug resistance. Cheng et al. detected high expression of STC2 in liver cancer tissues. STC2 overexpressing cell lines HepG2 and SMMC7721 were more resistant to paclitaxel [18]. The study of Wang et al. showed that STC2 expression was significantly upregulated in cervical cancer tissues and cell lines. Moreover, the STC2 expression level was significantly increased in cisplatin-resistant cervical cancer cells [28]. Overexpression of STC2 was also detected in castration-resistant prostate cancer and

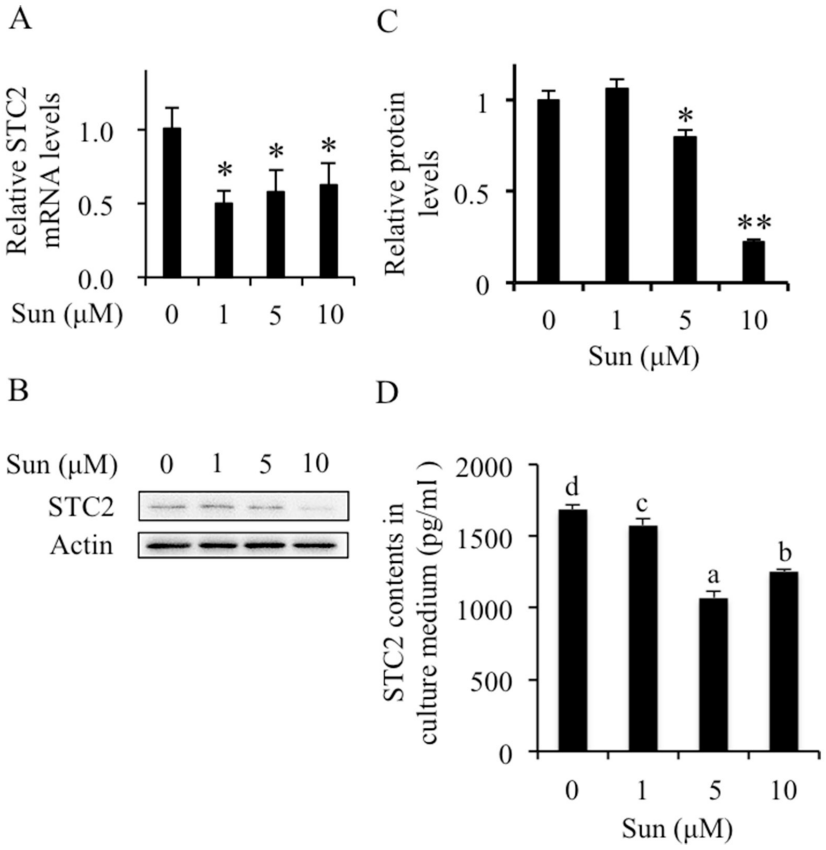

Figure 4. Effects of sunitinib on STC2 expression and secretion in Caki-1 cells. A) The mRNA expression levels of STC2 in Caki-1, 786-O, and 769-P cells in the absence (0) or presence of increasing concentrations of sunitinib (1, 5, 10 μM) were determined by RT-qPCR and normalized by β-actin. B, C) The protein levels of STC2 in Caki-1 cell lines were evaluated by western blotting after being treated with sunitinib (1, 5, 10 μM). β-actin was used as a loading reference. Relative protein levels were analyzed by ImageJ. D) The STC2 contents in the culture medium of Caki-1 cells treated with sunitinib (1, 5, 10 μM) were detected by ELISA. *p<0.05, **p<0.01

aggressive prostate cancer [21]. These results suggest that high expression of STC2 may enhance tumor resistance. GEPIA analysis based on TCGA database for kidney renal clear cell carcinoma (TCGA-KIRC) showed that the STC2 expression was significantly higher in the tumor tissues of patients with ccRCC than in the normal population. Our results here also showed that the expression levels of STC2 in ccRCC cells and STC2 contents in the culture medium were significantly higher than that in normal renal cells. Interestingly, sunitinib treatments reduced the expression of STC2 on both mRNA and protein levels. Although the secretion of STC2 was suppressed by the sunitinib treatments, there was unexpectedly a little increase by high dose compared with low dose treatment. This may explain that the higher sunitinib resistance of Caki-1 cells under high dose treatment.

Current studies have proved that downregulation of STC2 can reduce tumor drug resistance. In cisplatin-resistant cervical cancer cells, siRNA regulation of STC2 expression can reduce cisplatin resistance [28]. In nasopharyngeal carcinoma cells, STC2 knockout can enhance the sensitivity of cancer cells to X radiation [29]. Overexpression of STC2 in sensitive non-small cell lung cancer cells leads to epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) resistance. In contrast, STC2 gene silencing makes EGFR-TKI resistant cells more sensitive to EGFR-TKIs [30]. Yuan et al. showed that STC2 silencing could promote the chemical sensitivity of metastatic colorectal cancer cells to oxaliplatin, while exogenous STC2 upregulated the multi-drug resistance protein P-glycoprotein through the PI3K/Akt signaling pathway, inducing oxaliplatin resistance in colorectal cancer cells [31]. The present study confirmed that sunitinib resistance of ccRCC cells could be reduced by neutralizing antibodies to block secreted STC2 but increased by the addition of recombinant human STC2.

Studies have shown that resistance to sunitinib in ccRCC is due to the lysosomal isolation and inhibition of autophagy flow [26, 27]. However, the underlying mechanism is still unclear. Sunitinib is sequestrated in the lysosome might due to the destroyed acidic environment of the lysosome by sunitinib, thereby inhibition of its release and efficacy. Our

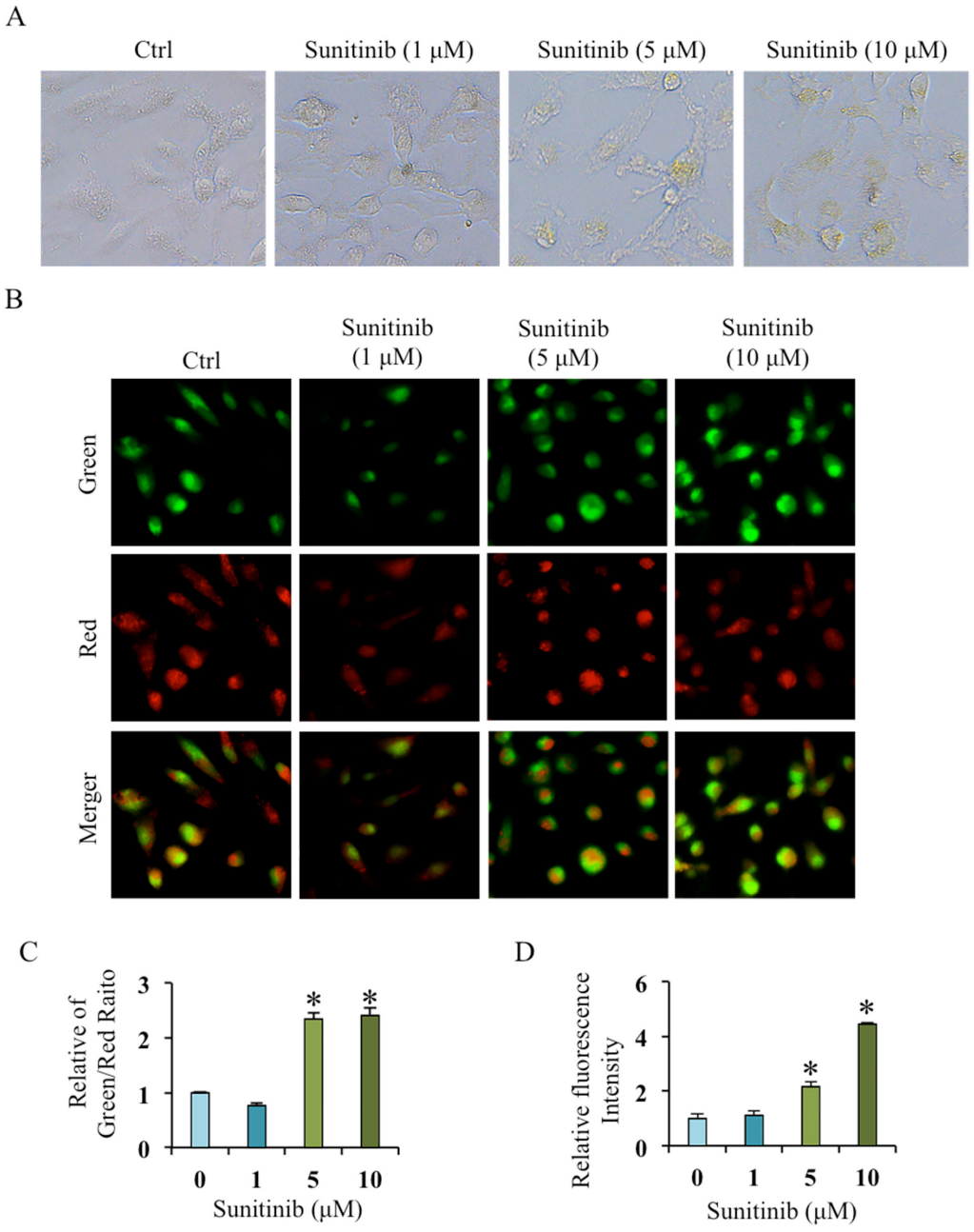

Figure 5. Sunitinib accumulated and destroyed lysosomal acidity in Caki-1 cells. A) Phase-contrast microscopy showing accumulation of yellow granules (sunitinib) in Caki-1 cells incubated in the absence (0) or presence of increasing concentrations of sunitinib (1, 5, 10 μM) for 24 h. B, C) AO staining indicates the lysosome pH in Caki-1 cells incubated in the absence (0) or presence of increasing concentrations of sunitinib (1, 5, 10 μM) for 24 h. The acidity region appears red. Quantification of the ratio between the red and green signal of AO was performed by the software ImageJ. D) The fluorescence of the lysosomal probe (LysoTracker™ Green DND-26) in Caki-1 cells incubated in the absence (Ctrl) or presence of increasing concentrations of sunitinib (1, 5, 10 μM) for 24 h. *p<0.05

results supported that sunitinib was accumulated in cells and destroyed the lysosome pH in a dose-dependent manner. But STC2 neutralizing antibody suppressed sunitinib accumulation was independent on restoring the lysosome pH, which might be due to reducing sunitinib capture when STC2 endocytosis. On the other hand, due to the lysosomal degradation, clinically used antibodies have severe immunotherapy-related adverse effects and low cancer immunotherapeutic effects [32]. Here, our results showed that lysosomal acid pH was destroyed by sunitinib when co-treated with an STC2 neutralizing antibody. Therefore, the STC2 neutralizing antibody could effectively block the STC2. Previous

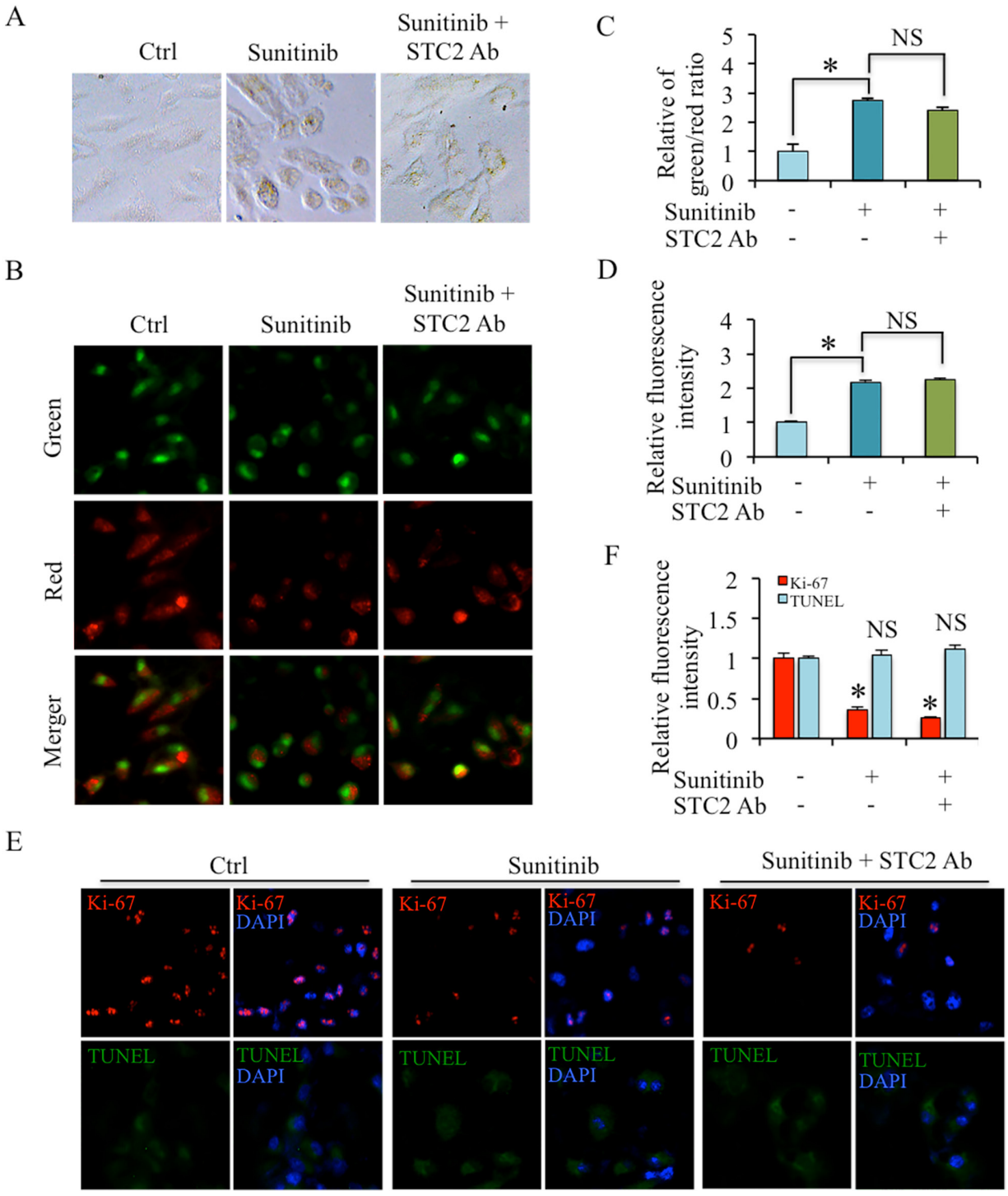

Figure 6. Effects of the STC2 neutralizing antibody on sunitinib accumulation, lysosomal pH, cell proliferation, and apoptosis in Caki-1 cells. A) Phase-contrast microscopy showing accumulation of yellow granules in Caki-1 cells incubated in the absence (Ctrl) or presence of sunitinib (5 μM), 5 μM sunitinib+0.2 μg/ml STC2 neutralizing antibody for 24 h. B, C) AO staining indicates the lysosome pH in Caki-1 cells incubated in the absence (Ctrl) or presence of sunitinib (5 μM), 5 μM sunitinib+0.2 μg/ml STC2 neutralizing antibody for 24 h. Quantification of the ratio between the red and green signal of AO was performed by the software ImageJ. D) The fluorescence of the lysosomal probe (LysoTracker™ Green DND-26) in Caki-1 cells incubated in the absence (Ctrl) or presence of sunitinib (5 μM), 5 μM sunitinib+0.2 μg/ml STC2 neutralizing antibody for 24 h. E, F) The immunofluorescence staining of cell proliferation marker Ki-67 and cell apoptosis detection by TUNEL staining in Caki-1 cells incubated in the absence (Ctrl) or presence of sunitinib (5 μM), 5 μM sunitinib+0.2 μg/ml STC2 neutralizing antibody for 24 h. Quantification of Ki-67 and TUNEL fluorescence was performed by the software ImageJ. NS: no significant difference between control with treatments. *p<0.05

studies have suggested that STC2 promotes cell proliferation of various cancer cells, such as cervical, breast, ovarian, and liver cancer cells [17, 28, 33], or promotes endometrial epithelial cell apoptosis [34]. The present study demonstrated that neutralizing the secreted STC2 could aggravate the inhibitory effect of sunitinib on cell proliferation instead of promoting cell apoptosis.

In conclusion, we present data that the high expression of STC2 in ccRCC patients and cells may play a key role in the process of tumor resistance, and blocking secreted STC2 may be an effective therapeutic option. This study reveals that the ccRCC targeted drug sunitinib reduces STC2 expression and secretion, destroys the lysosomal pH, and accumulates in ccRCC cells. Furthermore, the STC2 neutralizing antibody suppresses sunitinib accumulation in the cells and aggravates the inhibition of sunitinib on cell proliferation. STC2 could serve as a potential novel target for the treatment of ccRCC, and our results may contribute to the development of effective therapies for renal cell carcinoma such as STC2 immunotherapy.

Supplementary information is available in the online version of the paper.

Acknowledgments: This work was supported by the Foundation of Health and Family Planning Commission 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).

References

[1] COHEN HT, MCGOVERN FJ. Renal-cell carcinoma. N Engl J Med 2005; 353: 2477–2490. https://doi.org/10.1056/NEJMra043172

[2] HSIEH JJ, PURDUE MP, SIGNORETTI S, SWANTON C, ALBIGES L et al. Renal cell carcinoma. Nat Rev Dis Primers 2017; 3: 17009. https://doi.org/10.1038/nrdp.2017.9

[3] RINI BI, CAMPBELL SC, ESCUDIER B. Renal cell carcinoma. Lancet 2009; 373: 1119–1132. https://doi.org/10.1016/S0140-6736(09)60229-4

[4]. SATO Y, YOSHIZATO T, SHIRAISHI Y, MAEKAWA S, OKUNO Y et al. Integrated molecular analysis of clear-cell renal cell carcinoma. Nat Genet 2013; 45: 860–867. https://doi.org/10.1038/ng.2699

[5] MEYER HA, TOLLE A, JUNG M, FRITZSCHE FR, HAENDLER B et al. Identification of stanniocalcin 2 as prognostic marker in renal cell carcinoma. Eur Urol 2009; 55: 669–678. https://doi.org/10.1016/j.eururo.2008.04.001

[6] GIRGIS AH, IAKOVLEV VV, BEHESHTI B, BAYANI J, SQUIRE JA et al. Multilevel whole-genome analysis reveals candidate biomarkers in clear cell renal cell carcinoma. Cancer Res 2012; 72: 5273–5284. https://doi.org/10.1158/0008-5472.CAN-12-0656

[7] KANELLIS J, BICK R, GARCIA G, TRUONG L, TSAO CC et al. Stanniocalcin-1, an inhibitor of macrophage chemotaxis and chemokinesis. Am J Physiol Renal Physiol 2004; 286: F356–362. https://doi.org/10.1152/ajprenal.00138.2003

[8] XIAO LJ, YUAN JX, SONG XX, LI YC, HU ZY et al. Expression and regulation of stanniocalcin 1 and 2 in rat uterus during embryo implantation and decidualization. Reproduction 2006; 131: 1137–1149. https://doi.org/10.1530/rep.1.01100

[9] WESTBERG JA, SERLACHIUS M, LANKILA P, ANDERSSON LC. Hypoxic preconditioning induces elevated expression of stanniocalcin-1 in the heart. Am J Physiol Heart Circ Physiol 2007; 293: H1766–1771. https://doi.org/10.1152/ajpheart.00017.2007

[10] WESTBERG JA, SERLACHIUS M, LANKILA P, PENKOWA M, HIDALGO J et al. Hypoxic preconditioning induces neuroprotective stanniocalcin-1 in brain via IL-6 signaling. Stroke 2007; 38: 1025–1030. https://doi.org/10.1161/01.STR.0000258113.67252.fa

[11] YEUNG BH, LAW AY, WONG CK. Evolution and roles of stanniocalcin. Mol Cell Endocrinol 2012; 349: 272–280. https://doi.org/10.1016/j.mce.2011.11.007

[12] CHU SJ, ZHANG J, ZHANG R, LU WW, ZHU JS. Evolution and functions of stanniocalcins in cancer. Int J Immunopathol Pharmacol 2015; 28: 14–20. https://doi.org/10.1177/0394632015572745

[13] DONDETI VR, WUBBENHORST B, LAL P, GORDAN JD, D'ANDREA K et al. Integrative genomic analyses of sporadic clear cell renal cell carcinoma define disease subtypes and potential new therapeutic targets. Cancer Res 2012; 72: 112–121. https://doi.org/10.1158/0008-5472.CAN-11-1698

[14] JOENSUU K, HEIKKILA P, ANDERSSON LC. Tumor dormancy: elevated expression of stanniocalcins in late relapsing breast cancer. Cancer Lett 2008; 265: 76–83. https://doi.org/10.1016/j.canlet.2008.02.022

[15] COULSON-GILMER C, HUMPHRIES MP, RAJAN SS, DROOP A, JACKSON S et al. Stanniocalcin 2 expression is associated with a favourable outcome in male breast cancer. J Pathol Clin Res 2018; 4: 241–249. https://doi.org/10.1002/cjp2.106

[16] WANG HX, WU KJ, SUN Y, LI YD, WU MY et al. STC2 is upregulated in hepatocellular carcinoma and promotes cell proliferation and migration in vitro. Bmb Reports 2012; 45: 629–634. https://doi.org/10.5483/bmbrep.2012.45.11.086

[17] WU F, LI TY, SU SC, YU JS, ZHANG HL et al. STC2 as a novel mediator for Mus81-dependent proliferation and survival in hepatocellular carcinoma. Cancer Letters 2017; 388: 177–186. https://doi.org/10.1016/j.canlet.2016.11.039

[18] CHENG HW, WU ZX, WU CS, WANG XY, LIOW SS et al. Overcoming STC2 mediated drug resistance through drug and gene co-delivery by PHB-PDMAEMA cationic polyester in liver cancer cells. Mat Sci Eng C-Mater 2018; 83: 210–217. https://doi.org/10.1016/j.msec.2017.08.075

[19] YOKOBORI T, MIMORI K, ISHII H, IWATSUKI M, TANAKA F et al. Clinical Significance of Stanniocalcin 2 as a Prognostic Marker in Gastric Cancer. Ann Surg Oncol 2010; 17: 2601–2607. https://doi.org/10.1245/s10434-010-1086-0

[20] IETA K, TANAKA F, YOKOBORI T, KITA Y, HARAGUCHI N et al. Clinicopathological significance of stanniocalcin 2 gene expression in colorectal cancer. Int J Cancer 2009; 125: 926–931. https://doi.org/10.1002/ijc.24453

[21] TAMURA K, FURIHATA M, CHUNG SY, UEMURA M, YOSHIOKA H et al. Stanniocalcin 2 overexpression in castration-resistant prostate cancer and aggressive prostate cancer. Cancer Sci 2009; 100: 914–919. https://doi.org/10.1111/j.1349-7006.2009.01117.x

[22] BIELECKA ZF, CZARNECKA AM, SOLAREK W, KORNAKIEWICZ A, SZCZYLIK C. Mechanisms of acquired resistance to tyrosine kinase inhibitors in clear - cell renal cell carcinoma (ccRCC). Curr Signal Transduct Ther 2014; 8: 218–228. https://doi.org/10.2174/1574362409666140206223014

[23] MAKHOV P, JOSHI S, GHATALIA P, KUTIKOV A, UZZO RG et al. Resistance to systemic therapies in clear cell renal cell carcinoma: mechanisms and management strategies. Mol Cancer Ther 2018; 17: 1355–1364. https://doi.org/10.1158/1535-7163.MCT-17-1299

[24] ORNSTEIN MC, WOOD LS, ELSON P, ALLMAN KD, BEACH J et al. A phase II study of intermittent sunitinib in previously untreated patients with metastatic renal cell carcinoma. J Clin Oncol 2017; 35: 1764–1769. https://doi.org/10.1200/JCO.2016.71.1184

[25] VENKATESAN P. Intermittent sunitinib for metastatic renal cell carcinoma. Lancet Oncol 2017; 18: e139. https://doi.org/10.1016/S1470-2045(17)30082-7

[26] GIULIANO S, CORMERAIS Y, DUFIES M, GREPIN R, COLOSETTI P et al. Resistance to sunitinib in renal clear cell carcinoma results from sequestration in lysosomes and inhibition of the autophagic flux. Autophagy 2015; 11: 1891–1904. https://doi.org/10.1080/15548627.2015.1085742

[27] GOTINK KJ, BROXTERMAN HJ, LABOTS M, DE HAAS RR, DEKKER H et al. Lysosomal sequestration of sunitinib: a novel mechanism of drug resistance. Clin Cancer Res 2011; 17: 7337–7346. https://doi.org/10.1158/1078-0432.CCR-11-1667

[28] WANG Y, GAO Y, CHENG H, YANG G, TAN W. Stanniocalcin 2 promotes cell proliferation and cisplatin resistance in cervical cancer. Biochem Biophys Res Commun 2015; 466: 362–368. https://doi.org/10.1016/j.bbrc.2015.09.029

[29] HE H, QIE S, GUO Q, CHEN S, ZOU C et al. Stanniocalcin 2 (STC2) expression promotes post-radiation survival, migration and invasion of nasopharyngeal carcinoma cells. Cancer Manag Res 2019; 11: 6411–6424. https://doi.org/10.2147/CMAR.S197607

[30] LIU YN, TSAI MF, WU SG, CHANG TH, TSAI TH et al. Acquired resistance to EGFR tyrosine kinase inhibitors is mediated by the reactivation of STC2/JUN/AXL signaling in lung cancer. Int J Cancer 2019; 145: 1609–1624. https://doi.org/10.1002/ijc.32487

[31] YUAN Q, ZHAN L, ZHANG LL, WANG Q, LIU J et al. Stanniocalcin 2 induces oxaliplatin resistance in colorectal cancer cells by upregulating P-glycoprotein. Can J Physiol Pharm 2016; 94: 929–935. https://doi.org/10.1139/cjpp-2015-0530

[32] ZHANG Y, DU X, LIU M, TANG F, ZHANG P et al. Hijacking antibody-induced CTLA-4 lysosomal degradation for safer and more effective cancer immunotherapy. Cell Res 2019; 29: 609–627. https://doi.org/10.1038/s41422-019-0184-1

[33] LAW AYS, WONG CKC. Stanniocalcin-2 is a HIF-1 target gene that promotes cell proliferation in hypoxia. Exp Cell Res 2010; 316: 466–476. https://doi.org/10.1016/j.yexcr.2009.09.018

[34] CUI JZ, LIU XR, YANG LC, CHE SC, GUO HR et al. MiR-184 combined with stc2 promotes endometrial epithelial cell apoptosis in dairy goats via RAS/RAF/MEK/ERK Pathway. Genes-Basel 2020; 11: 1052.