

Clinical significance of SCCRO (DCUN1D1) in prostate cancer and its proliferation-inhibiting effect on Lncap cells

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Abstract. – OBJECTIVE: SCCRO/DCUN1D1/DCN1 (squamous cell carcinoma-related oncogene/defective in cullin neddylation 1 domain containing 1/defective in cullin neddylation) is considered as an oncogene, but its role in the prostate cancer (PC) is still not clear. The current study aims to investigate the expression of SCCRO in PC tumor tissues, further its clinical significance, and proliferation inhibiting effect on PC cells *in vitro*.

PATIENTS AND METHODS: RT-PCR was used to detect the expression of SCCRO in PC tissue and corresponding adjacent normal tissues from 160 cases, and its relationship with clinical pathological characteristics was analyzed. Small interfering RNA (siRNA) expression plasmid targeting SCCRO gene was constructed and transferred into PC cell line Lncap. The effect on proliferation was observed by CCK8 assay, and its influence on invasion and migration of Lncap cells was studied by Transwell Matrigel assay after SCCRO gene was silenced. The expression of focal adhesion kinase (FAK) and matrix metalloproteinase-2 (MMP-2) influenced by SCCRO silencing were detected by Western blot.

RESULTS: mRNA expression of SCCRO protein increased significantly in cancer tissues compared to adjacent normal tissue, especially for T3+T4, N+, and III+IV patients ($p<0.05$). SCCRO expression was an independent prognostic factor ($p<0.05$). After SCCRO gene was knocked down by siRNA, the SCCRO protein level decreased 78.4% in the siRNA-3 group. By CCK8 assay, knocking down SCCRO in Lncap significantly reduced the cell proliferation, as well as its migration and invasion capability compared to siRNA-control group ($p<0.01$) by transwell invasion and migration assay. The expression of FAK and MMP-2 also reduced in siRNA-3 group compared to siRNA control group ($p<0.01$).

CONCLUSIONS: SCCRO is associated with progression and prognosis of PC. After SCCRO gene was transferred, the growth of Lncap cells was inhibited, and ability of invasion and migration decreased by reducing the expression of FAK and MMP-2. SCCRO has potential to become a new target for the treatment of PC.

Key Words:

Tumor gene, Prostate cancer, RNA, Focal adhesion kinase, Matrix metalloproteinase-2.

Introduction

Prostate cancer (PC) is the most common cancer among males in the Western world, with more than 1.11 million new cases diagnosed in 2012 and 307,000 deaths^{1,2}. The lifetime risk of developing PC is 1 in 8³. It is expected that the incidence will increase in the coming decades due to the aging population, which makes it a huge healthcare problem. The total economic costs of PC in Europe are estimated to exceed 8.43 billion⁴. Recently, with the economy improvement, the morbidity and mortality of PC have also been steadily increased in China^{5,6}. Thus, exploring the molecular biological mechanism of the occurrence and development of PC has important clinical significance in the diagnosis, treatment, and prognosis of the disease.

Squamous cell carcinoma-related oncogene (SCCRO/DCUN1D1) is a new cancer gene, located on chromosome 3q26.3, can be used as a transcription factor^{7,8}. Studies⁹⁻¹¹ have indicated that SCCRO plays an important role in the formation of lung cancer, glioma, and cervical cancer. Overexpression of SCCRO is very important in the malignant transformation of the tumor, and it is associated with poor clinical prognosis. However, there is still lack of enough information about SCCRO in prostate cancer. In this work, we used RT-PCR to detect the expression of SCCRO in PC tissues and adjacent tissues respectively, and aim to analyze whether its expression is correlated with prognosis and TNM staging of PC. With an *in vitro* study, we investigated the effect of knocking-down SCCRO gene by RNAi on the growth, migration and invasion of Lncap cells, to understand

the role of SCCRO in the development of PC. We aimed to understand SCCRO regulating pathway; the MMP2 and FAK expression change after SCCRO RNA interference in Lncap cells were tested, because a previous paper¹² has reported that SCCRO induced invasion involves activation of MMP2 in several kinds of cancer, and FAK is an important regulator of MMP2¹³.

Patients and Methods

Patients and Tumor Samples

PC patients with complete data in Tianjin Union Medical Center were retrospectively analyzed from January 2008 to November 2016, and 160 patients were taken up in this research. Tissue from the tumor and normal mucosa 2 cm beside tumor were taken into liquid nitrogen and kept at -80°C. All the patients had not been treated with radiotherapy and chemotherapy before the surgery, and postoperative pathological diagnosis confirmed PC. The data of the patients were shown in Table I. The patients were followed-up every 3 months to investigate the survival situation. We obtained the informed consent from all the patients. This study was approved by the Ethics Committee of Tianjin Union Medical Center and was conducted in accordance with the provisions of the Declaration of Helsinki, Good Clinical Practice guidelines, and local laws and regulations.

Real-time PCR

The total RNA from each prostate tissue and corresponding adjacent normal tissue was firstly extracted by the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), then cDNA was obtained by Reverse transcription polymerase (Toyobo, Tokyo, Japan). Real-time polymerase chain reaction (RT-PCR) method was used to measure SCCRO level in each prostate tumor tissues and the corresponding normal tissues. The total volume of RT-PCR reaction system (TIANGEN Biotech, Beijing, China) was 20 µl: reaction system contained 2× premix reagent 10 µl, forward primer 0.4 µl, reverse primer 0.4 µl, cDNA 2 µl and 6.2 µl of distilled deionized water. 1 µl 20×SYBR Green I was used as an intercalating dye (TIANGEN Biotech, Beijing, China). The CFX96 Touch™ Real-time PCR Detection System (BioRad, Hercules, CA, USA) was used for quantitative measurement with the following conditions: 95°C for 5 min, 95°C for 15 s, 60°C

for 1 min (40 cycles). The relative expression of SCCRO was calculated by methods mentioned by previous study¹⁴: $F = 2^{-\Delta Ct}$, $\Delta Ct = Ct_{SCCRO} - Ct_{GAPDH}$. CT means the number of cycles experienced by the fluorescent signals reached the threshold inside the reactor. The SCCRO primer sequence was following: forward was 5' GAAGCTGTA-CTTGGGGCTG 3', and the reverse primer was 5' TCCGCTGACAGATATGCCAA 3', the product size was 213bp. The GAPDH primers were following: forward 5' GCTCTCTGCTCCTCCT-GTTC', and reverse 5' ACGACCAAATCCGTT-GACTC'. All the primers were synthesized by life technology Inc. (Shanghai, China).

Vector Construction

Three pairs of siRNA specific sequence targeting human SCCRO gene and a pair of non-specific sequence were designed and synthesized by GenePharma (Shanghai, China). SCCRO siRNA-1 (forward: 5'-CCCTCAAATTGCTGG-GACA-3', reverse: 5'-UGUCCCAGCAAU-UUGAGGG-3'), SCCRO siRNA-2 (forward: 5'-GGAATTTGCACGCCCTCAA-3', reverse: 5'-UUGAGGGCGUGCAAUU CC-3'), SCCRO siRNA3 (forward: 5'-GCAGATGACATGTCTA-ATT-3', reverse: 5' -AAUUAGACAUGUCAU-CUGC-3'), siRNA negative control (forward: 5'-UUCUCCGAACGUGUCACGU-3', reverse: 5'-ACGUGACACGUUCGGAGAA-3'). The above RNA was cloned into a psiCHECK-2 vector, and the sequence correction was confirmed by direct gene sequencing.

Cell Culture and Transfection

Lncap cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) under the condition of 37°C, 5% CO₂ saturation humidity. The experiment was carried out using logarithmic growth phase cells. Small interfering RNA (siRNA) was stably transfected with Lncap cells by Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) for four weeks. Real-time PCR and Western blot was used for testing their knocking down efficiency. Groups were divided into siRNA-1, siRNA-2, siRNA-3, siRNA negative control (siRNA-NC) and blank control.

Real-time PCR and Western Blot to Assess the Knocking-down Efficacy

After three stable siRNA clones were obtained, the total RNA was extracted from cells,

and RT-PCR was performed as same procedure as above mentioned to assess the efficiency of siRNA knocking-down, GAPDH and SCCRO primer was used. Protein extraction was performed with RIPA lysis buffer (Applygen, Beijing, China), according to the manufacturer's protocol. Protein concentration was measured using BCA assay (Applygen, Beijing, China). The protein samples were boiled for 5 min with buffer and 40 µg total protein was used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, the protein in the gel was electric transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked with tris buffered saline-tween (TBS/T) (NaCl 500 mM, Tris 20 mM, pH7.5) containing 5% skim milk for 60 min, and then probed with the SCCRO primary antibodies (1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4°C. The secondary antibody (1:2000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was applied for 1 h. After incubation, the membrane was thoroughly washed with TBS/T at least three times, and then the membrane was treated with ECL enhanced solution (Thermo Fisher Scientific, Waltham, MA, USA) by Western blot and exposed to GE-ImageQuant-LAS-4000 system. β -actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used as internal reference. Using the same method, FAK polyclonal antibody (rabbit anti human, 1:1000, Proteintech, Chicago, IL, USA) and MMP-2 polyclonal antibody (rabbit anti human, 1:1000, Proteintech, Chicago, IL, USA) were used to examine their expression change in Lncap cells caused by siRNA transfection.

Proliferation, Invasion and Migration Assay of Lncap Cells with SCCRO Knocking Down

The proliferation of Lncap cells was detected by CCK8 assay (Beyotime Biotechnology, Shanghai, China). Briefly, the siRNA-control cells and siRNA-3- SCCRO cells were plated in 96-well plate and incubate for 96 hours. After that, CCK8 was added into each well for another 2 hours; then, the optical absorption was measured at a 450 nm wavelength in a spectrometer (EnSpire, Perkin Elmer, Waltham, MA, USA).

Invasion assay was performed using Boyden chamber system (Neuro Probe, Gaithersburg, MD, USA) with a fibronectin-precoated (0.5 mg/

ml) polycarbonate membrane (8 µm pore size). The lighter side of the polycarbonate membrane was precoated with 250 µg/ml matrigel (BD). The bottom chambers were filled with 30 µl RPMI1640 medium containing 2% bovine serum albumin (BSA) while the top chambers were filled with 50 µl RPMI-1640 serum-free medium containing 0.2% BSA. 5×10^4 cells per well were added to the top chamber, followed by a 15 h incubation at 37°C, 5% CO₂ incubator. Three independent experiments were performed with triplicate treatment. The cells were fixed in methanol and stained with hematoxylin. The top surface of the membrane was gently scrubbed with a cotton bud, the cells migrated to the lower side of the membrane was counted under the microscope, and the numbers of migrated cells were calculated as average plus standard deviation (SD).

Cell migration ability was evaluated by *in vitro* wound healing model. A horizontal line was drawn evenly in the 6-well culture plate with a marker-line distance of 0.5 cm. 5 lines were drawn in each well. 5×10^5 cells were inoculated in each well and reached to 100% fusion after cultured overnight. 10 µl of pipette tip was used to scratch cells along the drawn line on the bottom. The non-attached cells were removed by phosphate buffered saline (PBS) washing. Cells were cultured in 37°C incubator with 5% CO₂. Cell images were acquired at 0 h and 24 h, respectively.

Statistical Analysis

Data are shown as mean $\pm$ SD. The Kaplan-Meier method was used in survival analysis. Statistical significance was determined by χ^2 -test and Student's *t*-test, with a *p*-value of < 0.05 considered to be statistically significant.

Results

SCCRO is Highly Expressed in the Prostate Cancer

The average expression of SCCRO protein was significantly higher in PC tissues compared to adjacent normal tissue with statistical significance (Figure 1, mean value 2.90 ± 1.78 vs. 1.78 ± 0.71 , $p < 0.001$). SCCRO mRNA expression increased in T3+T4, N+, and III+IV patients compared to that in T1+T2, N0, and I+ II (Table I, $p = 0.002$, $p = 0.024$, $p = 0.003$, respectively). The expression of SCCRO had no significant difference with age, primary site, distant metastases, and preoperative smoking ($p > 0.05$, Table I).

Table I. Data of the patients and relationship between SCCRO expression and clinic pathological characteristics of prostate cancer.

	n	Expression of SCCRO		p-value
		Low (n, %)	High (n, %)	
Age (year-old)				
≤60	73	42 (57.5)	31 (42.5)	0.764
>60	87	38 (43.7)	49 (56.3)	
Gleason score#				
≤7 (3+4)	61	34 (39.3)	37 (60.7)	0.611
≥7 (4+3)	99	46 (35.4)	64 (64.6)	
Differentiation level				
High	68	31 (45.6)	37 (54.4)	0.885
Medium-low	92	49 (53.3)	43 (46.7)	
T Staging				
T1+T2	73	54 (74.0)	19 (26.0)	0.002
T3+T4	87	26 (29.9)	61 (70.1)	
Lymph node metastases				
N0	85	53 (62.4)	32 (37.6)	0.024
N+	75	27 (36.0)	48 (64.0)	
Distant metastasis				
M0	95	52 (54.7)	43 (45.3)	0.063
M1	65	28 (43.1)	37 (56.9)	
Clinical stage				
I+II	72	50 (69.4)	22 (30.6)	0.003
III+IV	88	30 (34.1)	58 (65.9)	

Note: The threshold of low and high expression of SCCRO was its median value, above median value was classified into higher expression group, and below the median value was considered as lower expression. All the data was analyzed by χ^2 -test.

Table II. Multivariate Cox analysis of prognostic factors in patients with prostate cancer.

Prognostic factors	Regression coefficient	Standard error	Wald	p-value	Relative risk	95% CI
T staging	1.542	0.361	18.258	0.000	4.674	2.304-9.481
Lymph node metastases	1.069	0.337	10.053	0.002	2.912	1.504-5.683
SCCRO expression	0.926	0.335	7.636	0.006	2.524	1.309-4.866

SCCRO is Correlated with Prognosis of Prostate Cancer

Median follow-up time was 54.0 (17-102) months, 22 patients were lost to follow-up, and the follow-up rate was 92% combined active review with passive follow-up. Survival analysis by Kaplan-Meier method and log-rank test showed that the median survival time with high SCCRO expression (relative expression was above median value, 2.289) was 45.4 months, which is significantly shorter than that with low expression (70.9 months, χ^2 value=6.312, $p=0.012$, Figure 2). Multivariate survival analysis by Cox regression model with age, primary site, differentiation level, T staging, lymph node metastases, distant metastasis, clinical stage and pre-operative smoking showed that T staging (RR=4.674, 95% CI 2.304-9.481, $p=0.000$), lymph node metastases (RR=2.912, 95% CI 1.504-5.683, $p=0.002$), and

SCCRO expression (RR=2.524, 95% CI 1.309-4.866, $p=0.006$) were independent prognostic factors for patients (Table II).

Knocking Down SCCRO Decreased the Proliferation, Invasion and Migration Capability of Lncap

The expression of SCCRO mRNA and protein decreased after three different SCCRO siRNAs were transfected into Lncap cells, compared with siRNA control (Figure 3). The silencing efficiency of siRNA-3 group was highest and almost reached to 75% inhibition rate, so siRNA-3 knocking-down cells were selected for the proliferation, migration and invasion assay.

Results showed that Lncap cells proliferation was significantly inhibited due to SCCRO siRNA-3 transfection by CCK8 assay with 72 h incubation (Figure 4A), compared to siRNA control

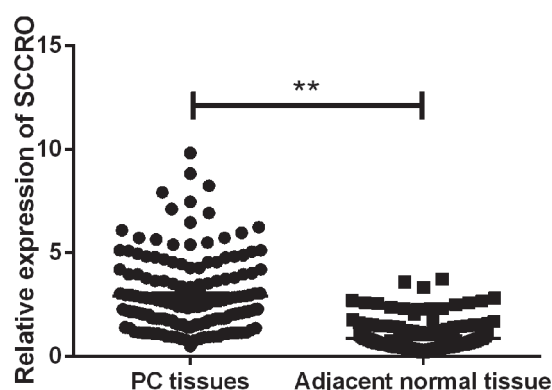


Figure 1. The relative expression of SCCRO in prostate cancer compared with adjacent normal tissue. The average SCCRO expression level in PC tissues was significantly higher than that from adjacent normal tissues ($p < 0.01$). **, $p < 0.01$.

group and blank control Lncap cells, and there was no difference between the blank group and the siRNA control group.

To study the role of SCCRO on the Lncap cell invasion and motility, which are the major characteristics of the metastasis, invasion assay, and migration assay were performed. In an invasion assay, we calculated the number of cells that migrated to the bottom side of the membrane on a chamber where the cells were seeded (Figure 4B). The data showed that the blank control cells and siRNA-control cells had more numbers of the migrated cells compared to siRNA-3 group ($p < 0.05$). These results suggested that the depletion of SCCRO significantly suppressed the migration ability of Lncap cells.

To examine whether the depletion of SCCRO has inhibitory effect on the motive ability of the cells, we performed a wound-healing experiment using Lncap cells transfected with control-siRNA and siRNA3. The data showed that the wild type Lncap cells had no difference in the relative wound closure with the cells transfected with control siRNA, but a significant slower closure speed was observed for the cells transfected with siRNA3 at 24 h, (Figure 4C, $p < 0.05$).

Protein Levels of FAK and MMP-2 Interfered by siRNA-3

The protein expression of FAK and MMP-2 among blank control, siRNA-control and siRNA-3 were studied by Western blot. The results demonstrated that knocking-down SCCRO significantly reduced the FAK and MMP2 protein level

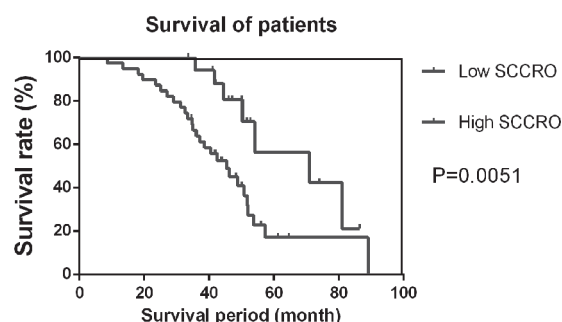


Figure 2. Survival curves of patients with different expression of SCCRO. By Log-rank (Mantel-Cox) test, $p = 0.0051$ between SCCRO high-expression and lower expression group.

compared with blank control and siRNA-control group ($p < 0.01$, Figure 5).

Discussion

Invasion and migration is a basic biology feature of a malignant tumor and is also an important cause of recurrence and death in most patients¹⁵. Prostate cancer is one of the most common malignant tumors in the male population. The current treatment is still dominated by surgery and radiotherapy. Although various treatment methods have been improved, the 5-year survival rate of prostate cancer have not significantly promoted in the past 30 years¹⁶. The invasion and metastasis of PC has serious impact on the living quality of patients and

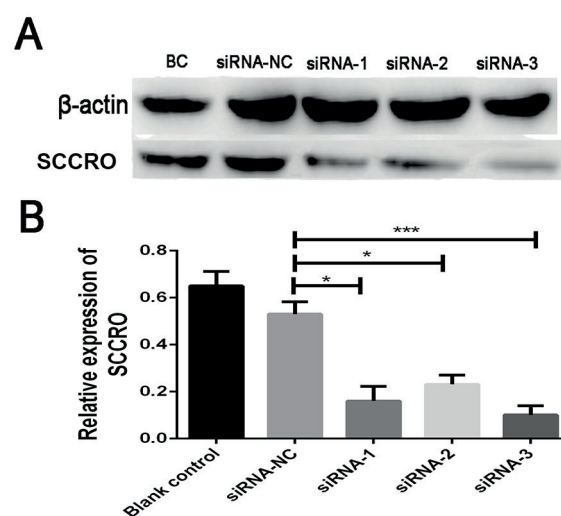


Figure 3. Expression of SCCRO protein by Western blot to evaluate siRNA interference efficiency. *, $p < 0.05$; ***, $p < 0.001$.

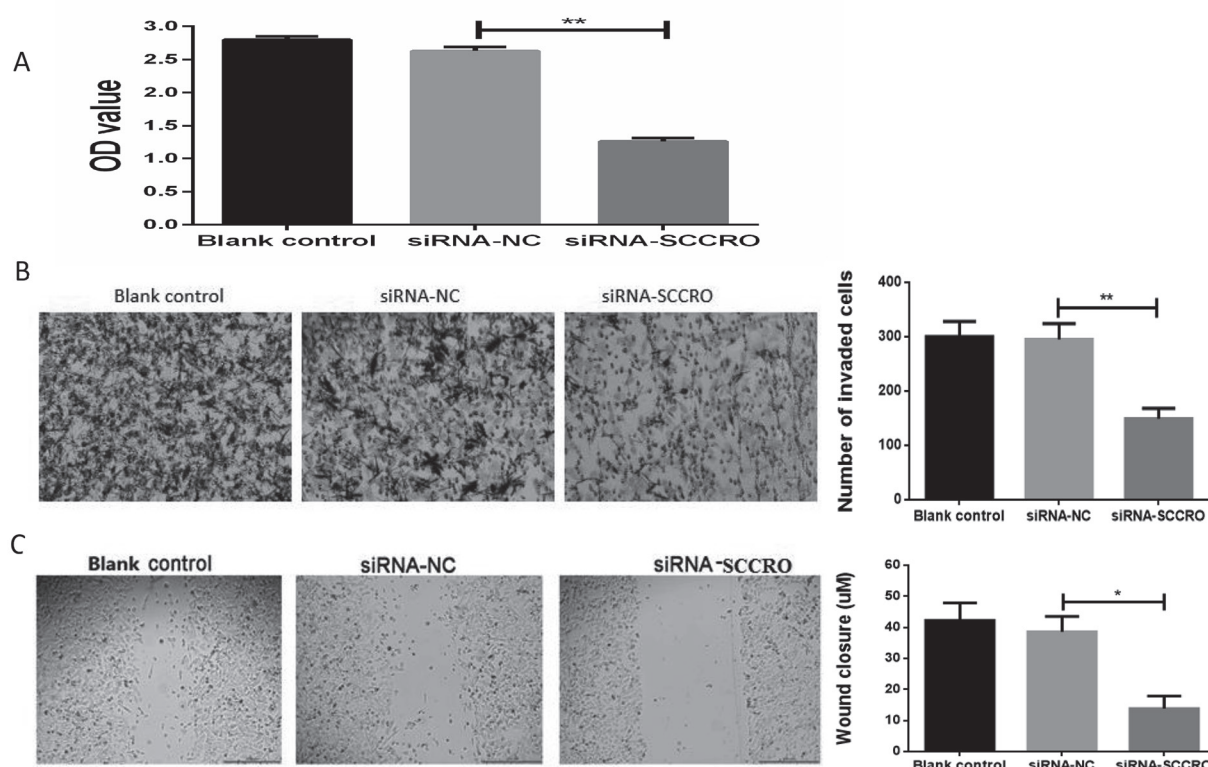


Figure 4. Proliferation, invasion, and migration of Lncap cells transfected by SCCRO siRNA-3. **A**, Proliferation ability of Lncap cells measured by CCK8 methods 72 hours after infection. **B**, Number of cells migrated to the lower side of the membrane by invasion assay. **C**, Wound closure measurement for testing Lncap migration capability among three groups. All the tests were conducted among three groups: blank control, siRNA-negative control (NC) and siRNA-SCCRO. *, $p < 0.05$; **, $p < 0.01$.

is the main cause of death¹⁷. Therefore, exploring the molecular biological mechanism of the invasion and migration of prostate cancer has great significance for diagnosis, treatment, and prognosis.

SCCRO is a cancer gene that can increase the effect like ubiquitination leading to a class of diseases including cancer^{18,19}. More and more evidence^{11,20} show that SCCRO is highly expressed in many malignant tumors, which can regulate the progression of the tumor, the sensitivity to chemotherapy, angiogenesis and metastasis. In this investigation, we found that high expression of SCCRO in PC was positively correlated with T staging, lymph node metastasis and clinical stage. Survival analysis by Kaplan-Meier method demonstrates average survival time with high expression is shorter than that with low expression. Multivariate Cox regression suggests that T staging, lymph node metastases, and SCCRO expression are independent prognostic factors for patients. Sarkaria et al²¹ find out that SCCRO can be used as a marker for the aggressive progression of bronchioloalveolar carcinoma and low expression in adjacent normal lung tissue present worse

survival rate, which may represent field cancerization or tumor-host effect. Estilo et al²² pointed out that over expression of SCCRO is associated with lymph node metastasis by amplification of chromosome 3q26, which means SCCRO may play a role in the pathogenesis of squamous cell carcinoma of the tongue, as a valuable predictor of regional tumor metastasis, tumor invasion, and clinical prognosis. O-charoenrat et al¹² also discovered SCCRO over-expression could increase the expression level and activity of MMP-2, which is necessary for induced invasion. The co-expression of SCCRO and MMP2 increased the incidence of regional lymph node metastasis. SCCRO is a potential molecular marker for progression of tumor metastasis. All of these studies indicated that SCCRO overexpression is associated with the development of human squamous cell carcinoma.

In this study, we designed three different siRNA targeting SCCRO, and all of them had been transfected into Lncap cells. The results show that the siRNA-3 can effectively reduce SCCRO protein level by 75%, which confirmed that siRNA-3 was most effective to inhibit its gene expression.

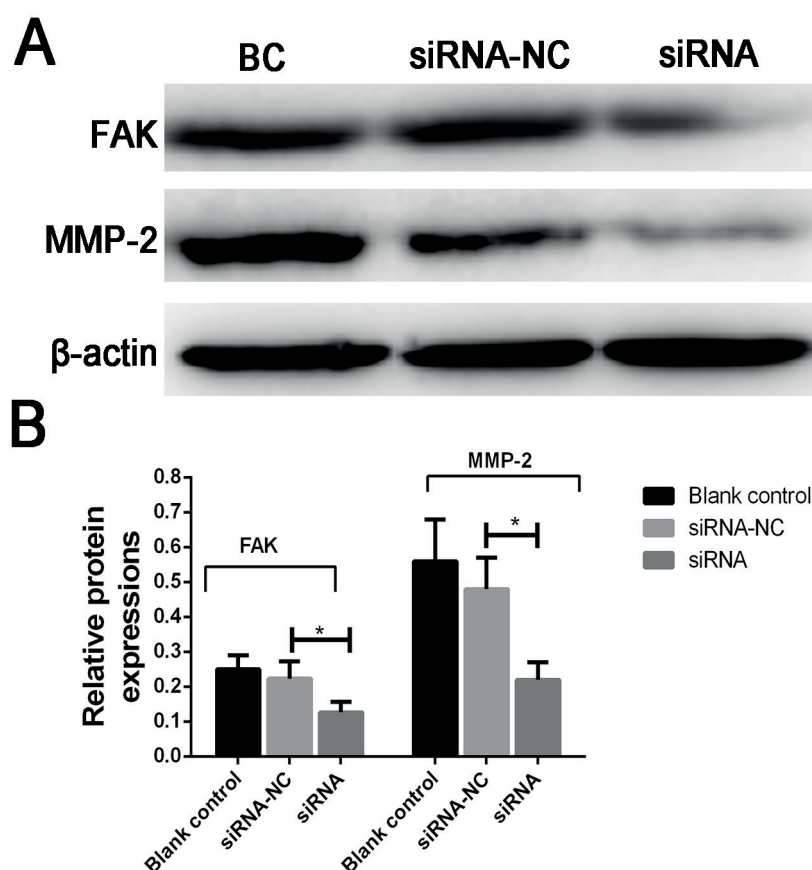


Figure 5. FAK and MMP2 protein level by Western blot. BC means Blank control (wild type Lncap cells); siRNA-NC refers to siRNA-negative control; siRNA refers to siRNA- SCCRO. *, $p < 0.05$.

The growth of Lncap cells was inhibited through the inhibition of SCCRO expression by RNA interference in Lncap cells. Transwell experiments proved that the migration and invasion of Lncap cells were also inhibited. About the mechanism of proliferation-inhibiting effect, Sarkaria et al²³ suggest that inhibition of SCCRO by siRNA can promote the cell apoptosis. The oncogenic potential of SCCRO is underscored by its ability to transform fibroblasts (NIH-3T3 cells) *in vitro* and *in vivo*²³. Studies²³ show that SCCRO is a new molecule of hedgehog signaling pathway, which can regulate the Gli1 (a key molecule of hedgehog pathway), and participate in the malignant transformation of squamous cell lines.

FAK can promote the spread of epicyte and is highly expressed in a variety of tumors, which plays an important role in cell adhesion, migration, invasion and dependent growth²⁴. Moreover, it can promote cell growth and invasion of tumor cells by tyrosine phosphorylation. MMP-2 can degrade extracellular matrix components and

is an important downstream molecule in FAK signaling pathway, which is related to the invasion and metastasis of tumor^{25,26}. We confirmed that knocking down SCCRO could down-regulate protein level of both FAK and MMP2, which might be one of the mechanisms of SCCRO promoting cancer cell migration and invasion.

SCCRO is expected to become a new target for the treatment of PC, which may provide new ideas for the prostate cancer gene therapy and drug development in the future.

Conclusions

SCCRO gene was highly expressed in the prostate cancer tissues, and this higher expression was correlated with prognosis. Knocking down SCCRO gene could inhibit the proliferation, invasion, and migration of Lncap cells. Its underlying mechanism might be due to enhancing FAK and MMP-2 expression in PC cells.

Conflict of interest

The authors declare no conflicts of interest.

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