Ubiquitin ligase SMURF1 functions as a prognostic marker and promotes growth and metastasis of clear cell renal cell carcinoma

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Smad ubiquitin regulatory factor 1 (SMURF1), a recently identified E3 ubiquitin ligase, targets substrate proteins for ubiquitination and proteasomal degradation. Previous studies have reported that SMURF1 also functions as an oncogene in human cancers. However, the clinical value of SMURF1 and its role in clear cell renal cell carcinoma (ccRCC) are unknown. SMURF1 expression was analyzed in 100 cases of ccRCC and matched tumor-adjacent specimens. SMURF1 was prominently overexpressed in ccRCC specimens compared with tumor-adjacent specimens. Increased levels of SMURF1 were also observed in ccRCC cell lines. Clinicopathological detection verified that SMURF1 expression was associated with advanced tumor node metastasis stage, large tumor size and vascular invasion of ccRCC patients. Moreover, Kaplan–Meier analysis found that SMURF1 elevation led to adverse overall survival and disease-free survival. Multivariate Cox regression analysis revealed that SMURF1 expression was an independent marker for prognosis prediction. Further experiments illustrated that SMURF1 knockdown significantly inhibited growth and metastasis of 769P cells, while SMURF1 overexpression promoted proliferation, migration and invasion in OSRC-2 cells. Mechanistically, SMURF1 inversely regulated the expression of DAB2 interacting protein, which negatively mediated the activation of both the ERK/RSK1 and PI3K/AKT/mTOR pathways in ccRCC cells. Taken together, these results suggest that SMURF1 might be a promising biomarker and target for novel treatment of human ccRCC.

Renal cell carcinoma (RCC) represents one of the commonest malignancies characterized by an adverse clinical outcome [1]. Approximately 75% of RCC patients have the clear cell renal cell carcinoma subtype (ccRCC) [2]. Radical surgery might be the only hope for curing RCC in the stage of precursor lesions [3]. However, even after surgical resection, radiotherapy and chemotherapy provide little benefit [4]. Previous studies have identified that a few molecular markers were correlated with prognosis, but the mechanism of RCC is still unqualified [5]. Therefore, there is an urgent need to identify an oncogenesis-associated biomarker that would be helpful for developing novel treatments for RCC.

Abbreviations
ccRCC, clear cell renal cell carcinoma; DAP2IP, DAB2 interacting protein; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IHC, immunohistochemistry; mTOR, mechanistic target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PI3K, phosphoinositide 3-kinase; qRT-PCR, quantitative real-time polymerase chain reaction; RCC, renal cell carcinoma; RSK1, ribosomal S6 kinase 1; shRNA, small hairpin RNA; SMURF1, Smad ubiquitin regulatory factor 1; TNM, tumor node metastasis.
Smad ubiquitin regulatory factor 1 (SMURF1), a recently identified E3 ubiquitin ligase, targets substrate proteins for ubiquitination and proteasomal degradation [6]. Increasing evidence has shown that SMURF1 exerts a promoting effect in carcinogenesis by targeting downstream proteins for proteolysis. Epidermal growth factor-induced SMURF1 overexpression promotes breast cancer cell migration and invasion by targeting RhoA [7]. Tumor necrosis factor receptor-associated factor 4 is also reported to be a substrate protein of SMURF1 and promotes migration of breast cancer cells [8]. SMURF1 was identified as a potential oncogene and good candidate for therapeutic target of pancreatic cancer [9,10]. The overexpression of SMURF1 is observed in human colorectal cancer, and contributes to tumor progression and poor prognosis [11]. Several studies have reported the upstream regulator of SMURF1, IQ motif containing GTPase activating protein 1, promotes the ubiquitination and degradation of transforming growth factor β receptor II by facilitating the targeting of SMURF1 to the plasma membrane in hepatic stellate cells [12]. Casein kinase-2 interacting protein-1 suppresses colon cancer cell growth and migration by inhibiting SMURF1 synthesis and facilitating SMURF1 autodegradation [13]. Furthermore, SMURF1 is recognized as a direct target of miR-497 in ovarian cancer cells and acts as a pro-metastatic factor [14]. But, the clinical significance of SMURF1 and its role in human ccRCC remain poorly investigated.

This study showed that elevation of SMURF1 expression predicted poorer survival. We also showed that SMURF1 promoted ccRCC cell growth and metastasis in vitro. Notably, SMURF1 regulated the expression of DAB2 interacting protein (DAP2IP) as well as the activation of the extracellular signal-regulated kinase (ERK)/ribosomal S6 kinase 1 (RSK1) and phosphoinositide 3-kinase (PI3K)/AKT/mechanistic target of rapamycin (mTOR) pathways. In conclusion, this work provided the first evidence that SMURF1 was a significant biomarker and a potential target for ccRCC.

Materials and methods

Patients

One hundred cases of ccRCC and matched tumor-adjacent specimens were obtained from the Department of Urology, Taizhou Hospital of Zhejiang Province, Wenzhou Medical University between January 2009 and December 2011, with a median follow-up time of 37.4 months. Tissue specimens were conserved in liquid nitrogen for quantitative real-time PCR (qRT-PCR) and 10% formalin for immunohistochemistry (IHC) until use. Informed consent for all samples was obtained before use. All cases were primary ccRCC and were reviewed by a pathologist. Tumor grade was assessed according to the Fuhrman nuclear grade [15]. Clinicopathological information for the patients is represented in Table 1. ccRCC was localized in 89 patients, and 11 patients had distant metastases at the time of diagnosis. Metastasis occurred in 12 cases of ccRCC patients during follow-up and 38 patients had died at last follow-up. The metastasized patients received sunitinib. The Ethics Committee of Wenzhou Medical University approved this study according to the Declaration of Helsinki.

Cell culture and transfection

ccRCC-derived cell lines (769P and OSRC-2) and a normal renal cell line (HK2) were obtained from the Cell Bank of Shanghai Institute of Cell Biology (Chinese Academy of Medical Science, Shanghai, China). Cell lines were cultured in DMEM with 10% fetal bovine serum (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) with antibiotics (Sigma-Aldrich, St. Louis, MO, USA) in an incubator containing a 5% CO2 humidified atmosphere at 37 °C.

Small hairpin RNA (shRNA) targeting SMURF1 and non-targeting (NT) shRNA were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). shRNA vectors were transferred into cells using lipofectamine 2000 (Thermo Fisher Scientific) on the basis of the manufacturer's recommendation. Retroviral vector pMMP-SMURF1 was...

Table 1. The correlation between SMURF1 and clinicopathological features in clear cell renal cell carcinoma patients. TNM, tumor node metastasis.

| Characteristic | Total | SMURF1 expression | |
|---------------|-------|-----------------|-----|
| Age           |       |                 |     |
| < 60 years    | 44    | 31              | 13  | 0.515|
| ≥ 60 years    | 56    | 36              | 20  |     |
| Gender        |       |                 |     |
| Male          | 64    | 45              | 19  | 0.348|
| Female        | 36    | 22              | 14  |     |
| TNM stage     |       |                 |     |
| I + II        | 79    | 49              | 30  | 0.040*|
| III + IV      | 21    | 18              | 3   |     |
| Grade         |       |                 |     |
| G1 + G2       | 81    | 51              | 30  | 0.076|
| G3            | 19    | 16              | 3   |     |
| Vascular invasion |     |                 |     |
| Positive      | 82    | 51              | 31  | 0.029*|
| Negative      | 18    | 16              | 2   |     |
| Tumor size    |       |                 |     |
| < 5 cm        | 61    | 36              | 25  | 0.034*|
| ≥ 5 cm        | 39    | 31              | 8   |     |

*Statistically significant.
generated by inserting the cDNA into pMMP. Retrovirus packaging and transduction were described previously [16].

Immunohistochemistry

The tissues that were previously formalin-fixed and paraffin-embedded were sliced into 4 µm sections, and underwent deparaffinization and then rehydration. Antigen retrieval, suppression of endogenous peroxidase activity and 10% skim milk blocking were performed before primary antibody incubation. SMURF1 (Abcam, Cambridge, MA, USA) antibody was used as primary antibody overnight at 4 °C. The slides were subsequently incubated with peroxidase conjugated secondary antibody (ZSGB Bio, Beijing, China) for 90 min, and a solution of peroxidase-labeled polymer, 2,4-diaminobutyric acid, was used for signal development for 5 min. The sections were counterstained with hematoxylin followed by dehydrating and mounting. Staining intensity was scored manually by two independent experienced pathologists as follows: no staining = 0, weak staining = 1, moderate staining = 2 and strong staining = 3.

Quantitative real-time PCR

Total RNA was drawn using Trizol (Invitrogen/Thermo Fisher Scientific) from fresh-frozen tissues. cDNAs were synthesized from 2 µg total RNA using TIANScript RT Kit (Tiangen Biotech, Beijing, China). Quantitative real-time PCR was performed using the SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) in a final volume of 10 µL in an ABI 7300 Fast PCR machine (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was employed as a reference gene to normalize the expression of SMURF1 mRNA. Data are presented as relative quantification based on the calculation of $2^{-\Delta\Delta CT}$. $\Delta CT$ was derived from subtracting the Ct value of reference cDNA from the Ct value of the cDNA of interest. The primers used for target genes were purchased from Sangon Biotech (Shanghai, China). Primer sequences were as follows: SMURF1 forward, 5'-CTA CCA GCG TTT GGA TCT AT-3'; reverse, 5'-TGT CTC GGG TCT GTA AAC T-3'. GAPDH forward, 5'-CAA GCT CAT TTC CTG GTA TGA C-3'; reverse, 5'-CAG TGA GGG TCT CTC TCT CCC T-3'.

Proliferation assay

Cells after transfection were gathered for cell growth assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay) following the manufacturer's recommendation (Roche, Indianapolis, IN, USA). In summary, cells were grown in 96-well plates containing 100 µL DMEM per well. After transfection for 24, 48, 72 and 96 h, respectively, 10 µL of MTT was added to each well; after incubation for 4 h the medium was undocked and subsequently 150 µL DMSO was added per well. The results represent the average of three independent replicates. For the colony formation assay, 2000 ccRCC cells were cultured on six-well plates, and 14–21 days after seeding, cell colonies stained with crystal violet and counted.

Wound healing assay

ccRCC cells transduced with corresponding vectors were seeded in six-well plates to form a single confluent cell layer. A wound was made with 100 µL pipette tip in the confluent cell layer; 0 and 24 h after wound scratching, the width of the wound was photographed with a phase-contrast microscope.

Transwell invasion assay

We determined the cell invasion capacities by using Transwell chambers of pore size 8 µm (Corning Costar, Cambridge, MA, USA). Twenty-four hours after transduction, 5 × 10^4 cells were cultured in the 1 : 9 diluted Matrigel-coated (BD Biosciences, Franklin Lakes, NJ, USA) upper chamber with 250 µL serum-free DMEM, and 700 µL DMEM with 10% fetal bovine serum was added in the lower chamber. After 24 h, the cells were fixed with paraformaldehyde and those in upper chamber were removed. Cells in the lower chamber were subsequently stained using 0.1% crystal violet solution and photographed.

Western blotting

Cancer cells were dissociated in RIPA lysis buffer (P0013D) (Beyotime, Haimen, China) and PMSF (ST506) (Beyotime). Split products were centrifuged at 18 500 g, and the supernatants were collected. A Bradford Protein Assay Kit (P0006) (Beyotime) was used to analyze protein concentration, and the proteins were subject to 10% SDS/PAGE electrophoresis. After separation the proteins were transferred onto poly(vinylidene difluoride) membranes (Sigma-Aldrich), and the membranes were blocked with 5% skim milk (Guangming, Shanghai, China) and incubated with primary antibody at 4 °C overnight. Then, specimens were hatched with secondary antibody conjugated with horseradish peroxidase (Cell Signaling Technology, Beverly, MA, USA). Signals were detected using a horseradish peroxidase chemiluminescent kit (Thermo Fisher Scientific) using an optional CCD camera and image processing system (Bio-Rad, Hercules, CA, USA). GAPDH (G8140, US Biological, Swampscott, MA, USA) was used as a loading control. SMURF1, DAB2IP and p-ERK1 (Ser380) were purchased from Abcam. p-AKT (Ser473), AKT, p-mTOR (Ser2448), mTOR, p-ERK (Thr202/
Tyr204), ERK and RSK1 were obtained from Cell Signaling Technology.

**Statistical analysis**

Data were presented as the mean ± SEM and analyzed by PRISM 5 software (GraphPad Software, Inc., San Diego, CA, USA) and SPSS version 13 (SPSS, Chicago, IL, USA). The chi-squared test was employed to explore the association between two variables. Student’s t test and ANOVA were carried out to analyze continuous variables. Survival curves were constructed and differences among groups were calculated using the Kaplan–Meier method and log-rank test. The multivariate Cox regression analysis was performed to reveal the prognostic significance of SMURF1 expression. The value of $P < 0.05$ was considered to have statistical significance.

**Results**

**SMURF1 is overexpressed in ccRCC**

One hundred samples of ccRCC and matched tumor-adjacent specimens were detected by immunohistochemistry for SMURF1 staining. Sections with IHC score ≥ 1 were considered to show positive expression of SMURF1. Of the ccRCC tissues, 67% of the cases (score 1: 18 cases; score 2: 32 cases; score 3: 17 cases) showed positive staining of SMURF1, while SMURF1 signal was detected in only 35% (score 1: 15 cases; score 2: 16 cases; score 3: 4 cases) of samples of tumor-adjacent specimens ($P < 0.05$, Fig. 1). Next, qRT-PCR demonstrated that the level of SMURF1 mRNA in ccRCC tissues was up-regulated compared with matched tumor-adjacent specimens ($P < 0.05$, Fig. 2A). The expression of GAPDH mRNA was comparable in ccRCC and tumor-adjacent tissues (Fig. S1). Moreover, the levels of SMURF1 expression in two ccRCC cell lines (769P and OSRC-2) were significantly elevated over that in normal human renal cells (HK2) ($P < 0.05$, respectively, Fig. 2B). Thus, overexpression of SMURF1 potentiates an oncogenic role in ccRCC.

**Clinical significance of SMURF1 in ccRCC patients**

To explore the clinical value of SMURF1, we correlated SMURF1 expression with clinical parameters of ccRCC patients. As shown in Table 1, patients with advanced tumor node metastasis (TNM) stage, vascular invasion and large tumor size showed a significantly higher positive rate of SMURF1 expression ($P < 0.05$, respectively). These results verify that elevation of SMURF1 expression may lead to ccRCC carcinogenesis. Notably, Kaplan–Meier analysis indicated SMURF1 positive expression significantly related to shortened overall and disease-free survival in our specimens ($P < 0.05$, respectively, Fig. 2C,D). Furthermore, the multivariate Cox regression analysis revealed that SMURF1 expression was an independent marker for 5-year overall and disease-free survival ($P < 0.05$ for each, Table 2). Our data revealed that SMURF1 may act as a promising prognostic indicator for ccRCC patients.

**Fig. 1.** Immunohistochemistry of SMURF1 protein in clear cell renal cell carcinoma (ccRCC) and normal tissues. Representative immunohistochemical staining showing (A) normal kidney proximal tubular epithelium, and (B) weak, (C) moderate and (D) strong staining of ccRCC. Scale bar: 50 μm.
SMURF1 knockdown restrains ccRCC cell growth and metastasis

Because SMURF1 was overexpressed in ccRCC compared with normal, we speculated that the biological functions of SMURF1 may include participating in controlling cell growth and metastasis. To verify this hypothesis, SMURF1 expression was silenced by a specific shRNA in 769P cells (\(P < 0.05\), Fig. 3A). MTT and colony formation assays were used to analyze the effect of SMURF1 on cell growth of ccRCC. We found that loss of SMURF1 restrained cell growth as compared with control in 769P cells (\(P < 0.05\), Fig. 3B,C). All results support that SMURF1 is involved in ccRCC cell growth. To disclose the potential role of SMURF1 in the metastasis of ccRCC, we analyzed the migration and invasion of ccRCC cells using wound healing and transwell assays. The results suggested that SMURF1 knockdown caused a prominent reduction of migration and invasion of 769P cells as compared with control cells (\(P < 0.05\), Fig. 3D,E). Taken together, the results prove that loss of SMURF1 can dramatically hold up ccRCC cell growth and metastasis in vitro.

SMURF1 overexpression leads to enhanced growth and metastasis of ccRCC cells

Next, OSRC-2 cells were infected with empty vector (EV) and SMURF1 retroviruses. SMURF1 overexpression was confirmed by western blotting in OSRC-2 cells (\(P < 0.05\), Fig. 4A). Overexpression of SMURF1 notably enhanced the proliferation of OSRC-2 cells (\(P < 0.05\), Fig. 4B,C). Furthermore, wound healing assays indicated SMURF1 overexpression facilitated the migration of OSRC-2 cells (Fig. 4D). In addition, SMURF1 overexpressing OSRC-2 cells showed increased invasive ability compared with control cells (\(P < 0.05\), Fig. 4E). Our data disclose that SMURF1 promotes the tumor growth and metastasis of ccRCC cells.
The ERK/RSK1 and PI3K/AKT/mTOR signaling axis may be involved in the role of SMURF1

Previous research has identified that SMURF1 promotes cancer cell proliferation and migration by negatively regulating DAB2IP [17]. Furthermore, DAB2IP knockdown leads to the activation of the ERK/RSK1 and PI3K/AKT/mTOR pathways in RCC cells [18]. Thus, we explored whether the DAB2IP-mediated ERK/RSK1 and PI3K/AKT/mTOR signaling axis was involved in the role of SMURF1 in ccRCC. Interestingly, we found that the expression of DAB2IP was increased and the levels of phosphorylated AKT, mTOR, ERK and RSK1 were remarkably decreased after SMURF1 knockdown in 769P cells. SMURF1 overexpression led to a decreased level of DAB2IP and increased activation of the ERK/RSK1 and PI3K/AKT/mTOR signaling pathways (Fig. 5). Therefore, these results indicated that the DAB2IP-mediated ERK/RSK1 and PI3K/AKT/mTOR signaling axis...
might be involved in the function of SMURF1 in ccRCC cells.

**Discussion**

ccRCC is the most common subtype of RCC with increasing incidence, high metastatic potential and high mortality rate [19]. Accumulating evidence has demonstrated that SMURF1 participates in many malignancies [7,9,10,13,14,17]. Dysregulation of SMURF1 is a common event found in various cancers including pancreatic cancer [9,10], breast cancer [7,8], ovarian cancer [14] and colorectal cancer [13]. To the best of our knowledge, few articles in the literature have focused on the clinical value and role of SMURF1 in ccRCC up to now. Thus, further study of SMURF1 in ccRCC may be helpful in relation to this deadly disease. This work demonstrated the pattern of
SMURF1 expression in ccRCC carcinogenesis and its potential prognostic significance in ccRCC patients, and further investigated its effect and mechanism in ccRCC cells.

The present study identified SMURF1 as a promising biomarker for prognosis of ccRCC patients. The expression of SMURF1 in ccRCC tissue was notably elevated compared with the tumor-adjacent specimens, and its elevation correlated with advanced TNM stage, vascular invasion and large tumor size. Notably, multivariate Cox regression analysis revealed that SMURF1 expression was an independent marker for prognosis prediction. Additionally, we provided the first evidence that upregulation of SMURF1 in ccRCC was correlated with adverse outcome. Thus, SMURF1 potentially functions as a prognostic marker in ccRCC.

Then, we explored the biology of SMURF1 in ccRCC cells and showed that loss of SMURF1 expression could inhibit cell growth and metastasis in vitro. In turn, SMURF1 overexpression significantly promoted cell proliferation as well as migration and invasion in ccRCC cells. The prominent ability of SMURF1 to promote tumorigenesis supports that it plays an oncogenic role in ccRCC. Therefore, targeting SMURF1 may represent a favorable therapeutic strategy for ccRCC treatment. Next, we explored potential target genes participating step-wise. We have shown that loss of SMURF1 up-regulated DAB2IP expression and down-regulated phosphorylated AKT, mTOR, ERK and RSK1. In contrast, SMURF1 overexpression reduced DAB2IP expression and enhanced the activation of the ERK/RSK1 and PI3K/AKT/mTOR signaling pathways in ccRCC cells. The expression of DAB2IP mRNA is regulated by DNA methylation in RCC, and DAB2IP CpG1 confers a poor survival of patients [20]. Furthermore, DAB2IP has been previously identified as a tumor suppressor via restraining RCC cell growth and invasion by regulating the activation of the ERK/RSK1 and PI3K/AKT/mTOR pathways [21]. The ERK/RSK1 pathway plays a critical role in determining the response of RCC cells to mTOR inhibitors via regulating hypoxia-inducible factor 2α/p21 [18]. The mTOR pathway has been reported to be implicated in promoting RCC growth and metastasis [22]. In addition, Yang et al. [23] revealed that infiltrating macrophages increase RCC cell invasion through induction of the epithelial–mesenchymal transition and increased cancer stem cell-like populations via activation of AKT/mTOR signaling. Here, we provide a preliminary exploration showing that SMURF1 regulates the DAP2IP-mediated ERK/RSK1 and PI3K/AKT/mTOR pathways, resulting in the induction of growth and metastasis in ccRCC cells.

In conclusion, we have provided the first evidence that SMURF1 has an oncogenic function important for ccRCC. Firstly, our results demonstrated that SMURF1 expression was elevated in ccRCC cell lines and tissues. Then, our clinical data suggested that SMURF1 may be used as a novel predictor for ccRCC. Moreover, SMURF1 overexpression in ccRCC cells not only resulted in growth and metastasis, but also increased the activation of the ERK/RSK1 and PI3K/AKT/mTOR pathways via DAB2IP inhibition. Taken together, our results verify that SMURF1 may serve as a potential target for cancer therapeutics of ccRCC.

**Conclusions**

SMURF1 has been previously found to be implicated in cancer cell growth and metastasis, but its clinical value and biological function have remained poorly known in ccRCC. Here, we presented evidence that the level of SMURF1 in ccRCC tissues was notably
elevated compared with normal specimens. Accordingly, the levels of SMURF1 were obviously up-regulated in ccRCC cell lines compared with HK2 cells. The positive expression of SMURF1 was notably higher in patients with advanced TNM stage, vascular invasion and large tumor size. Importantly, survival analysis revealed that SMURF1 expression was an independent prognostic indicator for predicting overall and disease-free survival of ccRCC patients. Knock-down of SMURF1 in 769P cells prominently inhibited growth and metastasis of cancer cells in vitro, while SMURF1 restoration had opposite effects on these behaviors in OSRC-2 cells. Mechanically, SMURF1 exerted an oncogenic function by suppressing DAB2IP and subsequently enhanced the activation of the ERK/RSK1 and PI3K/AKT/mTOR pathways in ccRCC cells. Together this suggests that SMURF1 can potentially act as a prognostic predictor and a drug target for ccRCC patients.

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Author contributions

MK, LM, WL, XZ and FL carried out the cell biology and molecular biology experiments, participated in the sequence alignment and drafted the manuscript. MK and HY participated in the design of the study and performed the statistical analysis. HY conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Fig. S1. Expression of GAPDH mRNA in ccRCC and normal tissues.