Research paper

SH3BGRL2 inhibits growth and metastasis in clear cell renal cell carcinoma via activating hippo/TEAD1-Twist1 pathway

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ABSTRACT

Background: Clear cell renal cell carcinoma (ccRCC) is one of the most prevalent malignancies in the world, and tumor metastasis is still the main reason for disease progression. Accumulating evidence shows that SH3BGRL2 may play a key role in tumor progression and metastasis. However, the role of SH3BGRL2 in ccRCC has not been systematically investigated and remains elusive.

Methods: The clinical significance of SH3BGRL2 was evaluated by bioinformatic analysis and tissue microarray (TMA) samples. SH3BGRL2 expression was determined by RT-PCR, western blot and immunohistochemistry staining. Tumor suppressive effect of SH3BGRL2 was determined by both in vitro and in vivo studies. Western blot, chromatin immunoprecipitation assay and luciferase report assay were applied for mechanism dissection.

Findings: SH3BGRL2 was crucial for epithelial-mesenchymal transition (EMT) progression and metastasis in ccRCC. Clinically, SH3BGRL2 was identified as an independent prognostic factor for ccRCC patients. Gain- and loss-of-function results suggested that SH3BGRL2 played a critical role in cell proliferation, migration and invasion. Mechanistically, we found that SH3BGRL2 acted as a tumor suppressor through Hippo/TEAD1 signaling, then TEAD1 altered Twist1 expression at the transcriptional level via directly binding to its promoter region.

Interpretation: Our findings established that SH3BGRL2 performed as a tumor suppressor and modulator via Hippo/TEAD1-Twist1 signaling in ccRCC, and the alteration of SH3BGRL2 could serve as a functional response biomarker of tumor progression and metastasis in ccRCC.

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1. Introduction

Renal cell carcinoma (RCC) is one of the most common cancers in the world, accounting for 4.2% of all new cancer cases [1]. Clear cell renal cell carcinoma (ccRCC), which also known as kidney renal clear cell carcinoma (KiRCC), is the most common subtype of RCC and accounts for 80% of all kidney cancers [2]. Although several treatments may be beneficial for patients with localized ccRCC, but tumor metastasis is still the main reason for disease progression, and about 30% of patients will relapse after initially surgical resection [3, 4]. Therefore, exploring new molecules involved in ccRCC progression and metastasis is the key to finding new targets for ccRCC treatment. SH3BGRL2, was first identified in 2002, belong to SH3BGR family, which includes SH3BGR, SH3BGRL, SH3BGRL2 and SH3BGRL3 [5]. Recent study found the expression of SH3BGR family varies significantly at different developmental stages and organ types, suggesting that abnormal expression of SH3BGR members may lead to a variety of diseases [6]. However, biological functions of SH3BGR family members are still largely unclear. Previous study reported SH3BGR suppresses cell migration and angiogenesis in Kaposi’s Sarcoma [7]. Interesting, Wang et al. suggested SH3BGRL may be an oncogene in mice, but as a tumor suppressor gene in human [8]. Xu et al. found SH3BGRL3 as a novel prognostic biomarker is down-regulated in acute myeloid leukemia [9]. Our previous study and another group also found SH3BGRL3 could be as a biomarker in kidney and bladder.
cancer [10, 11]. SH3BGR2L, as a new member of the SH3BGR family, containing a Src homology 3 (SH3) and an ENA/VASP Homology 1 (EVH1) domain, located in the nucleus and in the perinuclear region [5]. Considerable evidence suggests that SH3 and EVH1 domain plays a central role in cell growth, adhesion and migration [12, 13]. FLNC, a well-known protein involved in mechanosensitive signaling, has been shown to be a downstream target of the Hippo signaling pathway [14]. The results of the present study, which is the first to investigate the biological mechanism of SH3BGR2L in ccRCC, are in agreement with previous studies [5, 10].

2. Materials and methods

2.1. Clinical tissues

Three independent cohorts of ccRCC surgical specimens were obtained from Shanghai Tenth People’s Hospital. For group 1, tissue microarrays (TMAs) of 112 ccRCC and 30 adjacent non-tumor tissues were collected. For group 2, 16 fresh clinical samples were collected for further PCR, western blot (WB) and Immunohistochemistry (IHC) detection. For group 3, 5 patients suffered recurrence were enrolled. Their matched adjacent normal tissues, primary tumor, and recurrent tumor tissues were collected for further WB assay. The tissue samples used for the study were confirmed to the 1975 Declaration of Helsinki and was approved by the ethics committee of Shanghai Tenth People’s Hospital, School of Medicine in Tongji University. Informed consent was obtained from all patients.

2.2. Cell culture and lentivirus transfection

HK2, A498, 769-P, ACHN, 786-O, Caki-1and Caki-2 were obtained from Cell Bank of the Chinese Academy of Sciences. HK2 cells were cultured in Keratinocyte Serum Free Medium and the other tumor cell lines were cultured in Dulbecco’s modified Eagle’s medium. All cells were supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and cultured in humidified chamber at 37 °C under 5% CO2. The lentivirus packaging were using 293T cells. Briefly, using ECL western blot kit. The detailed antibodies are listed in Supplemental Information, methods and materials.

2.3. Western blot

Western blot assay were performed as described previously [23]. Briefly speaking, cells were lysed in RIP A lysis buffer containing PMSF on ice. The concentrations of proteins were determined using a BCA protein kit (Beyotime, China) and whole lysates were mixed with 6 × SDS loading buffer, heated at 100 °C for 10 min. A total of 30 μg of protein from each sample were loaded, running and then transferred to PVDF membranes. Immunoreactive bands were visualized using ECL western blot kit. The detailed antibodies are listed in Supplemental Information, methods and materials.

2.4. RNA extraction and quantitative real-time PCR

Total RNA was extracted using an RNeasy mini kit (Qiagen, Germany). The RNA concentration was measured using Nanodrop 2000 (Thermo Scientific, USA). cDNA was synthesized using the Quantitect Reverse Transcription Kit (Qiagen) with the primers (Sangon Biotech, Shanghai, China) according to the manufacturer’s instructions. GAPDH was used to correct the difference in template input. The relative RNA expression was calculated using the 2^−ΔΔCT method. The detailed primers are listed in Supplemental Information, methods and materials.

2.5. Cell proliferation, colony formation, wound healing and Matrigel invasion assays

Cell proliferation was measured by CCK-8 Kit according to the manufacturer’s protocols. For colony formation assay, cells were seeded in 6-well plates at a density of 5 × 102 cells per well then
stained and photographed after 2 weeks. Wound healing assay were used to detect cell migration ability as previously describe [24]. Cell invasion assay were performed using matrigel-coated chamber (Corning, USA). In brief, \(5 \times 10^4\) ccRCC cells with serum-free media were seeded into the upper chambers and stained after 24 h.

### 2.6. Tissue microarray and immunohistochemistry

The production of tissue microarray (TMA) was as described previously [25]. Briefly, a 0.6 mm diameter cylinder was used to take the core from the representative area of each tissue and the tissue cylinder was placed in a paraffin block. Immunohistochemistry (IHC) experiments were performed as previously described [26]. In general, tissue sections were incubated with primary antibodies and placed at 4 °C overnight, then secondary antibodies were incubated at room temperature. IHC staining was independently assessed by three experienced pathologists. The immunoreactive score representing the proportion of positively stained tumor cells were graded as: 0 (<10%); 1 (11%–25%); 2 (26%–50%); 3 (51%–75%) and 4 (>75%). The intensity of staining was determined as: 0 (no staining); 1 (weak staining = light yellow); 2 (moderate staining = yellow brown); and 3 (strong staining = brown). A semiquantitative scoring criterion was used, in which the staining index (values 0–12) were calculated by multiplying the staining intensity and the positive cells proportion. Finally, cases were classified into two different groups: low expression cases (score 0–6) and cases with high expression (score 7–12).

#### 2.7. Chromatin immunoprecipitation assay

The CHIP assay were in accordance with the Cold Spring Harbor protocol [27]. Briefly speaking, cells were treated with 1% formalde-hyde at room temperature for 10 min to cross-link DNA. After sonica-tion, cross-linked chromatin was pre-cleared with A/G-agarose beads, then immunoprecipitated with anti-TEAD1 antibody over-night at 4 °C. For the negative control, IgG was used. Specific primers for amplifying the target sequence of the human Twist1 promoter are listed in Supplemental Information, Methods and Materials. PCR products were identified by agarose gel electrophoresis.

#### 2.8. Luciferase reporter assay

Human Twist1 promoter region was constructed into pGL3-basic vector (Promaga, USA). Then cells were seeded in 24-well plates and transfected with CdNA via Lipofectamine 3000 (Invitrogen). pRL-TK was used as negative control. The luciferase activity measured were based on the manufacturer’s manual (Promega).

#### 2.9. Xenograft tumor and metastasis assay

The animal experiment was approved by the animal committee of Tongji university. For tumor growth model, \(2 \times 10^6\) cells with Luciferase reporter were injected subcutaneously into the flanks of nude mice. The tumor growth was measured every week and mice were sacrificed four weeks later. The tumor volume was calculated by length \(\times\) width\(^2\) \(\times\) 0.5. To generate tumor metastasis model, \(2 \times 10^8\) cells were suspended in 100 \(\mu\)l of PBS and injected into the tail vein of nude mice. Metastases were monitored through IVIS Lumina II sys-tem (Caliper Life Sciences, Hopkinton, MA). The bilateral lung tissues were excised and photoed, then fixed with 4% paraformaldehyde at room temperature and analyzed by HE staining. Mouse survival was monitored after tumor transplantation.

#### 2.10. Statistical analysis

Statistical analysis was performed using SPSS 23.0 software (IBM, USA). For comparisons, the Chi-square (\(\chi^2\)), Student’s t-test and One way ANOVA were used, as appropriate. The optimal cut-off value was determined by a ROC curve analysis in MedCalc software (MedCalc, Korea). Survival curves were calculated using the Kaplan-Meier method and differences were assessed by log-rank test. The Cox proportional hazards model was used to determine risk factors, which were screened by univariate analysis. Statistical significance was indicated by p values *\(p < 0.05\), **\(p < 0.01\) and ***\(p < 0.001\).

### 3. Results

#### 3.1. The pattern and significance of SH3BGRL2 in TCGA

To determine the role of SH3BGRL2 in ccRCC, we firstly detected the expression level of SH3BGRL2 in TCGA and GEO database. Interestingly, SH3BGRL2 was downregulated in tumor tissues compared with normal kidney tissues from TCGA database (Fig. 1a left). In addition, decreasing of SH3BGRL2 was observed in ccRCC metastatic-PDX tissues when compared with primary-PDX tissues (Fig. 1a right). Subsequently, the TCGA data showed that patients with lower expression levels of SH3BGRL2 had worse survival (Fig. 1b) and shorter disease-free survival rate (Fig. 1c). Furthermore, statistical analysis revealed that SH3BGRL2 expression was correlated with TNM stage (Fig. 1d–f), pathology histologic grade (Fig. 1g), clinical stage (Fig. 1h) and tumor recurrence (Fig. 1i) in TCGA patients (Supplementary Table 1), and 3 studies in Oncomine (www.oncomine.org) also confirmed these findings (Supplementary Fig. 1a–h). Besides, we also found the expression level of SH3BGRL2 in ccA subtype was higher than ccB subtype (Supplementary Fig. 2), which suggested a better long-term prognosis [28]. More importantly, according to the pathological grade and clinical stage classification, patients in each stage or grade with SH3BGRL2 low expression had poorer survival with those with SH3BGRL2 high expression (Supplementary Fig. 3a–h).

To evaluate whether the expression levels of SH3BGRL2 was asso-ciated with prognosis of ccRCC patients, based on the optimal cut-off values of SH3BGRL2 (Supplementary Fig. 4a–b), the 533 ccRCC patients were divided into two groups: SH3BGRL2 high expression group and SH3BGRL2 low expression group (Supplementary Table 1). Univariate Cox proportional hazards regression analysis showed that advanced TNM stage, pathological grade, clinical stage, recurrence status and low expression of SH3BGRL2 were correlated with poorer overall survival (Fig. 1j, Supplementary Table 2). In addition, the multivariate Cox regression analysis revealed SH3BGRL2, combined with tumor metastasis and advanced clinical grade stage, were independent prognostic factors for ccRCC patients (Fig. 1k, Supplementary Table 2). These data suggested that SH3BGRL2 played a vital role in tumor progression and metastasis in ccRCC.

Moreover, analysis of TCGA database showed that SH3BGRL2 was significantly decreased in various types of human cancer, such as breast, uterus, esophagus, brain, blood, lung and skin cancer (Supple-mentary Fig.5a), and SH3BGRL2 down regulation correlated with poor prognosis in human cancers, including ovarian cancer, thyroid carcinoma, pheochromocytoma and paraganglioma, lung adenocar-cinoma, head-neck squamous cell carcinoma and breast cancer(Supple-mentary Fig.5b), which indicated that SH3BGRL2 might be a critical tumor suppressor gene in various tumors.

#### 3.2. Dysregulated SH3BGRL2 in ccRCC indicates a poor prognosis

Considering the SH3BGRL2 expression level in TCGA database was detected by RNA sequencing. Thus, we analyzed a large cohort of clinical samples both in RNA and protein level to verify the results in TCGA database. Consistently, SH3BGRL2 expression was lower in 62.5% ccRCC patients than paired adjacent normal tissues (Fig. 2a). WB assays also confirmed the RT-PCR findings (Fig. 2b). Moreover, the expression levels of SH3BGRL2 showed a decreasing trend in matched adjacent non-tumor tissue, primary tumor, and recurrence
samples from 5 recurrent patients (Fig. 2c), which consistent with TCGA analysis results.

Next, the correlation between SH3BGRL2 and ccRCC prognosis was evaluated. Immunohistochemistry (IHC) staining showed SH3BGRL2 expression was shrinking continuously in clinical Fuhrman stage (Fig. 2d and 2f), and also downregulated in 112 ccRCC tissues compared with 30 normal kidney tissues (Fig. 2e). Moreover, patients with lower SH3BGRL2 expression were correlated with the advanced tumor stage (Fig. 2g, Table 1) and metastasis status (Fig. 2h, Table 1). Kaplan–Meier analysis showed that the 5-year overall survival rate for ccRCC patients with lower SH3BGRL2 expression significantly reduced when compared with the patients with higher
SH3BGR2 expression (Fig. 2i). More importantly, expression of SH3BGR2 was correlated with OS \( (p = 0.031, \text{hazard ratio (HR)}= 0.329, \text{Table 2}) \), which was consistent with our previously TCGA results (Fig. 1k and 1l).

Taken together, combined public RNA-seq data with our clinical data, these findings indicated that downregulation of SH3BGR2 might play a potential role to promote the malignant progression of ccRCC.

3.3. SH3BGR2 inhibited proliferation, migration and invasion of ccRCC cells

Next, the biological functions of SH3BGR2 in ccRCC progression were investigated. In order to choose the most suitable RCC cell lines, we detected SH3BGR2 expression level in human renal proximal tubular epithelial cells HK2, clear cell renal cell carcinoma (ccRCC) line A498, 769-P, 786-O, Caki-1 and papillary renal cell carcinoma (pRCC) cell lines ACHN, Caki-2. Real-time PCR and western blot showed that SH3BGR2 mRNA and protein expression, respectively, were markedly downregulated in all RCC cell lines compared to primary normal HK2 cells (Fig. 3aa and 3b). At last, we chose to establish SH3BGR2 stable knockdown (choose the high efficiency SH1) in 786-O (Fig. 3c left, which has more endogenous SH3BGR2) and overexpression in A498 cell lines (Fig. 3c right, which has less endogenous SH3BGR2). Strikingly, CCK-8 assay showed SH3BGR2 depletion enhanced 786-O cell line proliferation (Fig. 3d left), and SH3BGR2 overexpression reduced it in A498 cell line (Fig. 3d right). The colony formation assay also demonstrated these findings (Fig. 3e and 3f). Results of both wound-healing assay and transwell assay revealed that the 786-O sh-SH3BGR2
and invasion. We next explored SH3BGR2 function in vivo. The 786-O/sh-SH3BGR2 cells were inoculated into the flank of nude mice. As In Vivo Imaging Systems (IVIS) showed, SH3BGR2 knocked-down significantly promoted tumor proliferation (Fig. 4a), evidenced by larger tumor volume (Fig. 4b) and heavier tumor weight (Fig. 4c).

To further explore the effects of SH3BGR2 in tumor metastasis, 786-O cells labeled with luciferase were injected into tail vein of nude mice. Increased luciferase signal in tumors of the sh-SH3BGR2 group was detected by the IVIS, showing SH3BGR2 knocked-down promoted ccRCC cell metastases (Fig. 4d). HE analysis also showed an increase of pulmonary nodules metastasis in sh-SH3BGR2 group compared with control group (Fig. 4e and 4f). Consistently, metastatic foci volume of the sh-SH3BGR2 group in lung and liver were larger than the control group (Fig. 4g and 4h), and more lung and liver metastases occurred in sh-SH3BGR2 group (Fig. 4i and 4j). More importantly, SH3BGR2 knocked-down impaired mouse survival (Fig. 4k). Collectively, these observations demonstrated that inhibition of SH3BGR2 might promote the growth and metastasis of ccRCC cells in vivo.

### 3.5. SH3BGR2 inhibited epithelial–mesenchymal transition (EMT) of ccRCC cells

To dissect the detailed mechanism of SH3BGR2, we analyzed the ccRCC RNA expression profiles from TCGA database. Interestingly, GO enrichment showed that SH3BGR2 might participate in membrane related processes, such as hippo signaling, cell junction and endothelium development (Fig. 5a; Supplementary Fig. 6a-f; Table S3). Gene set enrichment analysis (GSEA) showed SH3BGR2 expression was significantly positive with renal system process (Fig. 5b) and cell-cell junction (Fig. 5c), which means SH3BGR2 might play pivotal role in renal system and tumor EMT progression.

Previous studies reported that most of ccRCC were derived from renal tubular epithelial cells, and epithelial-mesenchymal transition (EMT), is a key event that occurs during the cancer invasion and metastasis with epithelial origin [29, 30]. Based on this, the expression of EMT markers was detected both in mRNA and protein level. The results indicated that ZO-1 and E-cadherin, which epithelial marker, were decreased, whereas the levels of mesenchymal markers including N-cadherin, Vimentin and Twist1 were all increased in 786-O sh-SH3BGR2 cells (Fig. 5d and f). Conversely, upregulated of SH3BGR2 in A498 cells led to upregulation of ZO-1 and E-cadherin but downregulation of N-cadherin, Vimentin and Twist1 (Fig. 5e and g). The immunofluorescence assay also confirmed these results (Fig. 5h).

Therefore, SH3BGR2 acted as a suppressor of EMT in ccRCC cells.

### 3.6. SH3BGR2 regulated ccRCC cell proliferation and EMT via Hippo signaling pathway

Hippo signaling pathway plays a vital role in many physiological processes including cell proliferation, differentiation, survival and metastasis [19, 31, 32]. Moreover, GO enrichment (Fig. 5a) and GSEA analysis (Fig. 6a) showed SH3BGR2 expression was positively with hippo signaling pathway. The pathway enrichment analysis by GeneMANIA [33] also showed that the hippo signaling pathway was significantly enriched with SH3BGR2 (Fig. 6b). Additionally, SH3BGR2 expression was positively correlated with LAT51 and LAT52 expression in TCGA database (Fig. 6c-d). Therefore, we wondered whether SH3BGR2 functioned in ccRCC through regulating hippo signaling pathway. As expected, western blot showed that LAT51, LAT52 and phosphorylation of YAP were significantly downregulated in 786-O sh-SH3BGR2 cells (Fig. 6e). Conversely, upregulating SH3BGR2 in A498 cells led to upregulation of ZO-1 and E-cadherin but downregulation of N-cadherin, Vimentin and Twist1 (Fig. 5e and g). The immunofluorescence assay also confirmed these results (Fig. 5h).

Therefore, SH3BGR2 acted as a suppressor of EMT in ccRCC cells.

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### Table 1

| Clinical characteristics | No. of patients (n = 112) | SH3BGR2 low (n = 64) | SH3BGR2 high (n = 48) | P value* |
|-------------------------|---------------------------|----------------------|-----------------------|---------|
| Age (years) ≤50        |                           |                      |                       | 0.544   |
|                         | 64                        | 35                   | 29                    |         |
|                         | >60                       | 48                   | 29                    |         |
| Gender                  |                           |                      |                       |         |
| Male                    | 74                        | 46                   | 28                    | 0.134   |
| Female                  | 38                        | 18                   | 20                    |         |
| Tumor size ≤4 cm        |                           |                      |                       | 0.784   |
|                         | 52                        | 29                   | 23                    |         |
| >4 cm                   | 60                        | 35                   | 25                    |         |
| Laterality              |                           |                      |                       | 0.091   |
| Left                    | 55                        | 27                   | 28                    |         |
| Right                   | 57                        | 37                   | 20                    |         |
| Fuhrman grade           |                           |                      |                       | 0.269   |
| G1-2                    | 47                        | 24                   | 23                    |         |
| G3-4                    | 65                        | 40                   | 25                    |         |
| T stage                 |                           |                      |                       | 0.001   |
| I-II                    | 71                        | 32                   | 39                    |         |
| III-IV                  | 41                        | 32                   | 9                     |         |
| Metastasis              |                           |                      |                       | 0.019*  |
| No. of patients (n = 112) | Yes                      | 18                   | 4                     | 14      |
|                         |                           |                      |                       |         |
|                         |                           |                       |                       |         |

* Statistically significant (p < 0.05).

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### Table 2

|                  | Univariate analysis | Multivariate analysis |
|------------------|---------------------|-----------------------|
|                  | HR (95% CI)         | P value               | HR (95% CI)         | P value               |
| Age (years) ≤50  | 1.00                | 0.012*                | 1.00                | 0.020*                |
| >50              | 2.500(1.229–5.290)  |                       | 2.500(1.152–5.423)  |                       |
| SH3BGR2 Low      | 1.00                | 0.001*                | 1.00                | 0.031*                |
| SH3BGR2 High     | 0.195(0.075–0.510)  |                       | 0.329(0.119–0.905)  |                       |
| Gender Male      | 0.782(0.360–1.701)  |                       | NA                  |                       |
| Female           | 1.00                | 0.536                 | NA                  |                       |
| Tumor size ≤4 cm | 1.00                | 0.430                 | NA                  |                       |
| >4 cm            | 1.338(0.649–2.761)  |                       | NA                  |                       |
| Laterality Left  | 1.00                | 0.283                 | NA                  |                       |
| Right            | 0.675(0.330–1.383)  |                       | NA                  |                       |
| Fuhrman grade G1-2| 1.00                | 0.002*                | 1.00                | 0.335                 |
| G3-4             | 4.598(1.756–12.036) |                       | 1.769(0.554–5.645)  |                       |
| T stage I-II     | 1.00                | -0.0001*              | 1.714(0.637–4.610)  | 0.286                 |
| III-IV           | 6.134(2.730–13.784) |                       |                     |                       |
| Metastasis No. of patients (n = 112) | Yes                  | 7.739(3.743–16.000)  | 4.233(1.766–10.148) | 0.001*               |

CI confidence interval, HR hazard ratio.

* Statistically significant (p < 0.05).
Consistent with those studies, SH3BGRL2 knockdown promoted (Fig. 6g and 6h) and overexpression of SH3BGRL2 inhibited (Fig. 6i and 6j) nuclear translocation of YAP and TEAD1 enrichment. Furthermore, LATS1 and LATS2 were significantly decreased in metastasis ccRCC (Fig. 6k and 6n), advanced pathological grade (Fig. 6i and 6o) and clinical stage (Fig. 6m and 6p) in TCGA database. The Kaplan–Meier analysis also revealed that patients with higher LATS1 or LATS2 expression in ccRCC had significantly long OS and DFS (Supplementary Fig. 7a-b).

To illustrate whether hippo signaling pathway was critical for SH3BGRL2 mediated ccRCC cells growth and metastasis, we treated 786-O/sh-SH3BGRL2 and A498/OE-SH3BGRL2 cells with YAP-TEAD1 specific inhibitor Peptide 17 and OE-TEAD1. The results revealed that the Peptide 17 or OE-TEAD1 significantly alleviated...
or promoted the effects of SH3BGRL2 on proliferation (Fig. 7a and 7b, Supplementary Fig. 8), migration (Fig. 7c and 7d) and invasion (Fig. 7e and 7f, Supplementary Fig. 9) in ccRCC cells. Meanwhile, western blot assay indicated that the EMT related marker protein level were reversed in 786-O sh-SH3BGRL2 cells treated with Peptide 17(Fig. 7g) and in A498 cells co-transfected with SH3BGRL2 and TEAD1(Fig. 7h). Collectively, these results demonstrated that SH3BGRL2 inhibited the proliferation, migration and invasion abilities of ccRCC cells through activating LATS1/2-YAP-TEAD1 signaling pathway.

Fig. 4. SH3BGRL2 suppressed the growth and metastasis of ccRCC cells in vivo. a. Representative images of BALB/c nude mice injected with 786-O cells subcutaneously. b. Analysis of tumor volume of mice measured every week (n = 4 per group). c. Analysis of tumor weight of xenograft tumors(n = 4 per group). d. Representative images of metastasis by an in vivo bioluminescence imaging system. e. HE images of pulmonary micro-metastases. f. the number of pulmonary metastasis in each group. g-h. Macroscopic appearance of lung and liver metastatic nodule. i-j. The number of mice with lung(i) and liver(j) metastases in each group (n = 8 per group). k. Mice survival curves (n = 8 per group). Data are given as mean ± SD.*P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t-test, Chi-square test).
3.7. TEAD1 promoted EMT by transcriptional up-regulation TWIST1

TEAD1, as a co-transcriptional activator of YAP, mediates YAP-induced cell growth, oncogenic transformation, and EMT in many cancers [35-37]. Our above data indicated SH3BGRL2 inhibited ccRCC cells EMT through YAP-TEAD1 pathway. In addition, we found SH3BGRL2 (a) influence Twist1 expression at the mRNA and protein levels (Fig. 5d-g). In particular, Twist1, as a key regulator in the EMT program [38], were much higher in ccRCC tumor tissues than in adjacent normal tissues (Fig. 8a). Moreover, Twist1 expression was higher in advanced pathological grades (Supplementary Fig. 10a) and clinical stages (Supplementary Fig. 10b). The Kaplan–Meier analysis also revealed that patients with higher Twist1 expression in ccRCC had significantly short OS (Fig. 8b) and DFS (Fig. 8c). Thus, these results suggested that Twist1 is a key factor of EMT in ccRCC and Twist1 might the target gene of the co-transcriptional activators YAP/TEAD1.

To test this hypothesis, we firstly analyzed the Twist1 promoter using JASPAR database [39] to search for potential TEAD1 response elements. Later, we found SH3BGRL2 promoted EMT by transcriptional up-regulation TWIST1.

Fig. 5. SH3BGRL2 inhibited epithelial–mesenchymal transition (EMT) of ccRCC cells. a. Chord graph representing 18 differentially expressed genes between SH3BGRL2 high expression group and SH3BGRL2 low expression group and the association of these genes to the corresponding raft-related GO terms. b–c. GSEA plot showed SH3BGRL2 level was positively correlated with renal system process (b) and cell-cell junction (c) in the TCGA dataset. d–e. mRNA expression of EMT-related markers were detected in 786-O cells (d) and A498 cells (e). f–g. Protein expression of EMT-related markers were detected in 786-O cells (f) and A498 cells (g). h. Immunofluorescence assays detected ZO-1 expression in 786-O and A498 cells. Data are given as mean ± SD.*P < 0.05 (Student’s t-test).
Fig. 6. SH3BGRL2 regulated ccRCC cell proliferation and EMT via Hippo signaling pathway. a. GSEA plot showed SH3BGRL2 level was positively correlated with Hippo signaling pathway in TCGA dataset. b. GeneMANIA analysis of SH3BGRL2 regulated hippo pathway genes. c-d. TCGA database indicated SH3BGRL2 was positively associated with LATS1(e) and LATS2(d). e-f. The protein expression of LATS1, LATS2, YAP and TEAD1 in 786-O cells (e) and A498 cells (f). g, i. Immunoblotting analysis of YAP in cytoplasm and nucleus in 786-O cells (g) and A498 cells (i) separately. h, j. Co-immunoprecipitation with precipitating YAP or IgG antibodies and immunoblotting with YAP and TEAD1 in 786-O cell (h) and A498 cell (j). k, n. Compared the expression of LATS1(k) and LATS2(n) in adjacent normal tissue, primary-tumor and metastasis tumor tissues in TCGA database. i-m. The expression of LATS1 in different grades(i) and stages(m) in TCGA database. o-p. The expression of LATS2 in different grades(o) and stages(p) in TCGA database. (Student's t-test, one-way ANOVA).
Fig. 7. SH3BGR12 suppressed ccRCC proliferation, migration and invasion via modulating Hippo signaling. a-f. Colony formation assay(a, b), wound healing assay(c, d) and transwell assay(e, f) using YAP-TEAD1 specific inhibitor Peptide17 or overexpressing TEAD1 to demonstrate the functional connection of SH3BGR12 and Hippo signaling pathway in ccRCC cell lines. g-h. Western blot demonstrated that inhibit TEAD1 could rescue the EMT progression after knockdown of SH3BGR12 in 786-O cells(g), while promotion of TEAD1 could partially hinder the decreasing of EMT protein levels after overexpressing SH3BGR12 in A498 cells(h). Data are given as mean ± SD.*P < 0.05, **P < 0.01 (Student’s t-test).
elements (TEAD1Es) within proximal 1Kb promoter region (Fig. 8d), and found six putative TEAD1Es (Fig. 8e). ChIP assay revealed that TEAD1 could bind to TEAD1E I and TEAD1E V, but not TEAD1E II, III, IV or VI (Fig. 8f). Then, we cloned this 1 kb SH3BGRL2 promoter into pGL3-basic luciferase reporter vector (Fig. 8g). The results revealed that OE-TEAD1 significantly increased the wild-type and Mut1 group promoter luciferase reporter activity, but no change was observed in Mut2 group or Mut1&2 group (Fig. 8h). In contrast, adding Peptide 17 could decrease the luciferase reporter activity (Fig. 8i), suggesting TEAD1 modulated the Twist1 expression via binding to its promoter.

To investigate the clinical correlation between SH3BGR2, hippo pathway and Twist1, immunohistochemical staining (IHC) were tested in ccRCC and adjacent normal tissues (Fig. 9a). We found that SH3BGR2, LATS1/2 and P-YAP tended to have higher expression in normal tissues, while the expression of the TEAD1 and Twist1 were higher in ccRCC samples (Fig. 9b). Pairwise correlation showed...
Fig. 9. Associations of SH3BGRL2 expression with Hippo signaling and Twist1 in ccRCC. a. Representative images of IHC staining for SH3BGRL2, LATS1/2, P-YAP, TEAD1 and Twist1 in ccRCC tissues and paired normal tissues. b. The IHC score of SH3BGRL2, LATS1/2, P-YAP, TEAD1 and Twist1 in ccRCC tissues and paired normal tissues (n = 16). Data are given as mean ± SD, (Student’s t-test). c. Pairwise Spearman rank correlation among SH3BGRL2, LATS1/2, P-YAP, TEAD1 and Twist1 expression. Blue indicates positive and red negative correlation as reflected by the color legend below. d. Schematic representation for the mechanism of SH3BGRL2/Hippo-TEAD1 axis as a switch that regulates EMT in human ccRCC progression and metastasis by targeting Twist1 network.
SH3BGR2 was positively correlated with LATS1/2, P-YAP, and was negatively correlated with TEAD1 and Twist1 (Fig. 9c).

4. Discussion

Tumor metastasis leads to a poor prognosis for patients with ccRCC, but the mechanisms are largely unknown. Accumulating evidence indicates that SH3BGR2 might play critical roles in the initiation and progression of human cancers [15, 40, 41]. However, the biological function and molecular mechanisms of SH3BGR2 in ccRCC still elusive. In this study, we firstly reported that SH3BGR2 was downregulated in ccRCC tumor, especially in metastatic tissue, and was associated with poor clinical prognosis. Moreover, overexpression or knockdown SH3BGR2 could suppress or promote ccRCC cell proliferation and metastasis both in vitro and in vivo. Mechanically, SH3BGR2 activated Hippo-TEAD1 signaling and suppressed the transcription of Twist1 to intermediate ccRCC cells metastasis.

SH3BGR2, located on chromosome 6q13-15 [5], contains four exons and three introns spread over 72 Kb of genomic DNA. This genomic region is associated with several solid tumors, such as prostate cancer [42], hepatocellular carcinoma [43] and breast cancer [44]. Previous studies showed that SH3BGR2 was downregulated in esophageal squamous cell carcinoma [14], anaplastic thyroid carcinoma [45], ovarian chemotherapy resistance cells [15] and endometrial stromal cells [46]. Our study also found SH3BGR2 was downexpression in ccRCC tumor than adjacent normal tissues. Clinically, the level of SH3BGR2 in tumor tissues of ccRCC patients with metastasis or recurrence were lower than those of patients without, and downregulation of SH3BGR2 significantly correlated with adverse pathological and clinical features. In addition, Kaplan-Meier survival curves showed that ccRCC patients with lower SH3BGR2 expression had poorer overall survival and disease-free survival. Furthermore, the effects of SH3BGR2 on tumor growth and metastasis were directly shown in our in vitro and in vivo studies. These clinical and experimental evidences suggested that SH3BGR2 could be involved in ccRCC progression and metastasis and could be used as an independent prognostic marker for patients with ccRCC.

The reason for the decrease of SH3BGR2 expression in ccRCC remains unclear. As we all known, most of ccRCC are associated with loss of von Hippel-Lindau tumor suppressor (VHL) function and deregulation of hypoxia (HIF) pathways [47, 48]. Considering SH3BGR2 contains two protein binding domains, a Src homology 3 (SH3) and an ENA/VASP Homology 1 (EVH1) domain [5]. We hypothesis that HIF and/or VHL might interacted with these two domains. Interestingly, previous study reported HIF could regulated VHL-PTP1B-Src signaling axis in metastatic RCC [49]. Tang et al. also found HIF-1α acted at downstream of TNF-α to inhibit VASP expression and to modulate the acute pulmonary inflammation process [50]. But the detailed relationship of HIF-VHL and SH3BGR2, needs to be further studied.

Accumulating studies suggests that Epithelial-Mesenchymal Transition (EMT) plays an important role in tumor invasion and metastasis [29, 51]. However, the driving force of the EMT of ccRCC remains poorly understood. Through functional studies and bioinformatics analyses, our study found SH3BGR2 target multiple EMT related genes including ZO-1, E-cadherin, N-cadherin and Twist1, and inhibited the ccRCC EMT process. Twist1, a basic-helix-loop-helix transcription factor, is a key player in tumor metastasis by inducing EMT and promoting invadopodia-mediated extracellular matrix degradation [38, 52]. Previous studies found Twist1 could be as biomarker for prediction of poor prognosis in nasopharyngeal carcinoma [53], oral cancer [54] and esophageal squamous cell carcinoma [55]. Consistently, our study also found the levels of Twist1 in tumor tissues of ccRCC patients with metastasis were higher than those of patients without, and higher expression of Twist1 showed a significant correlation with advanced pathological and clinical stage. Kaplan-Meier survival curves showed that the higher expression of Twist1 correlated with poor patients’ prognosis, suggesting a pivotal role of Twist1 in ccRCC progression and metastasis.

The Hippo signaling pathway is a highly conserved tumor suppressor pathway [56], including LATS 1/2, YAP and TEAD [57], in which YAP-TEAD induced transcriptional responses are essential in proliferation and metastasis of cancer cells [58, 59]. It has been reported that LATS1/2 has decreased expression in many solid tumors, such as hepatocellular carcinoma [60], breast cancer [61] and gastric cancer [62]. Our finding also demonstrated LATS1/2 was significantly suppressed in ccRCC metastasis tissues compared with primary tumor tissues. In addition, lower expression of LATS 1/2 indicated worse overall survival and disease free survival rate, but the mechanism by which the hippo pathway was still largely unknown. Our key finding was identified SH3BGR2 as a novel regulator of the hippo pathway in ccRCC. The functional studies showed SH3BGR2 could suppress ccRCC cell growth and metastasis through interacting with LATS1/2-YAP-TEAD1 axis. Through CHIP assay and luciferase report assay, we found that YAP1 contributed to EMT progression by transcriptionally activating the Twist1 expression via binding to TEAD1.

In summary, our findings revealed that SH3BGR2 expression was significantly decreased in ccRCC and correlated with poor prognosis of ccRCC patients. Mechanistically (Fig. 9d), SH3BGR2 inhibited ccRCC growth and metastasis via activating Hippo-YAP-TEAD1 signaling pathway, then though transcriptionally downregulating Twist1 and shifting EMT progression. Collectively, our finding suggested that SH3BGR2 might have considerable potential as a prognosis predictor and therapeutic target for ccRCC.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Author contributions

B Peng and L Yin designed the experiments. L Yin executed the experiments. H Yang and L Yin drafted the manuscript. WJ Li assisted with animal breeding, KY Wang, H Shi, AM Xu assisted with clinical samples collection. RH Wang assisted the bioinformatics study. All authors participated in revising the manuscript and agreed to the final version.

Ethics approval and consent to participate

The tissue samples used for the study were conformed to the 1975 Declaration of Helsinki and was approved by the ethics committee of Shanghai Tenth People’s Hospital, School of Medicine in Tongji University. Informed consent was obtained from all patients. All of the mice experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of the Tongji University.

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Supplementary materials

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