CAV2 promotes the growth of renal cell carcinoma through the EGFR/PI3K/Akt pathway

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Background: Caveolin-2 (CAV2) is reported to have an important role in cancer. The following study investigated the expression and function of CAV2 in kidney cancer in vitro and in vivo.

Materials and methods: Real-time PCR, immunohistochemistry and Western blotting analysis were used to determine CAV2, epidermal growth factor receptor (EGFR), phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) in kidney cancer cell line OS-RC-2 and clinical specimens. The role of CAV2 in maintaining kidney cancer malignant phenotype was examined by wound healing assay, Matrigel invasion assays and mouse orthotopic xenograft model.

Results: Higher expression of CAV2 was found in renal cell carcinoma tissue compared to normal tissue. Furthermore, increased expression of CAV2 was associated with cancer progression. Also, silencing of CAV2 inhibited the proliferation, migration and invasion, as well as the expression of EGFR, PI3K and p-Akt in OS-RC-2 cells in vitro, and OS-RC-2 xenograft growth in vivo.

Conclusion: Our results revealed that CAV2 promotes the growth of renal cell carcinoma through EGFR/PI3K/Akt pathway.

Keywords: Caveolin-2, CAV2, EGFR, PI3K, p-Akt, renal cell carcinoma, invasion

Introduction
Renal cell carcinoma (RCC) is the most common type of renal malignant tumor. Currently, surgery is still considered as the main treatment approach for most types of RCC; even though its efficacy remains controversial.¹⁻³ Drug resistance is common and represents a major cause of RCC death. RCC progression is usually accompanied with uncontrollable proliferation, distant metastasis and recurrence.⁴⁻⁷ However, the exact molecular mechanism of RCC is still unclear and needs to be further investigated.

Caveolin-2 (CAV2) is a member of the caveolae family which has an essential role in intracellular cell transport and signal transduction.⁸ Higher expression of CAV2 has been associated with different types of cancer progression including lung, prostate, renal and breast cancer.⁹⁻¹² In breast tumor, CAV2 expression has been strongly associated with high histological grade¹³ and poor prognosis.¹⁴ Conflicting observations have been reported by Sagara et al, who found that CAV2 expression was suppressed in breast cancer tissues compared to normal tissues and that the reduced CAV1 was significantly associated with increasing tumor size.¹⁵ In addition, a positive correlation between plasma CAV2 levels and progression of prostate cancer¹⁶ and RCC¹⁷ were also observed. Nevertheless, the exact role of CAV2 in RCC remains unexplored.
In this study, we found an abnormal expression of CAV2 in RCC tissues. In addition, we found that silencing of CAV2 inhibits tumor biological behavior in vitro and in vivo through the EGFR/PI3K/Akt pathway.

**Materials and methods**

**Immunohistochemistry**

The tissue chip, including 86 cancer tissues and 10 normal tissues were purchased from US Biomax (cat no KD2085, Xi An, China). The flow of immunostaining was performed by the streptavidin-peroxidase method. The staining intensity was scored as 0, 1, 2 or 3, while percentage of stained cells was scored as 1 (<25%), 2 (26%–50%), 3 (51%–75%), or 4 (>75%). The final score was obtained by multiplying the intensity and percentage scores. The score below six scores were defined low expression. The score over six scores were defined high expression. The use of human samples was approved by the Ethics Committee of Chongqing Medical University.

**RT-PCR (reverse transcription-polymerase chain reaction)**

RT-PCR was done according to previously described method. Briefly, total RNA was isolated from cancer cells using TRIzol reagent (cat no T9424, Sigma-Aldrich Co., Aldrich, MO, USA) according to the manufacturer’s protocol. All-in-One First-Strand cDNA Synthesis Kit was used to reverse transcribe the total RNA into cDNA (cat no 1708890, Bio-Rad Laboratories Inc., Hercules, CA, USA). Real-time PCR was performed with All-in-One™ qPCR mix (GeneCopoeia, Guanzhou, People’s Republic of China). The following primers were used: CAV2 (cat no HQP054857) and GAPDH (cat no HQP070342) purchased from GeneCopoeia. The experiments were performed in triplicate in the same reaction, and the results of the RT-PCR experiments were analyzed using the 2^{-ΔΔCT} method.

**Cell culture and reagents**

Human RCC cells OS-RC-2 were acquired from Cell bank of Chinese Academy of Sciences (TCHu 40). The cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and streptomycin in a humidified atmosphere with 5% CO₂ at 37°C. siRNA sequences were synthesized by GenePharma Co., Ltd (Shanghai, People’s Republic of China). The following sequences were targeted for CAV2: CAV2-1: 5′-GCAAAUAUGUAUAUGUACAAGU-3′; CAV2-2: 5′-GGAGAUUGGGAUACUGUAUAU-3′; and negative control siRNA: 5′-UUCUUCGAAUGGUGUCACGU-3′.

**CCK-8 assay**

The cellular proliferation was determined by the CCK-8 assay (cat no CK04, Japan) according to the manufacturer’s instruction.

**Cell migration and invasion assays**

The cell migration and invasion assays were performed in accordance with our previous studies. Briefly, OS-RC-2 cells were cultured in a six-well plate until reaching 80% confluency. The medium was replaced with serum-free medium. After the wounding, the distance between two wounds was measured at 0 and 72 hours. The invasion assays were performed as follows: the upper side was coated using Matrigel basement membrane matrix for 2 hours at 37°C. The OS-RC-2 cells were added into the top chamber, and then incubated for 48 hours; 6% paraformaldehyde was then used to fix the invasive cells. They were then stained in 0.5% crystal violet (Beyotime) and counted.

**Western blot**

Western blot analysis was performed as previously described. Three independent experiments in a certain condition were subjected to Western blot analysis. The CAV2 (ab3417), EGFR (ab52894), PI3K (ab40776), Akt (ab38449) and GAPDH (ab8245) antibody were purchased from Abcam Inc. The antibody was dilution at 1:1,000.

**Animals**

BALB/c male nude mice, 6–8 weeks old, weighing 20–25 g, were obtained from Vital River Laboratories, China. All the animals were housed in an environment with temperature of 22°C ± 1°C, relative humidity of 50% ± 1% and a light/dark cycle of 12/12 hours. All animal studies (including the mice euthanasia procedure) were performed in compliance with the Accreditation of Laboratory Animal Care International (AAALAC) and Institutional Animal Care and Use Committee (IACUC) of Chongqing Medical University guidelines and approved by the IACUC of Chongqing Medical University. Mice were randomly divided in two groups: LV3-shCAV2-1 group (n=10) and LV3-NC group (n=10). Human RCC cells OS-RC-2 were infected with LV3-shCAV2-1 or LV3-NC and injected (5×10⁶ cells per mouse in 200 μL) subcutaneously into the left armpit of nude mice. Then, 21 days later, animals were sacrificed under isoflurane anesthesia.

**Statistical evaluation**

All values were expressed as mean ± SEM. Statistical analysis was performed by Student’s t-test. A P-value of <0.05 was considered statistically significant.
**Results**

**CAV2 expression was increased in RCC tissue**

The ONCOMINE database was used to investigate differential genes expression.\textsuperscript{25–27} In this research, three independent studies from the ONCOMINE database were conducted to analyze the expression of CAV2 in RCC and normal kidney tissues. Three independent studies (Higgins Renal, Gumz Renal and Jones Renal) showed that the expression of CAV2 was higher in RCC compared to normal kidney tissues (fold changes were 5.716, 5.312, 8.918, 8.432, 2.618 and 4.156, respectively) \((P=4.27E-12, 8.61E-19, 3.75E-4, 9.06E-4, 0.005 \text{ and } 0.002, \text{ respectively})\) (Figure 1A–F).

Furthermore, the evaluation of the CAV2 expression with OS was performed using the Human Protein Atlas online tool. Briefly, we found a significant correlation between high...
CAV2 and poor overall survival (OS) in patients with invasive RCC ($P=3.44\times10^{-3}$; Figure 1G).

The increased expression of CAV2 was associated with cancer progression

Next, we investigated the location and expression of CAV2 in RCC tissues and found that CAV2 was primary localized on the plasma membrane and cytoplasm (Figure 2A). In addition, the CAV2 expression was high staining in kidney carcinoma (Figure 2B). CAV2 in tubules cells was medium staining. However, CAV2 was not detected in glomeruli cells (Figure 2A). Furthermore, the CAV2 expression was correlated to tumor stage, and its expression was significantly higher in advanced stage (stage III/IV) compared to early stage tumor (stage I/II) ($P<0.05$; Table 1). Moreover, the expression of CAV2 significantly correlated with the tumor grade (grades 2–3 vs 1, $P<0.05$; Table 1). However, the associations between CAV2 expression and age were not significant ($P>0.05$; Table 1).

Silencing of CAV2 inhibited proliferation, migration and invasion of OS-RC-2

The expression of CAV2 was reduced in LV3-shCAV2-1 and LV3-shCAV2-2 infected OS-RC-2 cells compared with LV3-NC infected OS-RC-2 cells (Figure 3A). The cell proliferation, migration and invasion of OS-RC-2 cells infected with LV3-shCAV2-1 and LV3-shCAV2-2 decreased compared to cells infected with LV3-NC ($P<0.05$; Figure 3B–F).

CAV2 regulated EGFR/PI3K/Akt pathway

The EGFR/PI3K/Akt pathway has an important role in RCC. We further investigated the correlation between CAV2 and EGFR, PI3K and Akt using an online tool (http://gepia.cancer-pku.cn/detail.php). Our results showed that the expression of CAV2 in RCC was positively correlated with EGFR, PI3K and p-Akt (Figure 4A–C). In addition, we found that silencing of CAV2 reduces the expression of EGFR, PI3K and p-Akt in OS-RC-2 (Figure 4D and E).

Table 1 Association of CAV2 expression with clinicopathological characteristics in 86 patients of kidney cancer

| Characteristics | No of patients (n=86) | CAV2 expression | P-value |
|-----------------|----------------------|-----------------|---------|
| Age (years)     |                      |                 |         |
| $<50$           | 36                   | 19 (52.78%)     | 17 (47.22%) | $>0.05$ |
| $\geq50$        | 60                   | 34 (56.67%)     | 26 (43.33%) |         |
| Normal tissues  | 10                   | 10 (100%)       | 0 (0%)   | $<0.05$ |
| Cancer tissues  | 86                   | 43 (50.00%)     | 43 (50.00%) |         |
| FIGO stage      |                      |                 |         |
| I/II            | 70                   | 41 (58.57%)     | 29 (41.12%) | $<0.05$ |
| III/IV          | 16                   | 2 (12.50%)      | 14 (87.50%) |         |
| Grade           |                      |                 |         |
| 1               | 56                   | 37 (66.07%)     | 19 (33.93%) |         |
| 2               | 22                   | 5 (22.73%)      | 17 (77.27%) |         |
| 3               | 8                    | 1 (12.50%)      | 7 (87.50%)  |         |
| Grade 2–3 versus 1 |           |                 |         |

Abbreviations: CAV2, Caveolin-2; FIGO, International Federation of Gynecology and Obstetrics.
Silencing CAV2 inhibited the growth of OS-RC-2 cells in vivo

To investigate the role of CAV2 in vivo, OS-RC-2 cells infected with LV3-NC and LV3-shCAV2-1 were injected in nude mice. Briefly, the average tumor volume and weight were decreased in LV3-shCAV2-1 group compared to LV3-NC group ($P<0.05$) (Figure 5A–C). In addition, lower expression of EGFR, PI3K and p-Akt were found in tumors derived from LV3-shCAV2-1 compared to that in the LV3-NC group (Figure 5D).

Discussion

In this study, we found that CAV2 was abnormally expressed in RCC. The expression of CAV2 was related to the stage and grade of RCC, while high expression of CAV2 suggested a poor prognosis. We also found that CAV2 regulated the EGFR/PI3K/Akt signaling pathway.

Previous studies have found that CAV2 is associated with the occurrence and development of tumors. The expression of CAV2 has been observed in 5.9% of all breast cancer, while CAV2 expression has been reported as being strongly
CAV2 regulates the EGFR/Pi3K/Akt signaling pathway.

Notes: (A–C) The correlation between CAV2 and EGFR/Pi3K/AKT were analyzed using the Online Tool (http://gepia.cancer-pku.cn). (D) Silencing CAV2 inhibited the expression of EGFR, Pi3K and p-AKT. (E) The relative protein expression. Error bars represent standard error. *P<0.05; **P<0.001.

Abbreviations: CAV2, Caveolin-2; EGFR, epidermal growth factor receptor; Pi3K, phosphatidylinositol 3-kinase; Akt, protein kinase B.

CAV2 high expression was significantly correlated with poor OS in all patients with invasive RCC. Silencing of CAV2 caused reduction in cell proliferation and growth with retarded entry into the S phase. Our data revealed that silencing CAV2 inhibited the cellular proliferation, migration and invasion in RCC. Our results furthermore indicated that high expression of CAV2 promoted the progression of RCC.

The expression of EGFR correlates with prognosis in patients with clear cell RCC. Suppression of the EGFR signaling pathway retards RCC progression. The PI3K/AKT pathway is highly activated in RCC progression. This pathway is a promising drug target. In this research, we found that silencing CAV2 inhibited the expression of EGFR, Pi3K and p-AKT in vitro and in vivo. We also found that the expression of CAV2 was positively correlated with EGFR, Pi3K and AKT. Therefore, CAV2 may promote the malignant behavior through the EGFR/Pi3K/AKT pathway in RCC. Inhibition of this pathway could serve as a promising target in RCC.
To sum up, our findings indicated that CAV2 played a role in promoting the growth of RCC. Inhibition of the EGFR/PI3K/AKT signaling pathway could be used as a potential approach for the treatment of RCC.

Disclosure
The authors report no conflicts of interest in this work.

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