Pathological significance of C3aR signaling in the tumor cells of clear cell renal cell carcinoma: a clinical observation

CURRENT STATUS: UNDER REVIEW

Jing-Min Zheng
zhengjingmin@enzemed.com
Corresponding Author
ORCiD: https://orcid.org/0000-0002-2344-7089

Xiong Tian
Taizhou Hospital of Zhejiang Province
ORCiD: https://orcid.org/0000-0002-8354-8447

Mei-Fu Gan
Taizhou Hospital of Zhejiang Province

Hong-Yuan Yu
Taizhou Hospital of Zhejiang Province

Li-Cai Mo
Taizhou Hospital of Zhejiang Province

DOI:
10.21203/rs.3.rs-16351/v1

SUBJECT AREAS
Cancer Biology

KEYWORDS
Clear cell renal cell carcinoma, C3aR, C3a, Prognostic analysis
Abstract

**Background** Increasing evidences suggest that anaphylotoxin-induced signaling is involved in tumor pathogenesis, but the exact role of C3a/C3aR signaling in clear cell renal cell carcinoma (ccRCC) still remains to be investigated. The aim of the study was to investigate the pathological significance of C3a/C3aR signaling in ccRCC.

**Methods** The expression of C3aR and C3 mRNA in the tumor tissues of ccRCC patients were analyzed by using the data from TCGA database. The expression of C3aR and C3c protein in the tumor tissues of another 129 ccRCC patients were examined by immunohistochemistry.

**Results** Compared with the normal controls, both C3aR and C3 mRNA increased in the tumor tissues. Patients with higher C3 mRNA had shorter survival time. Immunostaining for C3aR and C3c also increased in the tumor tissues when compared with the adjacent normal renal tissues. Higher level of C3aR in the tumor cells and C3c in the tumor tissues were found to be associated with indices reflecting poor prognosis including higher tumor grade, the presence of necrosis in tumor tissues and shorter survival time. Besides, the level of C3aR in the tumor cells and C3c in the tumor tissues were found to correlate with the level of Vimentin, E-Cadherin and the ratio of Ki-67 positive tumor cells.

**Conclusions** These results support the idea that C3aR signaling is over-activated in the tumor cells and may contribute to the progression of ccRCC. Inducing EMT and promoting the proliferation of tumor cells might be among the mechanisms underlying the role of C3aR signaling in ccRCC.

**Background** Renal cell carcinoma (RCC) is one of the most common malignant tumors in the urinary system. It is among the top ten most commonly diagnosed cancers worldwide [1]. Clear cell renal cell carcinoma (ccRCC) is the major histological subtype of RCC, comprising more than 70% of all RCC cases [2]. ccRCC is commonly resistant to conventional chemotherapy and radiotherapy. Early surgical resection is the preferred therapy. However, up to 40% of ccRCC patients with localized disease will eventually suffer a relapse or develop metastatic disease even after nephrectomy [3–4]. In the last two decades, some adjuvant therapies including immunotherapy and target therapy have been introduced to treat patients with metastatic ccRCC. However, these therapies either have limited effect and severe side
role or develop resistance quickly. Clinically, improvement in the effect of ccRCC treatment is limited. Therefore, there is an urgent need to found new prognosis marker and therapeutic target for the disease.

The complement system is an effector arm of innate immunity, which is evolved as a safeguard against non-self elements and consists of more than 30 soluble and membrane-bound plasma proteins. It can be activated mainly through three pathways: the classical, the alternative and the lectin pathways. All the three pathways converge into the generation of C3 convertase, which splits complement component C3 into C3a and C3b. C3b binding to C3 convertase assembles the C5 convertase that cleaves C5 into C5a and C5b. In combination with C6, C7, C8 and C9, C5b assembles the membrane attack complex C5b-9, which mediates targeted cell lysis. Traditionally, complement activation is believed to be advantageous in tumor control. However, this longstanding dogma is challenged now. Increasing evidences from recent years suggest that complement enhances rather than inhibits tumor growth and metastasis and thus promote tumor progression [5–6]. It has been reported that the role of complement in promoting tumor progression is mediated mainly by anaphylotoxins, the bioactive complement activation products C3a and C5a. Of note, most of the previous studies are based on in vitro or animal experiments. The exact clinical significance and underlying mechanism still remain to be elucidated in specific diseases. Besides, most of the previous reports have been focused on the role of C5a/C5aR, studies about the role of C3a/C3aR in tumors is still limited.

The expression of C5a and its receptor C5aR1 in the tumor tissue of patients with ccRCC has been reported by several studies [7–9]. However, to our knowledge, no study has reported the clinical significance of C3a/C3aR signaling in patients with ccRCC. Here, we investigated the expression of C3aR and the production of its ligand in the tumor tissue of ccRCC patients and associated them with various clinicopathological indices.

Methods And Materials

Patients

Two cohorts of patients were included. The cohort of TCGA patients included 530 ccRCC patients with
RNA-seq data available in TCGA database (TCGA-KIRC). All the normalised mRNA expression data and clinical data were obtained from the TCGA website (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). mRNA expression data of 72 normal controls were also obtained from the database. This cohort included 344 male and 186 female patients with a median age of 61 years (ranging from 26 to 90 years).

The other cohort of patients included 129 patients, who were hospitalized at Department of Urology, Taizhou hospital, Wenzhou medical university from 2004 to 2013. All the patients were histologically confirmed ccRCC after partial or radical nephrectomy with no other malignancy history and no history of anticancer therapy. Patients with mixed histological type and died in a month after surgery were excluded. The patient cohort included 88 males and 41 females with a median age of 58 years (ranging from 24 to 78 years). Among them, 20 patients have lost follow-up. The clinical and pathological data were collected from medical records and follow-up records. The time interval between surgery and the date of death or the last visit was calculated and defined as overall survival (OS). The interval between primary surgery and death from ccRCC or the last follow-up visit was defined as disease-specific survival (DSS). For the analysis of disease-specific mortality, deaths as a result of other causes were censored. Informed consent has been obtained from all the participants. All research work with human participants was in accordance with the ethical standards of the responsible committee on human experimentation and with the Declaration of Helsinki. The Taizhou Hospital Ethics Committee approved the present study (No. K20191201).

**Immunohistochemical staining and analysis**

Immunohistochemical staining was performed on formalin-fixed Paraffin sections. Briefly, the sections were deparaffinised in xylene, rehydrated with graded ethanols, autoclaved for antigen repair and treated with 3% hydrogen peroxide solution to inactivate the endogenous peroxidase. After blocking for 30 min in 10% fetal calf serum and rinsed in PBS, the sections were incubated overnight at 4°C with different first antibodies, such as rabbit anti-human C3aR antibody (Catalogue number LS-C382362, LifeSpan, WA, USA, 1:200), rabbit antihuman C3c antibody (Catalogue number LS-B7932, LifeSpan, WA, USA, 1:200), rabbit anti-human E-Cadherin antibody (Catalogue number 20874-I-AP,
Proteintech, Wuhan, China, 1:400), mouse anti-human Vimentin antibody (Catalogue number YM6529, Immunoway, TX, USA, 1:500) and mouse anti-human Ki-67 antibody (Catalogue number YM6189, Immunoway, TX, USA, 1:300). Then, the sections were washed for three times, incubated with the second antibody for 30 min, washed again and developed with diaminobenzidine. Finally, each section was counterstained with haematoxylin. Normal homologous serum was used to replace the first antibody as a negative control.

According to the immunostaining intensity, the level of C3aR, C3c, E-Cadherin and Vimentin in each section was scored: 1, weak; 2, intermediate, 3, strong. In survival analysis, the level of C3aR and C3c was divided into lower and higher group according to the immunostaining intensity of C3aR in the tumor cells and C3c in the tumor tissues respectively. To estimated Ki-67 positive tumor cell ratio, fifteen non-overlapped high-power fields in each section were randomly selected and photographed. Then, the number of Ki-67 positive nucleus and total nucleus in each photograph were counted and the ratio of Ki-67 positive nucleus to the total nucleus was calculated. The average ratio of the fifteen pictures was obtained and used as Ki-67 positive tumor cell ratio for the section.

**Statistical analysis**

The data were analyzed with the SPSS software, version 17.0. Mann-Whitney U test was used to compare the difference between two groups while Kruskal-Wallis test was used to compare difference among three groups. The Spearman correlation analysis was used to explore the correlation of C3aR and C3c level (represented by score) with the clinicopathological indices and the level of other molecules. The Kaplan-Meier method and log-rank test were used for survival analysis. The Cox proportional hazards model was used to determine which variables influenced survival. The variables that significantly impacted survival in univariate analyses were included in multivariate analyses. All statistical tests were two tailed, and P values <0.05 were considered significant.

**Results**

**C3aR and C3 mRNA expression in the TCGA cohort and their association with overall survival**

A total of 611 files of normalized mRNA expression data were obtained. These include 539 files of
ccRCC patients and 72 files of normal controls. After integration of the mRNA expression data from the same patients (four patients were found to have three files each and one patient was found to have 2 flies), a total of 530 mRNA expression data corresponding to 530 ccRCC patients were obtained. Compared with the normal controls, the expression level of C3aR and C3 mRNA increased significantly in the tumor tissues of ccRCC patients (Figure 1A-B).

To analyze the association of C3aR and C3 mRNA level with overall survival, patients were divided into lower C3aR mRNA and higher C3aR mRNA, lower C3 mRNA and higher C3 mRNA group according to the median level of C3aR and C3 mRNA level, respectively. As shown in Figure 1C-D, patients with higher C3 mRNA level were found to have shorter overall survival while no significant difference in overall survival was observed between patients of lower C3aR mRNA and higher C3aR mRNA.

**Results of immunohistochemistry for C3aR and C3c in the tumor tissue of ccRCC patients**

Positive immunostaining for C3aR was observed in all the tumor tissue samples. As shown in Figure 2, immunostaining for C3aR could be observed in both infiltrating cells and cancer cells. C3aR was found to be distributed in the plasma membrane, cytoplasm and nucleus in the tumor cells. Compared with the normal renal tubular epithelial cells, the cells from which the tumor cells were believed to derive, the expression level of C3aR in the tumor cells of ccRCC patients increased significantly (Figure 2). C3aR can only exert its roles when it is activated. To determine whether C3aR in ccRCC cells could be activated, it is necessary to examine whether the ligand for C3aR, C3a, could be produced in the tumor tissues. As no proper antibody against C3a could be found, we examined the presence of C3c, a product of C3 activation in the tumor tissue of ccRCC patients. As shown in Figure 3, C3c was found extensively expressed in the tumor tissue of ccRCC patients. And compared with the normal renal tissues, the expression level of C3c in ccRCC tumor tissues increased markedly.

**Association of C3aR and C3c protein level with clinicopathological parameters**

The level of C3aR in the tumor cells and C3c in the tumor tissue of each patient were scored and their associations with the baseline clinical and pathological characteristics were examined. As shown in Table1, no significant difference in C3aR expression was observed among patients of different ages and genders. Also, the expression level of C3aR in ccRCC tumor cells was not significantly influenced
by tumor stage, tumor size and the presence of diabetes mellitus and hypertension. However, higher tumor cell C3aR level was observed in patients with higher Fuhrman grade and patients with necrosis within the tumor (Table 1). Results of bivariate correlation analysis also showed a significant correlation between the level of C3aR in the tumor cells and tumor grade (r=0.454, p<0.01) and the presence of necrosis in the tumor (r=0.298, p<0.001).

The level of C3c in the tumor tissues was also found to be associated with tumor grade and the presence of necrosis in the tumor tissue.

**Results of survival analysis based on C3aR level in the tumor cells and C3c level in the tumor tissue of ccRCC patients**

Patients were divided into lower C3aR group and higher C3aR group according to the immunostaining intensity for C3aR in the tumor cells, and lower C3c group and higher C3c group according to the immunostaining intensity for C3c in the tumor tissues. As shown in Figure 4, patients with higher C3aR level in the tumor cells or C3c in tumor tissues were found to have a shorter OS and DSS when compared with patients with lower C3aR or lower C3c. Analysis based on Cox regression revealed that higher C3c level in the tumor tissue is an independent risk factor along with tumor stage and tumor grade for both OS and DSS of ccRCC patients. However, the significance of the association of C3aR level with both OS and DSS disappeared in the multivariable Cox analysis (Table 2 and Table 3).

**Association of C3aR and C3c expression with E-Cadherin, Vimentin and Ki-67 in tumor tissues**

The upregulation of C3aR in tumor cells and increased activation of C3 (as reflected by increased C3c) in tumor tissues indicate that C3aR signaling is over-activated in the tumor cells of ccRCC patients. Previously, activation of C3aR was reported to be able to induce epithelial-to-mesenchymal transition (EMT) in tubular epithelial cells [10], the cells from which ccRCC was believed to derive. Besides, activation of C3aR has been reported to promote proliferation of glomerular mesangial cells [11] and cutaneous squamous cell carcinoma cells [12]. To determine whether C3aR signaling also contributes to EMT and proliferation in ccRCC cells, we further examined the expression of the molecular markers for EMT (E-Cadherin and Vimentin) and cell proliferation (Ki-67) in the tumor specimens from ccRCC
patients and associate them with the level of C3aR and C3c. As shown in Figure 5, all the three molecules are expressed by tumor tissues with immunostaining for Ki-67 was only observed in the nucleus. Results of spearman coefficient analysis revealed that the expression level of C3aR and C3c correlated positively with the level of Vimentin \( r=0.434, p<0.01; r=0.374, p<0.01 \); respectively) and the ratio of Ki-67 positive tumor cells \( r=0.428, p<0.01; r=0.252, p<0.01 \); respectively) and negatively with the level of E-Cadherin \( r=-0.287, p<0.01; r=-0.179, p=0.042 \); respectively).

**Discussion**

In the present study, we explored the clinical and pathological significance of C3aR signaling in ccRCC by examining the expression of C3aR and the production of its ligand in the tumor tissue of ccRCC patients and their association with the disease. C3aR was found increasingly expressed in both mRNA and protein level in the tumor tissue of ccRCC patients. It was found to be distributed mainly in the tumor cells. In the meanwhile, the expression of C3 mRNA and the immunostaining for C3c, a product of C3 activation, were also found to increase, indicating increased production of C3a, the ligand for C3aR, in the tumor tissues. These results suggest the presence of over-activation of C3aR signaling in the tumor cells of ccRCC patients and its involvement in the pathogenesis of ccRCC. In accordance with this, higher C3aR level in the tumor cells and C3c level in the tumor tissues were found to be associated with higher tumor grade and the presence of tumor necrosis, two indices that were thought to be associated with worse prognosis in ccRCC patients \[13-14\]. Indeed, patients with higher C3aR or C3c level were found to have shorter OS and DSS. And higher C3c level in the tumor tissues was found to be an independent risk factor for worse prognosis. Together, these results strongly suggested that C3aR signaling have important role in the regulation of ccRCC progression and higher C3aR in tumor cells and C3c in the tumor tissues might be used as markers of worse prognosis of ccRCC.

As a member of G protein coupled receptor, C3aR was initially found to be expressed by some immune cells and to play important role in the regulation of inflammation by recruiting and activating inflammatory cells \[15\]. However, increasing evidence from the last two decades has demonstrated that C3aR is also expressed by many non-immune cells and participates in various physiological and
pathological processes. For examples, C3aR signaling was reported to participate in the regulation of eye morphogenesis [16], neural development [17–18], embryonic chick retina [19] and cardiac resident cell [20] regeneration, astroglial cell differentiation [21] and survival [22], diet-induced obesity and metabolic dysfunction [23], and tau hyperphosphorylation in Alzheimer's Disease [24]. Also, C3aR signaling was found to be involved in the induction of inflammatory cytokines [25], proliferation of mesangial [11] and carcinoma cells [12] and EMT of tubular epithelial cells [10]. Recently, C3aR signaling was believed to be an important factor in maintaining cellular homeostasis [26–27].

In the field of oncology, signaling through C3aR has been suggested to promote tumor progression. However, variable results about the role of C3aR signaling in tumor cells have been reported in previous studies. In 2014, Cho et al first reported that tumor cell derived anaphylotoxins (C3a and C5a) promoted proliferation, migration and invasiveness of ovarian, uterine and lung cancer cells [28]. Later, Maurer et al reported that activation of C3aR could stimulate proliferation of medulloblastoma cells [29]. In addition, Fan et al reported that activation of C3aR promoted proliferation, migration and stemness in cutaneous squamous carcinoma [12]. However, in other studies [30–31], C3aR signaling was reported to contribute to tumor progression through regulating the recruitment and the function of immune cells rather than influencing tumor cells themselves directly. The discrepancy about the role of C3aR signaling observed in different tumor models might be explained by the heterogeneity of tumor cells, for an example, the different expression level of C3 in different tumor cells [31]. However, it indeed has reflected the complexity of C3aR function in tumor pathogenesis and underlined the importance of understanding the exact role of C3aR signaling in a specific context. For the first time, we investigated the clinical and pathological significance of C3aR signaling in ccRCC. The finding of the association of tumor cell C3aR and tumor tissue C3c expression with the progression of ccRCC has stressed a direct role of C3aR signaling in tumor cells, through which over-activation of C3aR may contribute to the progression of ccRCC.

According to the functions of C3aR signaling revealed in previous studies, especially the roles of C3aR signaling in the regulation of EMT and cell proliferation, we supposed that C3aR signaling might also
have a role in inducing ccRCC tumor cell’s EMT and proliferation. To test this supposition, in the present study, we examine the association of C3aR and C3c with the expression level of E-Cadherin (an extensively accepted molecular marker for epithelial cells), Vimentin (a molecular marker for mesenchymal cells) and Ki-67 (a molecular marker for proliferating cells) in tumor cells. In accordance with our supposition, the expression level of C3aR and C3c was found to correlate positively with the level of Vimentin and negatively with the level of E-Cadherin, supporting the involvement of C3aR signaling in ccRCC tumor cell’s EMT. Also, tumor cell C3aR and C3c level was found to correlate positively with the ratio of Ki-67 positive tumor cells. Thus, we believe that probably through inducing proliferation and EMT in tumor cells, C3aR signaling contributes to the progression of ccRCC.

Limitations are present in the present study. First, in the analyzing of C3aR and C3 mRNA expression, we only analyzed the data from TCGA database. No further proving work, such as quantitative RT-PCR, was done. Secondly, in the analyzing of C3aR and C3c protein expression, only patients from our hospital were included and the patient population involved is somehow small. Given the high heterogeneity of ccRCC, bias due to both a single center study and a small population may be inevitable.

Conclusions
In summary, for the first time, the present study examined the pathological significance of C3aR signaling in the tumor cells of ccRCC patients. Our results indicated that C3aR signaling is over-activated in ccRCC tumor cells and might contribute to the progression of the disease. Promoting the proliferation and inducing EMT of the tumor cells might be two mechanisms underlying the role of C3aR signaling in ccRCC. High level of C3aR in tumor cells and C3c in tumor tissues might be used as markers for the worse prognosis of the disease and C3aR signaling pathway might be a useful target for treatment. However, further studies are still needed to confirm our hypothesis.

Declarations
Acknowledgements
The authors thank H. X. Zhou, who is affiliated to the Department of Urology, Taizhou Hospital, for her help in clinical data collection.
Author contributions JMZ designed the study. JMZ and XT performed the experiments, analyzed the data, and wrote the manuscript. HYY and LCM contributed to the collection of tissue specimens and the clinical datasets, and the revision of the manuscript. MFG contributed to the pathological analysis and the revision of the manuscript.

Funding
Not applicable

Availability of data and materials
All data generated or analyzed during this study are included in this article

Ethical approval and consent to participate
The experimental applications of patient specimens were approved by The Taizhou Hospital Ethics Committee (No. K20191201). Informed consent has been obtained from all the participants.

Consent of publication
All Authors have seen and approved the manuscript and consent publication.

Competing interests
The authors declare that they have no competing interests.

Abbreviations
ccRCC: clear cell renal cell carcinoma; OS: overall survival; DSS: disease-specific survival; EMT: epithelial-to-mesenchymal transition; HR: hazard ratio; CI: confidence interval.

References
1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin. 2018; 68(1):7-30. doi: 10.3322/caac.21442.
2. Linehan WM, Walther MM, Zbar B. The genetic basis of cancer of the kidney. J Urol, 2003;170(6 Pt 1):2163-2172.
3. Diamond E, Molina AM, Carbonaro M, Akhtar NH, Giannakakou P, Tagawa ST, Nanus DM. Cytotoxic chemotherapy in the treatment of advanced renal cell carcinoma in the era of targeted therapy. Crit Rev Oncol Hematol. 2015; 96(3):518-26. doi: 10.1016/j.critrevonc.2015.08.007
4. Blanco AI, Teh BS, Amato RJ. Role of radiation therapy in the management of renal cell cancer. Cancers (Basel). 2011; 3(4): 4010-23. doi: 10.3390/cancers3044010.

5. Afshar-Kharghan V. The role of the complement system in cancer. J Clin Invest. 2017;127 (3): 780-789. doi: 10.1172/JCI90962.

6. Ajona D, Ortiz-Espinosa S, Pio R. Complement anaphylatoxins C3a and C5a: Emerging roles in cancer progression and treatment. Semin Cell Dev Biol. 2019; 85: 153-163. doi: 10.1016/j.semcdb.2017.11.023. Epub 2017 Nov 23.

7. Xi W, Liu L, Wang J, Xia Y, Bai Q, Long Q, Wang Y, Xu J, Guo J. High Level of Anaphylatoxin C5a Predicts Poor Clinical Outcome in Patients with Clear Cell Renal Cell Carcinoma. Sci Rep.2016; 6: 29177. doi: 10.1038/srep29177.

8. Maeda Y, Kawano Y, Wada Y, Yatsuda J, Motoshima T, Murakami Y, Kikuchi K, Imamura T, Eto M. C5aR is frequently expressed in metastatic renal cell carcinoma and plays a crucial role in cell invasion via the ERK and PI3 kinase pathways. Oncol Rep. 2015; 33(4): 1844-50. doi: 10.3892/or.2015.3800.

9. Xi W, Liu L, Wang J, Xia Y, Bai Q, Xiong Y, Qu Y, Long Q, Xu J, Guo J. Enrichment of C5a-C5aR axis predicts poor postoperative prognosis of patients with clear cell renal cell carcinoma. Oncotarget. 2016; 7(49): 80925-80934. doi: 10.18632/oncotarget.13108.

10. Tang Z, Lu B, Hatch E, Sacks SH, Sheerin NS. C3a mediates epithelial-to-mesenchymal transition in proteinuric nephropathy. J Am Soc Nephrol. 2009;20(3):593-603. doi: 10.1681/ASN.2008040434.

11. Zhang Y, Yan X, Zhao T, Xu Q, Peng Q, Hu R, Quan S, Zhou Y, Xing G. Targeting C3a/C5a receptors inhibits human mesangial cell proliferation and alleviates immunoglobulin A nephropathy in mice. Clin Exp Immunol. 2017; 189 (1): 60-70. doi: 10.1111/cei.12961.
12. Fan Z, Qin J, Wang D, Geng S. Complement C3a promotes proliferation, migration and stemness in cutaneous squamous cell carcinoma. J Cell Mol Med. 2019; 23(5): 3097-3107. doi: 10.1111/jcmm.13959.

13. Taneja K, Williamson SR. Updates in Pathologic Staging and Histologic Grading of Renal Cell Carcinoma. Surg Pathol Clin. 2018; 11(4): 797-812.

14. Kim H, Inomoto C, Uchida T, Furuya H, Komiyama T, Kajiwara H, Kobayashi H, Nakamura N, Miyajima A. Verification of the International Society of Urological Pathology recommendations in Japanese patients with clear cell renal cell carcinoma. Int J Oncol. 2018; 52 (4): 1139-1148. doi: 10.3892/ijo.2018.4294.

15. Sacks SH1. Complement fragments C3a and C5a: the salt and pepper of the immune response. Eur J Immunol. 2010; 40(3): 668-70. doi: 10.1002/eji.201040355.

16. Grajales-Esquivel E, Luz-Madrigal A, Bierly J, Haynes T, Reis ES, Han Z, Gutierrez C, McKinney Z, Tzekou A, Lambris JD, Tsonis PA, Del Rio-Tsonis K. Complement component C3aR constitutes a novel regulator for chick eye morphogenesis. Dev Biol. 2017; 428 (1): 88-100. doi: 10.1016/j.ydbio.2017.05.019.

17. Coulthard LG, Hawksworth OA, Conroy J, Lee JD, Woodruff TM. Complement C3a receptor modulates embryonic neural progenitor cell proliferation and cognitive performance. Mol Immunol. 2018; 101:176-181. doi: 10.1016/j.molimm.2018.06.271.

18. Carmona-Fontaine C, Theveneau E, Tzekou A, Tada M, Woods M, Page KM, Parsons M, Lambris JD, Mayor R. Complement fragment C3a controls mutual cell attraction during collective cell migration. Dev Cell. 2011; 21 (6): 1026-37. doi: 10.1016/j.devcel.2011.10.012.

19. Haynes T, Luz-Madrigal A, Reis ES, Echeverri Ruiz NP, Grajales-Esquivel E, Tzekou A, Tsonis PA, Lambris JD, Del Rio-Tsonis K. Complement anaphylatoxin C3a is a potent inducer of embryonic chick retina regeneration. Nat Commun. 2013; 4:2312. doi:
20. Lara-Astiaso D, Izarra A, Estrada JC, Albo C, Moscoso I, Samper E, Moncayo J, Solano A, Bernad A, Díez-Juan A. Complement anaphylatoxins C3a and C5a induce a failing regenerative program in cardiac resident cells. Evidence of a role for cardiac resident stem cells other than cardiomyocyte renewal. Springerplus. 2012; 1 (1): 63. doi: 10.1186/2193-1801-1-63.

21. Hooshmand MJ, Nguyen HX, Piltti KM, Benavente F, Hong S, Flanagan L, Uchida N, Cummings BJ, Anderson AJ. Neutrophils Induce Astroglial Differentiation and Migration of Human Neural Stem Cells via C1q and C3a Synthesis. J Immunol. 2017; 199 (3): 1069-1085. doi: 10.4049/jimmunol.1600064.

22. Shinjyo N, de Pablo Y, Pekny M, Pekna M. Complement Peptide C3a Promotes Astrocyte Survival in Response to Ischemic Stress. Mol Neurobiol. 2016; 53 (5): 3076-3087. doi: 10.1007/s12035-015-9204-4.

23. Lim J, Iyer A, Suen JY, Seow V, Reid RC, Brown L, Fairlie DP. C5aR and C3aR antagonists each inhibit diet-induced obesity, metabolic dysfunction, and adipocyte and macrophage signaling. FASEB J. 2013; 27 (2): 822-31. doi: 10.1096/fj.12-220582.

24. Hu J, Yang Y, Wang M, Yao Y, Chang Y, He Q, Ma R, Li G. Complement C3a receptor antagonist attenuates tau hyperphosphorylation via glycogen synthase kinase 3β signaling pathways. Eur J Pharmacol. 2019; 850:135-140. doi: 10.1016/j.ejphar.2019.02.020.

25. Peng Q, Li K, Smyth LA, Xing G, Wang N, Meader L, Lu B, Sacks SH, Zhou W. C3a and C5a promote renal ischemia-reperfusion injury. J Am Soc Nephrol. 2012; 23 (9): 1474-85. doi: 10.1681/ASN.2011111072.

26. Liszewski MK, Kolev M, Le Friec G, Leung M, Bertram PG, Fara AF, Subias M, Pickering MC, Drouet C, Meri S. Intracellular complement activation sustains T cell homeostasis
and mediates effector differentiation. Immunity. 2013; 39 (6): 1143-57. doi:10.1016/j.immuni.2013.10.018.

27. Reichhardt MP, Meri S. Intracellular complement activation-An alarm raising mechanism? Semin Immunol. 2018; 38:54-62. doi: 10.1016/j.smim.

28. Cho MS, Vasquez HG, Rupaimoole R, Pradeep S, Wu S, Zand B, Han HD, Rodriguez-Aguayo C, Bottsford-Miller J, Huang J, Miyake T, Choi HJ, Dalton HJ, Ivan C, Baggerly K, Lopez-Berestein G, Sood AK, Afshar-Kharghan V. Autocrine effects of tumor-derived complement. Cell Rep. 2014; 6(6): 1085-1095. doi: 10.1016/j.celrep.2014.02.014.

29. Maurer AJ 1, Bonney PA, Toho LC, Glenn CA, Agarwal S, Battiste JD, Fung KM, Sughrue ME. Tumor necrosis-initiated complement activation stimulates proliferation of medulloblastoma cells. Inflamm Res. 2015; 64 (3-4): 185-92. doi: 10.1007/s00011-015-0796-y.

30. Kwak JW, Laskowski J, Li HY, McSharry MV, Sippel TR, Bullock BL, Johnson AM, Poczobutt JM, Neuwelt AJ. Complement Activation via a C3a Receptor Pathway Alters CD4+ T Lymphocytes and Mediates Lung Cancer Progression. Cancer Res. 2018; 78 (1): 143-156. doi: 10.1158/0008-5472.CAN-17-0240.

31. Zha H, Wang X, Zhu Y, Chen D, Han X, Yang F, Gao J, Hu C, Shu C, Feng Y, Tan Y, Zhang J, Li Y, Wan YY, Guo B, Zhu B. Intracellular Activation of Complement C3 Leads to PD-L1 Antibody Treatment Resistance by Modulating Tumor-Associated Macrophages. Cancer Immunol Res. 2019; 7 (2): 193-207. doi: 10.1158/2326-6066.CIR-18-0272.

Tables
Table 1 Association of C3aR and C3c level with clinicopathological parameters
| Variable             | C3aR level in tumor cells (score) | p   | C3c level in tumor tissue (score) | p   |
|----------------------|----------------------------------|-----|----------------------------------|-----|
|                      | 1      | 2      | 3      |                                | 1   | 2      | 3      |
| Age (years)          |        |        |        |                                |     |        |        |
| ≤58 (median)         | 20     | 27     | 22     | 0.218                           | 6   | 38     | 25     | 0.239 |
| >58 (median)         | 8      | 32     | 20     |                                | 10  | 32     | 18     |        |
| Gender               |        |        |        |                                |     |        |        |
| Male                 | 15     | 41     | 32     | 0.058                           | 9   | 48     | 31     | 0.319 |
| Female               | 13     | 18     | 10     |                                | 7   | 22     | 12     |        |
| Tumor size           |        |        |        |                                |     |        |        |
| ≤4 cm                | 13     | 33     | 20     | 0.929                           | 7   | 35     | 24     | 0.388 |
| >4 cm                | 15     | 26     | 22     |                                | 9   | 35     | 19     |        |
| Stage                |        |        |        |                                |     |        |        |
| III                  | 27     | 47     | 33     | 0.083                           | 12  | 57     | 38     | 0.192 |
| III vs IV            | 1      | 12     | 9      |                                | 4   | 13     | 5      |        |
| Fuhrman grade        |        |        |        |                                |     |        |        |
| 12                   | 25     | 41     | 16     | 0.0000066                       | 16  | 42     | 24     | 0.016 |
| 34                   | 3      | 18     | 26     |                                | 0   | 28     | 19     |        |
| Location             |        |        |        |                                |     |        |        |
| Left                 | 11     | 30     | 18     | 0.929                           | 10  | 31     | 18     | 0.269 |
| Right                | 17     | 29     | 24     |                                | 6   | 39     | 25     |        |
| Hypertension         |        |        |        |                                |     |        |        |
| Yes                  | 9      | 21     | 10     | 0.360                           | 4   | 27     | 9      | 0.247 |
| No                   | 19     | 38     | 32     |                                | 12  | 43     | 34     |        |
| Diabetes             |        |        |        |                                |     |        |        |
| Yes                  | 4      | 5      | 2      | 0.172                           | 2   | 7      | 2      | 0.252 |
| No                   | 24     | 54     | 40     |                                | 14  | 63     | 41     |        |
| Necrosis             |        |        |        |                                |     |        |        |
| Yes                  | 1      | 10     | 15     | 0.001                           | 1   | 9      | 16     | 0.001 |
| No                   | 27     | 49     | 27     |                                | 15  | 61     | 27     |        |

Table 2 Univariable and multivariable Cox regression analysis for overall survival

| Variables             | Univariate analysis | Multivariate analysis |
|-----------------------|---------------------|-----------------------|
|                       | HR (95.0% CI)       | p value               | HR (95.0% CI)       | p value               |
| Age                   | 2.65(1.09-6.45)     | 0.032                 | 2.50(1.02-6.12)     | 0.045                 |
| ≤58 vs >58 (years)    |                     |                       |                      |
| Stage                 | 3.42(1.43-8.15)     | 0.006                 | 3.34(1.34-8.35)     | 0.010                 |
| III vs III vs IV      |                     |                       |                      |
| Gender                | 1.24(0.49-3.16)     | 0.646                 |                       |                      |
| Male vs female        |                     |                       |                      |
| Tumor size ≤4 vs >4 (cm) | 1.48 (0.65-3.37)     | 0.354                 |                       |                      |
| Fuhrman Grade 12 vs 34 | 3.87 (1.63-9.19)     | 0.002                 | 2.76 (1.14-6.68)     | 0.025                 |
| C3aR level Higher vs lower | 3.00 (1.27-7.11)     | 0.013                 | 1.40 (0.54-3.64)     | 0.487                 |
| C3c level Higher vs lower | 2.55(1.05-6.22)     | 0.039                 | 3.09(1.23-7.76)     | 0.016                 |

HR, hazard ratio; CI, confidence interval.
Table 3 Univariable and multivariable Cox regression analysis for Disease specific survival

| Variables          | Univariate analysis |     | Multivariate analysis |     |
|--------------------|---------------------|-----|-----------------------|-----|
|                    | HR (95.0% CI)       | p   | HR (95.0% CI)         | p   |
| Age ≤58 vs >58 (years) | 2.16 (0.86-4.43)   | 0.101 | 4.69 (1.73-12.73)   | 0.002 |
| Stage III vs II IV | 4.38 (1.76-10.89)  | 0.002 | 4.69 (1.73-12.73)   | 0.002 |
| Gender Male vs female | 1.02 (0.39-2.65)  | 0.976 | 4.69 (1.73-12.73)   | 0.002 |
| Tumor size ≤4 vs >4 (cm) | 1.39 (0.58-3.37)   | 0.461 | 3.36 (1.25-9.05)    | 0.017 |
| Fuhrman Grade 12 vs 34 | 4.90 (1.87-12.85) | 0.001 | 3.36 (1.25-9.05)    | 0.017 |
| C3αR level Higher vs lower | 2.44 (0.99-6.00) | 0.052 | 3.60 (1.29-10.06)   | 0.015 |
| C3c level Higher vs lower | 2.65 (1.01-6.91) | 0.047 | 3.60 (1.29-10.06)   | 0.015 |

HR, hazard ratio; CI, confidence interval.

Table 3 Univariable and multivariable Cox regression analysis for Disease specific survival

| Variables          | Univariate analysis |     | Multivariate analysis |     |
|--------------------|---------------------|-----|-----------------------|-----|
|                    | HR (95.0% CI)       | p   | HR (95.0% CI)         | p   |
| Age ≤58 vs >58 (years) | 2.16 (0.86-4.43)   | 0.101 | 4.69 (1.73-12.73)   | 0.002 |
| Stage III vs II IV | 4.38 (1.76-10.89)  | 0.002 | 4.69 (1.73-12.73)   | 0.002 |
| Gender Male vs female | 1.02 (0.39-2.65)  | 0.976 | 4.69 (1.73-12.73)   | 0.002 |
| Tumor size ≤4 vs >4 (cm) | 1.39 (0.58-3.37)   | 0.461 | 3.36 (1.25-9.05)    | 0.017 |
| Fuhrman Grade 12 vs 34 | 4.90 (1.87-12.85) | 0.001 | 3.36 (1.25-9.05)    | 0.017 |
| C3αR level Higher vs lower | 2.44 (0.99-6.00) | 0.052 | 3.60 (1.29-10.06)   | 0.015 |
| C3c level Higher vs lower | 2.65 (1.01-6.91) | 0.047 | 3.60 (1.29-10.06)   | 0.015 |

HR, hazard ratio; CI, confidence interval.

Figures
Figure 1

Expression of C3aR and C3 mRNA in the tumor tissues of ccRCC patients and their association with overall survival. The expression data of C3aR and C3 mRNA and the clinical data were obtained from TCGA. A total of 530 patients of ccRCC and 72 normal controls were included. In the analysis of overall survival, Kaplan-Meier method was used and 23 patients which died within a month after operation were excluded.
Expression of C3aR in tumor tissues from ccRCC patients. Immunohistochemical staining was performed on formalin-fixed Paraffin sections. A total of 129 ccRCC patients were enrolled and 17 normal renal tissue samples were used as controls. A: A representative picture of normal renal tissue; B: A representative picture of tumor tissues showing weak C3aR expression in tumor cells (tumor cell C3aR level was scored as 1); C: A representative picture of tumor tissues showing intermediate C3aR expression in tumor cells (tumor cell C3aR level was scored as 2); D: A representative picture of tumor tissues showing strong C3aR expression in tumor cells (tumor cell C3aR level was scored as 3). A1, B1, C1 and D1 are the local magnification of A, B, C and D respectively. E: Negative control of immunohistochemistry; F: Results of semi-quantitative analysis. **p<0.01. Solid black line in F: median. Scale bar in A-E (black): 100 μm; Scale bar in A1-D1 (white): 25 μm.
Expression of C3c in tumor tissue from ccRCC patients. Immunohistochemical staining was performed on formalin-fixed Paraffin sections. A total of 129 ccRCC patients were enrolled and 17 normal renal tissue samples were used as control. A: A representative picture of normal renal tissue; B: A representative picture of tumor tissues showing weak C3c
expression (tumor C3c level was scored as 1); C: A representative picture of tumor tissues showing intermediate C3c expression (tumor C3c level was scored as 2); D: A representative picture of tumor tissues showing strong C3c expression (tumor C3c level was scored as 3).

A1, B1, C1 and D1 are the local magnification of A, B, C and D respectively. E: Negative control of immunohistochemistry; F: Results of semi-quantitative analysis. **p<0.01. Solid black line in F: median. Scale bar in A-E (black): 100 μm; Scale bar in A1-D1: 25 μm.
Kaplan-Meier survival analysis based on C3aR level in the tumor cells and C3c level in the tumor tissues of ccRCC patients. The expression of C3aR and C3c in tumor tissue samples from 129 ccRCC patients was detected by immunohistochemistry and the level of C3aR in the tumor cells of each sample and the level of C3c in each section were scored. According to the level of C3aR in tumor cells and C3c in tumor tissue, patients were divided into lower C3aR group and higher C3aR group, lower C3c group and higher C3c group. Then, the overall survival and Disease-specific survival were compared between the groups.
Expression of E-Cadherin, Vimentin and Ki-67 in the tumor tissue of ccRCC patients. The expression of E-Cadherin, Vimentin and Ki-67 in the tumor tissue samples from 129 ccRCC patients was detected by immunohistochemistry. A: Representative pictures of immunohistochemical staining for E-Cadherin; B: Representative pictures of immunohistochemical staining for Vimentin; C: Representative pictures of immunohistochemical staining for Ki-67. W: Weak staining; I: Intermediate staining; S: Strong staining. a1, b1, c1, d1, e1, f1, g1, h1 and i1 is the local magnification of a, b, c, d, e, f, g, g and i respectively. Scale bar in a-i (black): 100 μm; Scale bar in a1-i1 (white): 25 μm.
