CTLA4 has a profound impact on the landscape of tumor-infiltrating lymphocytes with a high prognosis value in clear cell renal cell carcinoma (ccRCC)

Shiyi Liu (✉ shiyiliu@whu.edu.cn)
Wuhan University Renmin Hospital  https://orcid.org/0000-0001-6139-8380

Feiyan Wang
Shanghai Skin Disease Clinical College of Anhui Medical University, Shanghai Skin Disease Hospital

Wei Tan
Wuhan University Renmin Hospital

Li Zhang
Wuhan University Renmin Hospital

 Fangfang Dai
Wuhan University Renmin Hospital

Yanqing Wang
Wuhan University Renmin Hospital

Yaqi Fan
Shanghai Skin Disease Hospital, Tongji University School of Medicine

Mengqin Yuan
Wuhan University Renmin Hospital

Dongyong Yang
Wuhan University Renmin Hospital

Yajing Zheng
Wuhan University Renmin Hospital

Zhimin Deng
Wuhan University Renmin Hospital

Yanxiang Cheng
Wuhan University Renmin Hospital

Yeqiag Liu
Shanghai Skin Disease Clinical College of Anhui Medical University, Shanghai Skin Disease Hospital

Primary research

Keywords: CTLA4, clear cell renal cell carcinoma, tumor microenvironment, tumor-infiltrating lymphocytes, Prognosis, CD8+ T cells, immune checkpoints
Abstract

**Background:** Cytotoxic T-lymphocyte associated protein 4 (CTLA4) inhibitors have been shown to significantly prolong the overall survival (OS) in a wide range of cancers. However, its application in clear cell renal cell carcinoma (ccRCC) is limited due to the therapy response, and the prognostic value of CTLA4 in ccRCC has not been investigated in detail.

**Methods:** By using immunohistochemistry, Kaplan-Meier (K-M) analysis, uni- and multi-variate Cox analysis, we comprehensively and systematically studied the prognostic value of CTLA4 in ccRCC. Then, we applied Gene Ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG) and CIBERSORT, ESTIMATE algorithm, ssGSEA and somatic mutation analyses to reveal the impact of CTLA4 on the landscape of tumor-infiltrating lymphocytes (TILs) infiltration and genetic mutation. Besides, given current concerns caused by combined immunotherapy, we also investigated the relationship between CTLA4 and other immune checkpoints.

**Results:** *In vitro* experiment and data mining showed that, CTLA4 was up-regulated in ccRCC tissues and closely related to the disease progression as well as a poor prognosis. Deeper researches demonstrated that CTLA4 regulates T cell activation and was significantly linked to TIL-abundant tumor microenvironment (TME), but was accompanied by an immunosuppressed phenotype. Mutation analysis showed that CTLA4 was associated with more frequent BRCA-associated protein 1 (BAP1) mutation. Moreover, we found that CTLA4 was markedly correlated with multiple immune checkpoints, which suggested that ccRCC patients with high expressed CTLA4 may benefit more from immune checkpoint blockades (ICBs) combined therapy.

**Conclusion:** CTLA4 has a profound impact on the landscape of TILs and genetic mutation, and can be used as the biomarker with high prognosis value in ccRCC.

1. **Background**

Clear cell renal cell carcinoma (ccRCC) is the most common and fatal histological subtype of renal cell carcinoma in adults, accounting for about 65-70% in RCC, characterized by abundant tumor-infiltrating lymphocytes (TILs) infiltration within the tumor microenvironment (TME) (1) (2). Since the symptoms were not obvious, 20% of patients were initially diagnosed with metastases and nearly 30% relapsed with metastasis after surgical excision (3). Therefore, the development of novel therapeutic strategies for ccRCC is necessary.

Along with advances in cancer immunology, the role of TME in ccRCC has attracted increased attention in recent years, consisting of cancer cells, fibroblasts, myofibroblasts, endothelial cells, TILs, and extracellular matrix (4). The composition of TILs within TME determines whether its phenotype is anti-tumor immunity or immune evasion. Anti-tumor immunity is characterized by CD8+ T cells, M1 macrophages, while immune evasion is characterized by mast cells, T cells regulatory (Tregs), and M2-macrophages. Macrophages in tumor tissues are biased towards M2 subtype (5). Furthermore, immune
checkpoint within the TILs is a crucial factor in maintaining immune evasion of TME (6). E.g. Tregs can inhibit the activation of CD8+ T cells through Cytotoxic T-lymphocyte associated protein 4 (CTLA4), triggering tumor immunosuppression (7). Numerous clinical studies have reported that the immune checkpoint would be an ideal target for driving T cell mediated anti-tumor immunity (8, 9). With tremendous progress, immune checkpoint blockades (ICBs) such as programmed death-ligand 1 (PD-L1) inhibitor Durvalumab and CTLA4 inhibitor Tremelimumab et al. have been shown to significantly prolong overall survival (OS) in a wide range of cancers (10-12).

CTLA4, immune checkpoint protein, has received extensive attention in immunotherapy. CTLA4 was highly expressed in CD8+ T cells and inhibited the T cells activation through competitively blocking the binding of CD28 with B7, leading to immune evasion (13). Since the CTLA4 inhibitor ipilimumab (the first ICB approved by the FDA) has significantly prolonged the OS of patients with metastatic melanoma, CTLA4 inhibitors have proven to be effective agents for many cancers (14). However, the clinical application of CTLA4 inhibitors in ccRCC is strictly limited, for that the therapeutic response and prognostic value of CTLA4 in ccRCC have not been investigated in detail. In the current work, we attempted to comprehensively analyze the prognostic value of CTLA4 in ccRCC and its impact on TILs and genetic landscape through bioinformatics and in vitro experiment, which may be beneficial to the therapeutic response of CTLA4 inhibitors in ccRCC patients.

2. Materials And Methods

2.1 Data

The mRNA-seq data from 533 ccRCC and 72 normal tissues and corresponding clinical information, as well as 336 ccRCC somatic mutation data, were retrieved from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/), patients with OS more than 30 days were retained. Ultimately, 494 ccRCC and 68 normal tissues were included for our analyses. RNA-array data GSE40435 (GPL10558) and GSE46699 (GPL570) were obtained from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), including 101 ccRCC and paired normal kidney tissues, 67 ccRCC and 63 normal tissues, respectively.

2.2 Immunohistochemistry

Formalin Fixed Paraffin Embedded (FFPE) ccRCC (n=5) and corresponding normal specimens (n=5) were collected from the department of pathology, Renmin Hospital of Wuhan University (China). Our study was approved by the Ethics Committee of Renmin Hospital of Wuhan University.

Immunohistochemistry with the rabbit monoclonal to CTLA4 (abcam, Shanghai, China) and the secondary antibody (Aspen, Wuhan, China) was performed following the manufacturer’s instructions (15). Images were obtained using a BX63 microscope (Olympus, Japan), and the density of CTLA4 expression was evaluated by the percentage of CTLA4 positive cells in the total cells.
2.3 Analysis of the functions of CTLA4

First, the CTLA4 related genes were screened based on Spearman correlation method with the absolute value of the correlation coefficient > 0.6 and \( p \) value < 0.05 as the threshold. Then, Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were carried out through R package clusterProfiler (16) to reveal the potential function of CTLA4 in ccRCC.

2.4 Genetic mutation analysis

The somatic mutation data of ccRCC patients from TCGA were analyzed by R package Maftool (17) to identify the impact of genetic mutation by CTLA4.

2.5 Immune landscape of TME

CIBERSORT was a gene-based deconvolution algorithm developed by Newman et al and was applied to predict the abundance of immune cells using complex gene expression data in this investigation (18). Furthermore, the immune score was calculated by ESTIMATE algorithm, and the immunosuppression score was obtained from ssGSEA(19, 20).

2.6 Statistics

Data analysis was conducted by using R 3.6.2 together with Graphpad prism 6. Difference of continuous variables between the two groups was calculated by the Wilcoxon test or student's t test according to Shapiro-test and Bartlett-test. Chi-square (\( \chi^2 \)) test was applied to assess the difference between categorical variables. Kaplan-Meier (K-M) analysis (Log-rank test based), uni- and multi-variate Cox analyses were used to evaluate the survival prognosis. Spearman method was utilized in correlation analysis. Data are presented as mean ± SD and \( P \) value < 0.05 was considered statistically significant (\( p<0.05 \ast, p<0.01 \ast\ast, p<0.001 \ast\ast\ast \)).

3. Results

3.1 High prognosis value of CTLA4 in ccRCC

In TCGA dataset, CTLA4 was highly expressed in tumor tissues (N=494) compared to normal tissues (N=68, \( p<0.001 \)), and the paired test also confirmed that CTLA4 was highly expressed in ccRCC (N=68, \( p<0.001 \)) (Figure 1A, B). Moreover, CTLA4 overexpression in ccRCC had also been reproduced in the GEO datasets GSE40435 and GSE46699 (Figure 1C).

The immunohistochemistry was utilized to further validate the above results, and the result showed that the density of CTLA4 expression was higher in ccRCC tissues compared with normal tissues (\( p<0.001 \), Figure 1D). It seems that CTLA4 was steadily up-regulated in ccRCC both in data mining and in vitro experiment.
In TCGA dataset, patients were initially classified into high and low groups based on CTLA4 level. The K-M curves showed that CTLA4 shortened the OS in ccRCC, with the median survival time being 63.73 and 91.73 months in high and low group, respectively \( (p<0.001) \) (Figure 1E). In addition, 512 cases from GEPIA also confirmed that CTLA4 was a risk gene with Hazard ratio (HR) >1 (HR = 1.5, \( p= 0.013 \), Figure 1F).

We next sought to investigate the role of CTLA4 in cancer progression, and the result suggested that the overexpressed CTLA4 was related to high grade \( (\chi^2=12.465, p<0.001) \), advanced stage \( (\chi^2= 22.510, p<0.001) \), patient with tumor state \( (\chi^2= 7.874, p=0.005) \) and death \( (\chi^2= 9.965, p=0.002, \text{Table 1}) \). We also found that CTLA4 had a positive correlation with high grade in GSE40435 cohorts \( (\chi^2=3.971, p=0.046) \), indicating that CTLA4 may function as an oncogene in the progress of ccRCC. Subsequently, uni- and multi-variate Cox analyses found that some variables, including CTLA4, age, tumor pathological grade and stage, were independent risk factors in ccRCC \( (p < 0.01) \) (Table 2). These results proved that CTLA4 contributed to the progression of ccRCC with a high prognosis value.

3.2 CTLA4 indicated a higher density of TILs in ccRCC tumor microenvironment, but an immunosuppressed phenotype.

To outline the corresponding function of the CTLA4 in ccRCC TME, we performed KEGG and GO analysis based on 200 CTLA4 related protein coding genes (Supplementary Table 1). 35 KEGG items were identified, including T cell mediated immune related pathway, natural killer cell mediated cytotoxicity, T helper cells differentiation, PD-L1 expression and programmed death-1 receptor (PD-1) checkpoint pathway in cancer (Table 3), and CTLA4 was positively correlated with T cell receptor signaling pathway \( \text{cor}=0.80 \), Natural killer cell mediated cytotoxicity \( \text{cor}=0.75 \), Th1 and Th2 cell differentiation \( \text{cor}=0.75 \) and Th17 cell differentiation \( \text{cor}=0.78 \) (Figure 2A). The biological processes of CTLA4 yielded from the GO analysis were associated with the activation and differentiation of T cells (Table 3) and positively correlated with T cell activation \( \text{cor}=0.79 \), regulation of lymphocyte activation \( \text{cor}=0.79 \) and T cell differentiation\( \text{cor}=0.80 \) (Figure 2B).

Previous studies have demonstrated that TILs within the TME can be regarded as a prognostic indicator in ccRCC (21). Besides, the results of GO and KEGG promoted us to continue to investigate the role of CTLA4 in TILs infiltration, which affected the ICBs’ response. Our results showed that CTLA4 was associated with a higher immune score, which was calculated by the ESTIMATE algorithm and represented the level of TILs, indicating that CTLA4 promoted the recruitment of immune cells into the TME (Figure 2C). Furthermore, there was a great difference in the composition of TILs between high and low CTLA4 groups. CTLA4 increased the infiltration of T Cells CD8+, Tregs, Macrophage M1, whereas Plasma cells, NK cells activated, Monocytes, Macrophage M2, Dendritic cells activated were less infiltrated in CTLA4 high group (Supplementary Table 2). The correlation analysis result was presented in Figure 2D, showing that CTLA4 was positively correlated with CD8+ T cells \( \text{cor}=0.50, p<0.001 \), Tregs \( \text{cor}=0.28, p<0.001 \) (Figure 2D). However, the immunosuppression score as well as the expression of CD8+ T cell exhaustion markers Hepatitis A virus cellular receptor 2 (HAVCR2), lymphocyte activation
gene-3 (LAG3), and T cell immunoglobulin and ITIM domain (TIGIT) was higher in CTLA4 high group (Figure 2E, F). All in all, CTLA4 changed the landscape of TILs in ccRCC TME, and indicated a higher density of TILs, especially the CD8+ T cells and Tregs, but faced an immunosuppressed phenotype.

3.3 Genetic altered by CTLA4 in ccRCC

Genetic changes include non-synonymous mutations, which are mainly composed of missense mutation, synonymous mutation, insertion or deletion, and copy number gain or loss (22-25). Tumor mutation burden (TMB) can be used as a biomarker to predict the efficacy of ICBs (26). Some studies have shown that the RCC was sensitive to ICBs, although the TMB in RCC was moderate (27, 28). To identify the somatic mutations that were altered by CTLA4 in ccRCC, we performed the mutation analysis and the result showed that overexpressed CTLA4 was correlated with BRCA-associated protein 1 (BAP1) mutation (p<0.05, Figure 3). The TMB in the high CTLA4 expression group tended to be higher than the low expression group, although it was not statistically significant. Moreover, Nonsense Mutation and In Frame Ins in the high CTLA4 expression group were higher than those in the low group (Table 4). BAP1 is a deubiquitinating enzyme and considered to be a tumor suppressor, and the loss of BAP1 contributes to the metastasis and poor prognosis in various cancers (29).

3.4 CTLA4 was highly related to other immune checkpoint molecules

Recently, the combined inhibition of PD-L1 and CTLA4 has attracted much attention (30). D Planchard et al reported that combination immunotherapy of PD-L1 and CTLA4 considerably prolonged the OS in advanced refractory colorectal cancer (31). Combination immunotherapy tends to replace monotherapy, for that the combinational usage of ICBs can produce higher synergistic anti-tumor efficiency and reduce side effects (32). Therefore, we continued to explore the correlation between CTLA4 and other immune checkpoint molecules, including PDCD1 (PD-1), CD274 (PD-L1), LAG3, indoleamine-2,3-dioxygenase-1 (IDO1), and TIGIT (33) (34). The results showed that CTLA4 was highly and positively related to PD-1, PD-L1, LAG3, IDO1, and TIGIT (Figure 4).

4. Discussion

CTLA4, as a transmembrane protein expressed in activated CD4+ T and CD8+ T cells, has received a lot of attention for its interaction with cancer. CTLA4 negatively regulates T cell activation by blocking the function of costimulatory signal and differentiation cluster CD28:B7 binding (35). CTLA4 inhibitors reverse inhibitory immune signal and restore the anti-cancer response by blocking the interaction between CTLA4 and the ligand expressed by antigen presenting cells (32). With the approval of CTLA4 inhibitor Ipilimumab for clinical applications, it has been used for metastatic melanoma after the first-line treatment (14). Furthermore, Romano E et al proved that Ipilimumab can exert a therapeutic effect by targeting Tregs in tumors (36).

Several studies have shown that CD8+ T cells are in a state of abnormal activation when they turn to exhausted phenotypes due to the elimination of tumor cells, which can not only up-regulate the
expression of immunosuppressive cytokines, but also directly lead to immunosuppression (37). Exhausted CD8+ T cells continue to activate the expression of CTLA4 and other immune checkpoint receptors under the chronic stimulation of tumor antigens, which further promoting tumor invasion (38). Here, we confirmed that CTLA4 was up-regulated in ccRCC tissues through data mining and in vitro experiment, and revealed that CTLA4 was correlated with poor prognosis. Corresponding to other studies, this study confirmed that CTLA4 played an important role in the regulation of T cells and represented more TILs infiltration to the TME, especially the CD8+ T cells and Tregs. However, the phenotype of the TME trended to immunosuppression, and the infiltrating CD8+ T cells biased to exhaustion in the CTLA4 high group, synergizing with Tregs, ultimately leading to tumor metastasis and progression.

We noticed that tumors with high TMB were sensitive to ICBs, contributing to a better outcome (26). Therefore, we tried to outline the relationship between somatic mutation and CTLA4. We found that the Nonsense Mutation and In Frame Ins were markedly higher, and the BAP1 mutation was more frequent in the CTLA4 high group. As a tumor suppressor gene, the loss of BAP1 tended to cause poor prognosis and higher TMB, suggesting that patients with high CTLA4 expression might be more sensitive to ICBs. Furthermore, the somatic mutation of BAP1 is more abundant in highly metastatic tumors, such as uveal melanoma (39). BAP1 is located at chromosome 3p21, adjacent to 3p25 where VHL is located (40). Considering that 3p deletions in ccRCC are common, we think that 3p deletions might cause the inactivation of these tumor suppressor genes. Nevertheless, further explorations are needed to reveal the underlying mechanisms between CTLA4 and BAP1.

Finally, we revealed that CTLA4 was highly related to other immune checkpoints: PD-1, PD-L1, LAG3, IDO1, and TIGIT. Activation of the PD-1/PD-L1 signaling pathway contributes to TME with immune evasion, and its inhibitors are representative, which have been used in lots of solid tumors (41). LAG3 can negatively regulate the activation and function of T cells, and its antagonists have been applied clinically (42). At present, some studies have been devoted to the synergism between LAG3 and PD-1 in enhancing the efficacy of immunotherapy (43). IDO1 is overexpressed in cancer cells, inhibiting the function of effector T cells and promoting the infiltration of Tregs. Studies have demonstrated that IDO1 is a promising target for improving patient outcomes in the field of immune-oncology (44). The above results suggested that the CTLA4 inhibitor combined with other ICBs like PD-1 inhibitor nivolumab or LAG3 inhibitor may obtain a better therapeutic response in ccRCC, since that preclinical and clinical studies have provided evidence that combination inhibitor of CTLA4 and other ICBs can enhance the anti-tumor efficiency of CD8+ T cells (45, 46).

5. Conclusion

We comprehensively and systematically studied the prognostic value of CTLA4 in ccRCC and its impact on the landscape of TME TILs infiltration and genetic mutation, finding that CTLA4, acted like an oncogene, can accelerate the progression of ccRCC with a high prognostic value, and that CTLA4 was associated with more TILs infiltrated TME but had an immunosuppressed phenotype. Besides, patients
with high CTLA4 levels may benefit more from the combined ICBs therapy. However, the potential role of CTLA4 in the progression of ccRCC needs further verification in vitro and in vivo.

Abbreviations

CTLA4  Cytotoxic T-lymphocyte associated protein 4
ccRCC  Clear cell renal cell carcinoma
TME  Tumor microenvironment
TILs  Tumor-infiltrating lymphocytes
OS  Overall survival
BAP1  BRCA-associated protein 1
ICBs  Immune checkpoint blockades
Tregs  T cells regulatory
PD-1  programmed death-1 receptor
PD-L1  Programmed death-ligand 1
TCGA  The Cancer Genome Atlas
GEO  Gene Expression Omnibus
FFPE  Formalin Fixed Paraffin Embedded
GO  Gene Ontology
KEGG  Kyoto Encyclopedia of Genes and Genomes
K-M  Kaplan-Meier
HR  Hazard ratio
HAVCR2  Hepatitis A virus cellular receptor 2
LAG3  lymphocyte activation gene-3
TIGIT  T cell immunoglobulin and ITIM domain
TMB  Tumor mutation burden
Declarations

Acknowledgements

We would like to thank all the researchers who participated in the study for their contributions.

Authors' contributions

Conception: SL, FW; Acquisition of data: LZ, FD; Analysis, Interpretation, Validation of data: SL, FW, YW, YF, MY, DY; Statistical analysis: YZ, ZD; Methodology: SL, MY, DY; Writing - original draft: FW, WT; Writing - review & editing: SL, FW; Supervision and Management: YL, YC; All authors read and approved the final manuscript.

Funding

This project was supported by the National Natural Science Foundation of China (Grant No. 81860276) and the Scientific Research Projects Fund Project of Hubei Health Commission (Grant No. WJ2019M179).

Availability of data and materials

All analyzed data are included in this published article and its supplementary information file. The original data used during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Our study was approved by the Ethics Committee of Renmin Hospital of Wuhan University (NO.2018017).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests in this work.

References

1. Inamura K. Renal Cell Tumors: Understanding Their Molecular Pathological Epidemiology and the 2016 WHO Classification. International journal of molecular sciences. 2017;18(10).
2. Owens B. Kidney cancer. Nature. 2016;537(7620):S97.
3. Posadas EM, Limvorasak S, Figlin RA. Targeted therapies for renal cell carcinoma. Nature reviews Nephrology. 2017;13(8):496-511.

4. Hui L, Chen Y. Tumor microenvironment: Sanctuary of the devil. Cancer letters. 2015;368(1):7-13.

5. Cervantes-Villagrana RD, Albores-Garcia D, Cervantes-Villagrana AR, Garcia-Acevez SJ. Tumor-induced neurogenesis and immune evasion as targets of innovative anti-cancer therapies. Signal transduction and targeted therapy. 2020;5(1):99.

6. Eun Y, Kim IY, Sun JM, Lee J, Cha HS, Koh EM, et al. Risk factors for immune-related adverse events associated with anti-PD-1 pembrolizumab. Scientific reports. 2019;9(1):14039.

7. Topalian SL, Taube JM, Anders RA, Pardoll DM. Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. Nature reviews Cancer. 2016;16(5):275-87.

8. Pardoll DM. Immunology beats cancer: a blueprint for successful translation. Nature immunology. 2012;13(12):1129-32.

9. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nature reviews Cancer. 2012;12(4):252-64.

10. Antonia SJ, Villegas A, Daniel D, Vicente D, Murakami S, Hui R, et al. Overall Survival with Durvalumab after Chemoradiotherapy in Stage III NSCLC. The New England journal of medicine. 2018;379(24):2342-50.

11. Nishijima TF, Muss HB, Shachar SS, Moschos SJ. Comparison of efficacy of immune checkpoint inhibitors (ICIs) between younger and older patients: A systematic review and meta-analysis. Cancer treatment reviews. 2016;45:30-7.

12. van de Ven K, Borst J. Targeting the T-cell co-stimulatory CD27/CD70 pathway in cancer immunotherapy: rationale and potential. Immunotherapy. 2015;7(6):655-67.

13. Gough SC, Walker LS, Sansom DM. CTLA4 gene polymorphism and autoimmunity. Immunological reviews. 2005;204:102-15.

14. Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP. Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. The Journal of experimental medicine. 2009;206(8):1717-25.

15. Lee SJ, Jun SY, Lee IH, Kang BW, Park SY, Kim HJ, et al. CD274, LAG3, and IDO1 expressions in tumor-infiltrating immune cells as prognostic biomarker for patients with MSI-high colon cancer. Journal of cancer research and clinical oncology. 2018;144(6):1005-14.

16. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. Omics : a journal of integrative biology. 2012;16(5):284-7.

17. Mayakonda A, Lin DC, Assenov Y, Plass C, Koeffler HP. Maftools: efficient and comprehensive analysis of somatic variants in cancer. Genome research. 2018;28(11):1747-56.

18. Hao Y, Yan M, Heath BR, Lei YL, Xie Y. Fast and robust deconvolution of tumor infiltrating lymphocyte from expression profiles using least trimmed squares. PLoS computational biology. 2019;15(5):e1006976.
19. Kardos J, Chai S, Mose LE, Selitsky SR, Krishnan B, Saito R, et al. Claudin-low bladder tumors are immune infiltrated and actively immune suppressed. JCI insight. 2016;1(3):e85902.

20. Hanzelmann S, Castelo R, Guinney J. GSVA: gene set variation analysis for microarray and RNA-seq data. BMC bioinformatics. 2013;14:7.

21. Zhang C, Li Z, Qi F, Hu X, Luo J. Exploration of the relationships between tumor mutation burden with immune infiltrates in clear cell renal cell carcinoma. Annals of translational medicine. 2019;7(22):648.

22. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature. 2013;499(7457):214-8.

23. Chalmers ZR, Connelly CF, Fabrizio D, Gay L, Ali SM, Ennis R, et al. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. Genome medicine. 2017;9(1):34.

24. Zehir A, Benayed R, Shah RH, Syed A, Middha S, Kim HR, et al. Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. Nature medicine. 2017;23(6):703-13.

25. Cheng Q, Li J, Fan F, Cao H, Dai ZY, Wang ZY, et al. Identification and Analysis of Glioblastoma Biomarkers Based on Single Cell Sequencing. Frontiers in bioengineering and biotechnology. 2020;8:167.

26. Chan TA, Yarchoan M, Jaffee E, Swanton C, Quezada SA, Stenzinger A, et al. Development of tumor mutation burden as an immunotherapy biomarker: utility for the oncology clinic. Annals of oncology : official journal of the European Society for Medical Oncology. 2019;30(1):44-56.

27. Gunjur A. Nivolumab plus ipilimumab in advanced renal-cell carcinoma. The Lancet Oncology. 2018;19(5):e232.

28. Hammers HJ, Pлимack ER, Infante JR, Rini BI, McDermott DF, Lewis LD, et al. Safety and Efficacy of Nivolumab in Combination With Ipilimumab in Metastatic Renal Cell Carcinoma: The CheckMate 016 Study. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2017;35(34):3851-8.

29. Saiтоe DD, van Dijk WJ, Ekkebus R, Ovaa H, Sixma TK. BAP1/ASXL1 recruitment and activation for H2A deubiquitination. Nature communications. 2016;7:10292.

30. Planchard D, Reinmuth N, Orlov S, Fischer JR, Sugawara S, Mandziuk S, et al. ARCTIC: durvalumab with or without tremelimumab as third-line or later treatment of metastatic non-small-cell lung cancer. Annals of oncology : official journal of the European Society for Medical Oncology. 2020;31(5):609-18.

31. Chen EX, Jonker DJ, Loree JM, Kennecke HF, Berry SR, Couture F, et al. Effect of Combined Immune Checkpoint Inhibition vs Best Supportive Care Alone in Patients With Advanced Colorectal Cancer: The Canadian Cancer Trials Group CO.26 Study. JAMA oncology. 2020.

32. Liu F, Huang J, Liu X, Cheng Q, Luo C, Liu Z. CTLA-4 correlates with immune and clinical characteristics of glioma. Cancer cell international. 2020;20:7.
33. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nature immunology. 2009;10(1):29-37.

34. Munn DH. Indoleamine 2,3-dioxygenase, Tregs and cancer. Current medicinal chemistry. 2011;18(15):2240-6.

35. Huang J, Liu F, Liu Z, Tang H, Wu H, Gong Q, et al. Immune Checkpoint in Glioblastoma: Promising and Challenging. Frontiers in pharmacology. 2017;8:242.

36. Romano E, Kusio-Kbialka M, Foukas PG, Baumgaertner P, Meyer C, Ballabeni P, et al. Ipilimumab-dependent cell-mediated cytotoxicity of regulatory T cells ex vivo by nonclassical monocytes in melanoma patients. Proceedings of the National Academy of Sciences of the United States of America. 2015;112(19):6140-5.

37. Ma J, Zheng B, Goswami S, Meng L, Zhang D, Cao C, et al. PD1(Hi) CD8(+) T cells correlate with exhausted signature and poor clinical outcome in hepatocellular carcinoma. Journal for immunotherapy of cancer. 2019;7(1):331.

38. Speiser DE, Ho PC, Verdeil G. Regulatory circuits of T cell function in cancer. Nature reviews Immunology. 2016;16(10):599-611.

39. Harbour JW, Onken MD, Roberson ED, Duan S, Cao L, Worley LA, et al. Frequent mutation of BAP1 in metastasizing uveal melanomas. Science. 2010;330(6009):1410-3.

40. Hakimi AA, Chen YB, Wren J, Gonen M, Abdel-Wahab O, Heguy A, et al. Clinical and pathologic impact of select chromatin-modulating tumor suppressors in clear cell renal cell carcinoma. European urology. 2013;63(5):848-54.

41. Farhood B, Najafi M, Mortezaee K. CD8(+) cytotoxic T lymphocytes in cancer immunotherapy: A review. Journal of cellular physiology. 2019;234(6):8509-21.

42. Ruffo E, Wu RC, Bruno TC, Workman CJ, Vignali DAA. Lymphocyte-activation gene 3 (LAG3): The next immune checkpoint receptor. Seminars in immunology. 2019;42:101305.

43. Andrews LP, Marciscano AE, Drake CG, Vignali DA. LAG3 (CD223) as a cancer immunotherapy target. Immunological reviews. 2017;276(1):80-96.

44. Komiya T, Huang CH. Updates in the Clinical Development of Epacadostat and Other Indoleamine 2,3-Dioxygenase 1 Inhibitors (IDO1) for Human Cancers. Frontiers in oncology. 2018;8:423.

45. Mahoney KM, Rennert PD, Freeman GJ. Combination cancer immunotherapy and new immunomodulatory targets. Nature reviews Drug discovery. 2015;14(8):561-84.

46. Postow MA, Callahan MK, Wolchok JD. Immune Checkpoint Blockade in Cancer Therapy. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2015;33(17):1974-82.

Tables

Table 1 Correlation between CTLA4 and clinicopathological features in ccRCC
| Variables | CTLA4\textsuperscript{low} group | CTLA4\textsuperscript{high} group | $\chi^2$ | $P$ value |
|-----------|----------------|----------------|------|------|
| **Age**   |                 |                 |      |      |
| <60       | 119            | 113             | 0.244| 0.622|
| $\geq$60  | 127            | 134             |      |      |
| **Gender**|                 |                 |      |      |
| Female    | 97             | 72              | 3.971| 0.046|
| male      | 150            | 175             |      |      |
| **Grade** |                 |                 |      |      |
| 1-2       | 126            | 89              | 12.465| <0.001|
| 3-4       | 114            | 157             |      |      |
| **Satge** |                 |                 |      |      |
| I-II      | 175            | 122             | 22.510| <0.001|
| III-IV    | 71             | 123             |      |      |
| **tumor status** | | | | |
| Negative  | 177            | 149             | 7.874| 0.005|
| Positive  | 55             | 84              |      |      |
| **vital status** | | | | |
| Alive     | 183            | 150             | 9.965| 0.002|
| Death     | 62             | 96              |      |      |

Table 2 Univariate and multivariate Cox analyses.
### Table 3 top 10 items in functional enrichment analysis.

| KE经 | GO (BP)                                      |
|------|----------------------------------------------|
| T cell receptor signaling pathway | T cell activation                             |
| Primary immunodeficiency         | Regulation of T cell activation               |
| Natural killer cell mediated cytotoxicity | Leukocyte cell-cell adhesion                 |
| Th1 and Th2 cell differentiation | Regulation of lymphocyte activation           |
| Th17 cell differentiation        | Regulation of leukocyte cell-cell adhesion    |
| Cell adhesion molecules (CAMs)   | Positive regulation of T cell activation      |
| Chemokine signaling pathway      | Regulation of cell-cell adhesion              |
| PD-L1 expression and PD-1 checkpoint pathway in cancer | T cell differentiation |
| Hematopoietic cell lineage       | Positive regulation of leukocyte cell-cell adhesion |
| Human immunodeficiency virus 1 infection | Positive regulation of cell-cell adhesion  |
| Type of variants          | High group (n=56) | Low group (n=63) |
|---------------------------|-------------------|------------------|
|                           | Summary | Mean | Summary | Mean |
| Frame Shift Del           | 782     | 5.21 | 791     | 5.24 |
| Frame Shift Ins           | 938     | 6.25 | 211     | 1.4  |
| In Frame Del              | 97      | 0.65 | 114     | 0.75 |
| In Frame Ins*             | 331     | 2.21 | 13      | 0.09 |
| Missense Mutation         | 5922    | 39.48| 5783    | 38.3 |
| Nonsense Mutation**       | 772     | 5.15 | 385     | 2.55 |
| Nonstop Mutation          | 8       | 0.05 | 9       | 0.06 |
| Splice Site               | 212     | 1.41 | 223     | 1.48 |
| Translation Start Site    | 14      | 0.09 | 9       | 0.06 |
| Total                     | 9076    | 60.51| 7542    | 49.95|

Notes: wilcoxon.test * $p<0.05$, ** $p<0.01$