CTLA4 had a profound impact in the landscape of tumor-infiltrating lymphocytes with a high prognosis value in ccRCC

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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 is limited due to the therapy response and the prognostic value of CTLA4 in Clear cell renal cell carcinoma (ccRCC), which is the most common and fatal histological subtype in renal cell carcinoma, have not been investigated in detail.

**Methods** In this study, we comprehensively and systematically studied the prognostic value of CTLA4 in ccRCC and its impact on the landscape of tumor-infiltrating lymphocytes (TILs) infiltration and genetic mutation through in vitro experiment and data mining.

**Results** Our results showed that the CTLA4 was up-regulated in both ccRCC tissues and cell lines, and closely related to the disease progression as well as a poor prognosis. Deeper researches demonstrated that CTLA4 regulated the T cell activation and significantly linked to TIL-abundant tumor microenvironment (TME), but companies with an immunosuppressed phenotype, and CTLA4 was associated with more frequent BRCA-associated protein 1 (BAP1) mutation. Furthermore, given the current concerns caused by combined immunotherapy, we also 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 had a profound impact on the landscape of TILs and genetic mutation, as well as can be used as the biomarker with high prognosis value.

1. **Introduction**

Clear cell renal cell carcinoma (ccRCC) is the most common and fatal histological subtype of renal cell carcinoma in adults, and accounting for about 65-70% in RCC, characteristic with abundant tumor-infiltrating lymphocytes (TILs) 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.

It is becoming increasingly difficult to ignore the impact of the TME on the ccRCC progression, the TME consists 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. Furthermore, immune checkpoint within the TILs is a crucial factor in maintaining immune evasion of TME (5). E.g. Tregs can inhibit the activation of CD8+ T cells through Cytotoxic T-lymphocyte associated protein 4 (CTLA4), triggering tumor immunosuppression (6). Numerous clinical studies have reported that immune checkpoint would be an ideal target for driving T cell mediated anti-tumor immunity (7, 8). With
tremendous progress, immune checkpoint blockades (ICBs) such as Programmed death-ligand 1 (PD-L1) inhibitor Durvalumab and CTLA4 inhibitor Tremelimumab, etc. have been shown to significantly prolong overall survival (OS) in a wide range of cancers(9-11).

CTLA4, immune checkpoint protein, has received extensive attention for 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 immune evasion (12). 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 (13). 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 experiments, 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 Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was utilized to verify the expression of CTLA4 in vitro, ccRCC cell lines Caki-2, 769-P, and normal renal tubular cell line HKC were obtained from Chinese Academy of Sciences (Shanghai, China). All the cells were incubated in RMPI-1640 (Gibico) added with 10% fetal bovine serum, under 5% CO2, 37°C condition. The total RNA from the Caki-2, 769-P, and HKC was extracted according to the instructions of Total RNA Kit (Solarbio). The RNA was reverse transcribed into cDNA under the instruction of Reverse Transcription-PCR Kit (TaKaRa SYBR Premix Ex Taq), and the qRT-PCR was performed with cDNA as the template. GAPDH served as an internal reference.

Forward primer of CTLA4: 5’-CCAGTGATGCTAAAGGTTATTG-3’;
Reverse primer of CTLA4: 5’-CTGCTATGACCTCATCCAGTTT-3’;
Forward primer of GAPDH: 5’-GGAGCGAGATCCCTCCAAAAT-3’;
Reverse primer of GAPDH: 5'-GGCTGTTGTCATACTTCTCATGG-3';

2.3 Analyze 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(14) 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(15) 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 (16). Furthermore, the immune score was calculated by ESTIMATE algorithm, and the immunosuppression score was obtained from ssGSEA(17, 18).

2.6 Statistics

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 (χ²) 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. p value < 0.05 was considered statistically significant (p<0.05*, p<0.01**, p<0.001***).

3. Results

3.1 CTLA4 has high prognosis value in ccRCC

In TCGA data, 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, the result of that CTLA4 overexpressed in ccRCC had also been reproduced in the GEO datasets GSE40435 and GSE46699 (Figure 1C, D).

To further validated the above results in vitro, the qRT-PCR was utilized, and the result showed that CTLA4 was up-regulated in Caki-2 (p = 0.002) and 769-P (p = 0.021) compared with normal renal epithelial cell line HKC (Figure 1E). CTLA4 seemed to be steadily up-regulated in ccRCC both by experimental data and data mining.
In the TCGA dataset, patients were initially classified into high and low group based on CTLA4 level, the K-M curves showed that CTLA4 shortened the OS in ccRCC, with the median survival time was 63.73 and 91.73 months in high and low group, respectively ($p<0.001$) (Figure 1F). 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 1G).

We next sought to investigate the role of CTLA4 in cancer progression, and the result suggesting 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 ($\chi^2 = 9.965, p=0.002$, Table 1). The result that CTLA4 was associated with high grade also appeared in GSE40435 ($\chi^2=3.971, p=0.046$), indicating that CTLA4 may function as an oncogene in the progress of ccRCC. Subsequently, Uni- and multi-variate Cox analysis found that CTLA4 and 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, and to be significantly linked to a worse outcome.

### 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 terms 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 (cor=0.80), natural killer cell mediated cytotoxicity (cor=0.75), Th1 and Th2 cell differentiation (cor=0.75) and Th17 cell differentiation (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 (cor=0.79), regulation of lymphocyte activation (cor=0.79) and T cell differentiation (cor=0.80) (Figure 2B).

A number of reports have been published demonstrated that TILs within the TME can be regarded as a prognostic indicator in ccRCC (19). 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 (cor=0.50, $p<0.001$), Tregs (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 (20-23). Tumor mutation burden (TMB) can be used as a biomarker to predict the efficacy of ICBs (24). Some studies have shown that the RCC was sensitive to ICBs, although the TMB in RCC was moderate (25, 26). 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 tumors(27).

3.4 CTLA4 was highly related to other immune checkpoint molecules

Recently, the combined inhibition of PD-L1 and CTLA4 has attracted much attention(28). D Planchard et al reported that combination immunotherapy of PD-L1 and CTLA4 considerably prolonged the OS in advanced refractory colorectal cancer(29). Combination immunotherapy tends to replace monotherapy, for that the combinational usage of ICBs can produce higher synergistic anti-tumor efficiency and reduce side effects (30). 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 (31) (32). The results showed that CTLA4 was highly 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 regulated T cell activation by blocking the function of costimulatory signal and differentiation cluster CD28: B7 binding (33). CTLA4 inhibitors reverse the inhibitory immune signal and restore the anti-cancer response by blocking the interaction between CTLA4 and the ligand expressed by antigen presenting cells(30). With approval of CTLA4 inhibitor Ipilimumab for clinical applications, it has been used for metastatic melanoma after the first-line treatment (13). Furthermore, Romano E et al proved that Ipilimumab can exert a therapeutic effect by targeting Tregs in tumors (34).

Here, we confirmed that CTLA4 was up-regulated in both ccRCC tissues and cell lines, and revealed that CTLA4 correlated with poor prognosis. Corresponding to other studies, this study confirmed that CTLA4
played an important role in regulation of T cells and represented more TILs infiltration to the TME, especially the CD8+ T cells. However, the phenotype of the TME trended to immunosuppression, and the infiltrating CD8+ T cells biased to exhaustion in the CTLA4 high group, ultimately leading to tumor metastasis and progression.

We noticed that tumors with high TMB were sensitive to ICBs, contributing to a better outcome (24). Therefore, we try 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 the poor prognosis and higher TMB, suggesting that patients with high CTLA4 expression might have more frequent BAP1 mutation, and be more sensitive to ICBs.

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 representation, which have been used in lots of solid tumors(35). LAG3 can negatively regulate the activation and function of T cells, and its antagonists have been applied clinically(36). At present, some studies have been devoted to the synergism between LAG3 and PD-1 in enhancing the efficacy of immunotherapy(37). 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 (38). 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 (39, 40).

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 to accelerate the progression of ccRCC with a high prognostic value. And 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, there are still some limitations to our study, 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
qRT-PCR  Quantitative real-time PCR
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
IDO1  indoleamine-2,3-dioxygenase-1

Declarations

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Author 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.

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**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**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

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

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Figures
Figure 1

CTLA4 has high prognosis value in ccRCC. The expression of CTLA4 between ccRCC and normal renal tissues in (A) TCGA dataset and (D) GSE46699. The expression of CTLA4 between paired ccRCC and
normal tissues in (B) TCGA dataset and (C) GSE40435. (E) Verifying the expression of CTLA4 in ccRCC cell lines Caki-2, 769-P and normal renal epithelial cell line HKC through Quantitative real-time PCR (qRT-PCR). Survival analysis in ccRCC patients from (F) TCGA dataset and (G) GEPIA database. ccRCC; qRT-PCR, Quantitative real-time PCR;
Figure 2
CTLA4 indicated a higher density of TILs in ccRCC tumor microenvironment, but an immunosuppressed phenotype. (A) CTLA4 is positively correlated with T cell receptor signaling pathway; Natural killer cell mediated cytotoxicity; Th1 and Th2 cell differentiation and Th17 cell differentiation. (B) CTLA4 is positively correlated with T cell activation and T cell differentiation. (C) Highly expressed CTLA4 has a higher Immune score, indicating that there are more TILs within the TME. (D) CTLA4 is positively correlated with infiltrating CD8+ T cells and Tregs. (E, F) Highly expressed CTLA4 is accompanied by a higher Immunosuppression score and higher expression of T cell exhaustion markers, indicating an immunosuppressed phenotype. TILs, tumor-infiltrating lymphocytes; TME, tumor microenvironment; Tregs, T regulatory cells;

Figure 3

Genetic changes by CTLA4 in ccRCC. Genetic mutation analysis of CTLA4 between high and low CTLA4 groups, the result shows that the BAP1 mutation is more frequent in the CTLA4 high group.
Figure 4

CTLA4 was highly related to other immune checkpoint molecules. CTLA4 is highly related to other immune checkpoint molecules PDCD1(PD-1), CD274(PD-L1), LAG3, IDO1, TIGIT.

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