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ORIGINAL ARTICLE

Unraveling LGALS1 as a Potential Immune Checkpoint and a Predictor of the Response to Anti-PD1 Therapy in Clear Cell Renal Carcinoma

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Abstract

Immunotherapy base on immune checkpoint inhibitor had obtained significant progress in extending the survival of clear cell renal carcinoma (ccRCC) patients. In order to further improve the efficiency of immunotherapy, novel immune checkpoint inhibitors needed to be developed. Differentially expressed genes (DEGs) between healthy kidney tissues and ccRCC tissues had been found from GSE68417 by GEO2R online analysis tool. Correlation analysis and Kaplan–Meier survival analyses were based on UALCAN database. Analyses of the outcome of anti-PD1 treatment had been found from GSE67501 dataset. At first, 9 genes with higher expression were associated with shorter overall survival time. More importantly, higher expression of LGALS1 was correlated with a profitable outcome of anti-PD1 treatment and the combined the expression level of PD-L1 and LGALS1 together could more efficiently predict the outcome of anti-PD1 treatment than using PD-L1 alone. At last, the genes which correlated with LGALS1 expression in ccRCC patients were enriched in TNF alpha Signaling Pathway which is mainly correlated with T cell apoptosis and survival. Together, these suggest LGALS1 could be a potential immune checkpoint, which could promote tumor progression through affecting T cell survival.

Keywords Immunotherapy · PD-L1 · LGALS1 · Galectin1 · Clear cell renal carcinoma · Immune checkpoint

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Introduction

Renal cell carcinoma is the most common type of renal malignant tumor and around 70% of renal cell carcinoma is clear cell renal carcinoma (ccRCC) [1]. Approximately 50% of ccRCC patients developed metastases although effective treatments were given [2]. The 5-year survival rate of ccRCC with distant metastasis is less than 10% [1, 3]. As known, ccRCC is a highly vascularized malignant tumor. Although considerable progress such as antiangiogenic treatment that targeting the VEGF pathway and inhibitors targeting rapamycin (mTOR) in the treatment of ccRCC have been made, drug-resistant is still inescapable, and these targeted treatments are still palliative with median overall survival less than 20 months [4, 5].

Although ccRCC harbor a low burden of somatic mutations that is 1.1 mutations/Mb on average [6], immunotherapy such as high dose IL2 was proved to be an effective weapon against ccRCC long ago [7]. Recently, immunotherapies based on targeting immune checkpoint inhibitors such as nivolumab which is an antibody against programmed death

1 (PD-1) and ipilimumab which is an antibody against anti–cytotoxic T lymphocyte–associated antigen 4 (CTLA4) display an efficacy and overall survival benefit in the treatment of advanced ccRCC [7, 8]. Although promising treatment results had been observed, most patients are refractory to the treatment of immune checkpoint inhibitor blockade. In ccRCC, objective response rates (ORR) to anti-PD-1 therapy is around 20% [7, 9]. In addition, patients who had a favorable response to immune checkpoint inhibitor blockade do not show long-lasting remission [2]. To improve the efficacy of immunotherapy in ccRCC, combined treatment of different immune checkpoint inhibitor such as Nivolumab (anti-PD-1) combined with ipilimumab (anti-CTLA4) and atezolizumab (anti-PD-L1) combined with bevacizumab (anti-VEGF) achieved a significant improvement in OS and ORR of ccRCC patients [2, 8]. As limited immune checkpoint inhibitor had been found in ccRCC patients, novel immune checkpoints are urgently needed to be further developed.

In our research, GSE68417 were downloaded from Gene Expression Omnibus (GEO) database and Differentially expressed genes (DEGs) between healthy kidney tissues and ccRCC tissues were analyzed by GEO2R online tool. In order to find the potential immune checkpoint inhibitor, other methods including Gene Ontology (GO), enriched pathway analysis and overall survival (OS) analysis were used to filter screen DEGs. Next, we downloaded GSE67501 from GEO database to analyze which selected genes could predict the outcome of anti-PD1 immunotherapy. Correlation analysis and enriched pathway analysis were further carried out to elucidate the underlying immunoregulatory mechanism of selected immune checkpoint. This study provides important findings regarding potential immune checkpoint inhibitor and related immunoregulatory mechanism in ccRCC patients.

Materials and Methods

Data Collection

Microarray dataset (GSE68417) containing samples from patients with ccRCC ($N = 29$) and healthy kidneys ($N = 14$) was downloaded from Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68417) [10]. The dataset was analyzed on the platform of GPL8786 (Affymetrix Human Gene 1.0 ST Array). Another dataset of GSE67501 containing 11 samples from patients with ccRCC pretreated with anti-PD1 treatment was also downloaded from GEO database [11]. The dataset was performed on the platform of GPL149519 (Illumina HumanHT-12 WG-DASL V4.0 R2 expression beadchip). In this dataset, patients were separated into two groups (responders $n = 4$ or nonresponders $n = 7$) according to their response to anti-PD-1 therapy.

Screening out Differentially Expressed Genes

Differentially expressed genes present in normal kidneys and ccRCC in GSE68417 were screening out by using *GEO2R* which is a web-based tool (https://www.ncbi.nlm.nih.gov/geo/geo2r/) allowing researchers to compare two groups or more than 2 groups of samples in GEO series [12]. The cutoff criteria were $|\log FC| \geq 1$ and adjusted P value <0.05.

Gene Ontology Analysis

FunRich (http://www.funrich.org/) is a stand-alone software tool used mainly for functional enrichment and interaction network analysis of genes. We employed FunRich for the GO analysis, containing biological processes, cellular components and molecular functions.

Pathway Analysis and Tissue Protein Expression Analysis

EnrichR (https://amp.pharm.mssm.edu/Enrichr/) is web-based software which has many gene set libraries, such as Wikipathway and Tissue Protein Expression from Human Proteome Map [13, 14]. We employed EnrichR for pathway analysis and Tissue Protein Expression analysis.

Gene Expression, Correlation Analysis and Kaplan–Meier Survival Analysis Using UALCAN Database

UALCAN (http://ualcan.path.uab.edu/index.html) which is an interactive web-based tool to perform analyses of gene expression data from The Cancer Genome Atlas (TCGA) [15, 16]. We employed UALCAN database to analyze the expression levels of LGALS1 between healthy kidney tissue and tumor tissue from different stage, grade and age of patients with ccRCC. In addition to gene expression analysis, we also performed correlation analysis in UALCAN to find out which gene was correlated with LGALS1 expression. Pearson correlation coefficient (PearsonCC) >0.3 was considered a positive correlation and PearsonCC <−0.3 was considered a negative correlation. At last, correlations of gene expression level with patient survival could also be visualized in UALCAN. According to gene expression level, Samples in UALCAN were categorized into High expression group (expression level above upper quartile) and Low expression group (expression level below upper quartile). The survival curves of samples with high gene expression and low gene expression were compared by log rank test.

Statistical Analysis

Statistical analysis was performed by two-tailed, unpaired t-test on Prism 5.01 (GraphPad Software Inc., La Jolla, CA, USA). while differences with p values of less than 0.05 were treated as statistically significant (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$; ns indicates not significant). Linear regression analysis was performed using Prism 5.0 as wells.

Results

Screening out Potential Immune Checkpoints in ccRCC

In order to screen out the DEGs between healthy kidney tissue and ccRCC tissue, GEO2R online analysis tool was used. 747 upregulated genes and 1110 downregulated genes had been found under the cutoff criteria of |logFC| ≥1 and adjusted to P value <0.05 (Fig. 1a). Next, to define the biological function of overexpressed and underexpressed genes, GO enrichment analysis was investigated by FunRich software. The most overrepresented GO terms were shown (Fig. 1b). For overexpressed genes, the most enriched GO terms include plasma membrane of cellular component; receptor activity of molecular function and immune response of biological process. For underexpressed genes, the most enriched GO terms include exosomes of cellular component, catalytic activity of molecular function and metabolism of biological process. For the sake of finding potential immune checkpoint inhibitor, we mainly focused on overexpressed genes that are enriched in immune response of biological process and plasma membrane of cellular component, which could be easily blocked by the specific antibody or small molecule. There are 47 genes which were enriched both in immune response of biological process and plasma membrane of cellular component were needed to be further investigated.

Survival Analysis of Screened Potential Immune Checkpoint Inhibitor

47 gene had been screened out as candidate genes for potential immune checkpoints in renal clear cell carcinoma. In order to better demonstrate their important role in ccRCC

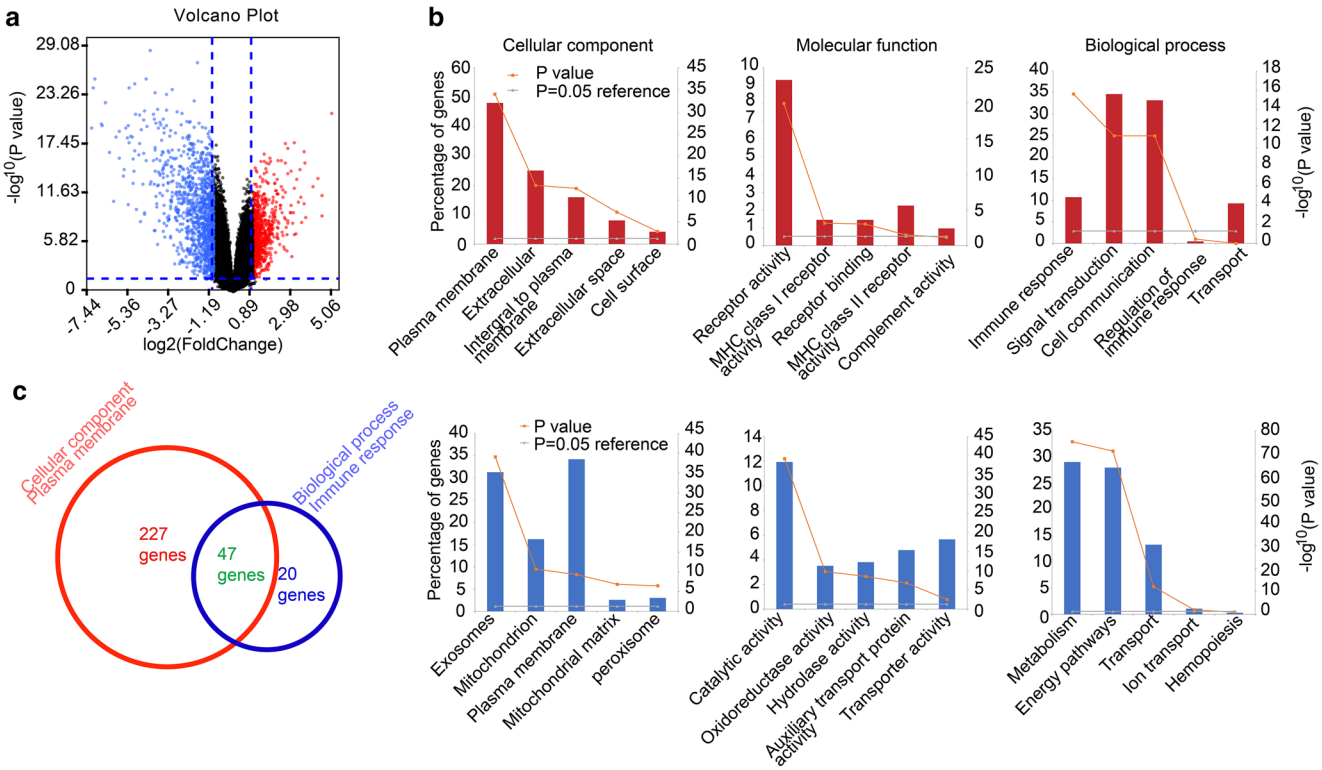

Fig. 1 Output of GO analysis of differentially expressed genes between normal kidney and ccRCC tissue. a Volcano plot show DEGs between the healthy kidney tissues and ccRCC tissue (|logFC| ≥1 and adjusted P value <0.05). The red dots represent the upregulated genes and blue dots represent the downregulated genes. **b** GO functions for differentially expressed genes. Enriched biological process, cellular component, and molecular function of the upregulated genes in ccRCC were shown (red); Enriched biological process, cellular component, and molecular function of the downregulated genes in ccRCC were shown (blue); **C,** Venn diagram showing 227 genes enriched only in plasma membrane of cellular component (red circle), 20 genes enriched only immune response of biological process (blue circle) and 47 genes enriched in both groups (intersection)

development, Kaplan–Meier survival analyses were developed based on UALCAN database. 9 genes (LGALS1, CD276, FCER1G, FCGR2A, IL1RAP, ALOX5AP, LILRB2, TREM1 and VSIG4) with higher expression significantly correlated with shortened overall survival time (Fig. 2). 3 genes (CD93, HLA-DQB2 and IGSF6) with higher expression significantly correlated with shortened overall survival time. Other genes expression level did not correlated with survival time (data not shown). As our objective is to develop novel immune checkpoint inhibitor in renal clear cell carcinoma, we mainly focus on 9 genes which overexpression could shorten patient's survival time.

High Expression of LGALS1 Associated with a Preferable Outcome of Anti-PD1 Treatment

GSE67501 containing 11 pretreatment samples from ccRCC patients downloaded from GEO database and patients were divided into responders or nonresponders to anti–PD1 treatment. Our results showed that higher expression level of LGALS1 correlated with a preferable outcome of anti-PD1 treatment. The expression level of the remaining 8 genes did not correlate with the outcome of anti-PD1 treatment (Fig. 3a). These further suggest LGALS1 is an important immune checkpoint in ccRCC and tumor immunosuppressive microenvironment induced by LGALS1 could be recovered by anti-PD1 treatment. In addition, no strong correlation was observed between the expression levels of LGALS1 and PD-L1 (Fig. 3b). At last, we found that combined the PD-L1 expression level with LGALS1 together could more efficiently predict the outcome of anti-PD1 treatment than PD-L1 alone. We divided the expression level of PD-L1 and LGALS1 into high expression group (expression level above median expression level) and Low expression group (expression level below median expression level). We found that patients with both high expressed genes of LGALS1 and PD-L1 show a favorable outcome to anti-PD1 treatment and both low expressed genes show a resistance outcome to anti-PD1 treatment (Fig. 3c). Together, these convinced us that

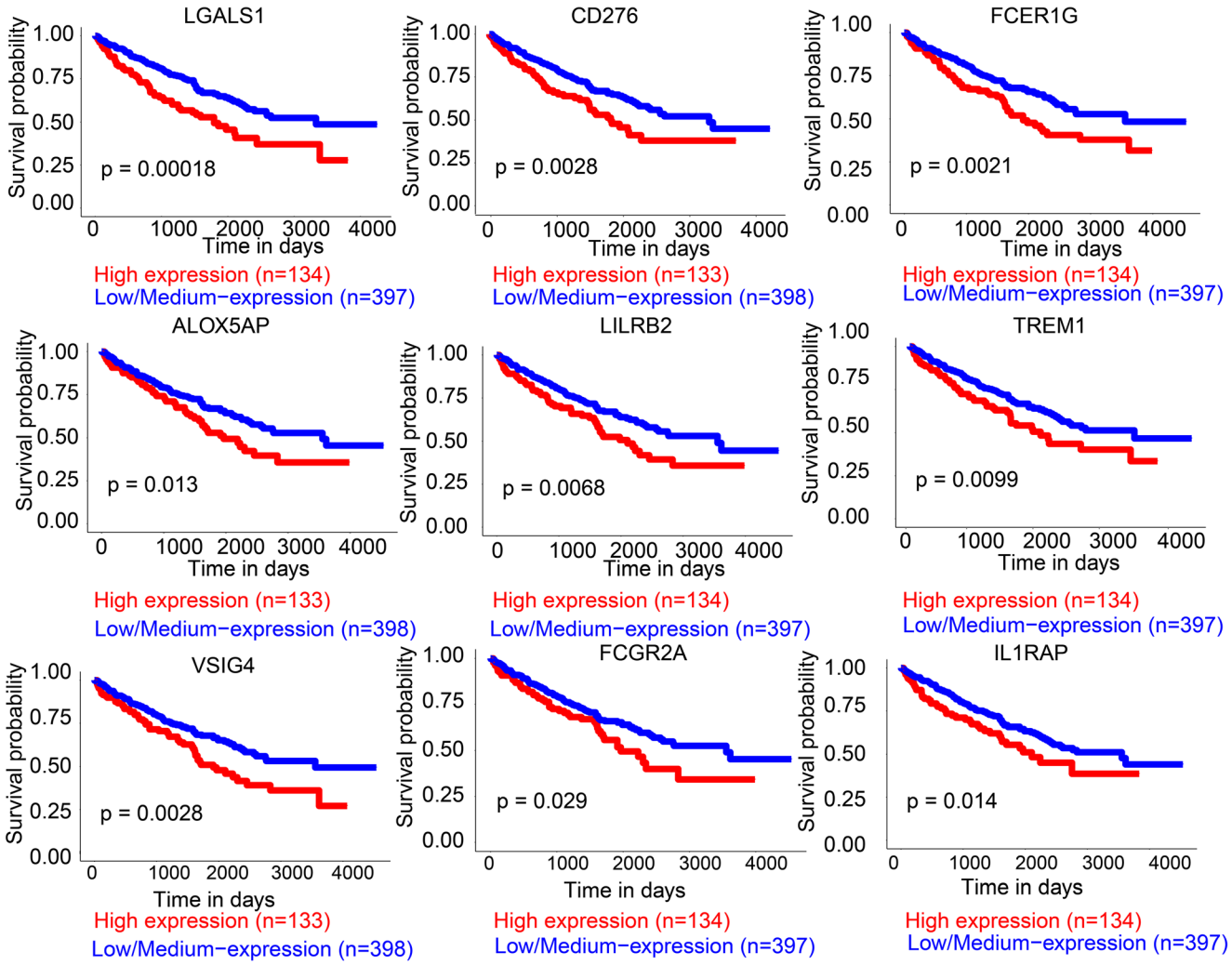

Fig. 2 Kaplan–Meier survival curve of genes selected as potential immune checkpoint inhibitors

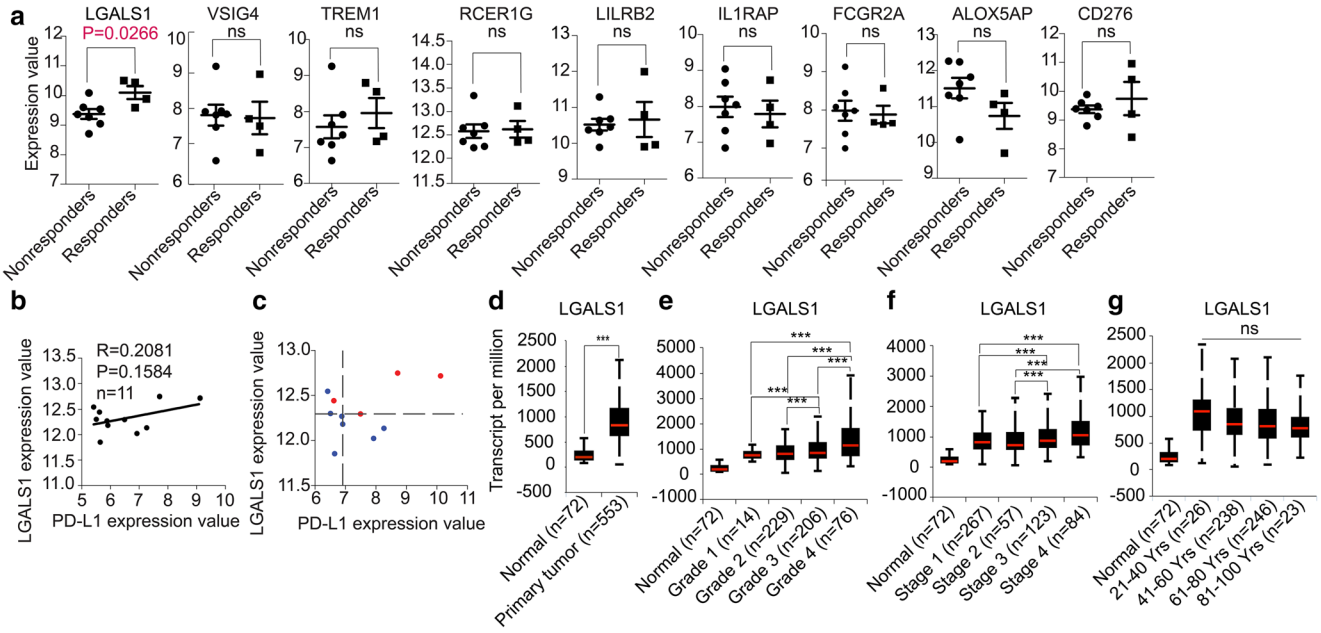

Fig. 3 Correlation between the expression value of target genes and the outcome of anti-PD1 treatment. a CcRCC patients were divided into responders and nonresponders according to their response to anti-PD1 treatment and the mRNA expression level of different genes selected as potential immune checkpoint inhibitor in patient's pretreatment specimens was shown. **b** Correlation between the expression value of LGALS1 and PD-1. **c** Scatter diagram show the distribution of responders (red spot) and nonresponders (blue spot) according to their expression value of LGALS1 and PD-1. **d** The mRNA expression level of LGALS1 in normal kidney tissue and ccRCC tissue was shown base on UALCAN database (**e**) The mRNA expression level of LGALS1 in different grade was shown base on UALCAN database. F, The mRNA expression level of LGALS1 in different stage was shown base on UALCAN database. **g** The mRNA expression level of LGALS1 in different age was shown base on UALCAN database. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$; ns indicates not significant

LGALS1 is an important immune checkpoint and predictor of the outcome of anti-PD1 therapy in ccRCC patients.

LGALS1 Expression Level and Clinical Features

Higher expression of LGALS1 had been found in renal clear cell carcinoma tissue than normal kidney tissue (Fig. 3d). Besides, a higher expression level of LGALS1 had been found in higher stage or grade tumor compare with lower stage or grade tumor (Fig. 3e). Although LGALS1 expression level had an upward tendency in younger people, no significance had been found (Fig. 3f).

Mechanisms of LGALS1 as a Potential Immune Checkpoint Inhibitors

In order to further investigate the underlying mechanism of LGALS1 affect the development of ccRCC. We analyzed the genes which correlated with LGALS1 expression in ccRCC based on UALCAN database. There were 814 genes positively correlated with LGALS1 expression (PearsonCC>0.3) and 1241 genes negatively correlated with LGALS1 expression (PearsonCC<−0.3). Gene set enrichment analysis by EnrichR showed the positively correlated genes were mainly enriched in FOXM1 transcription factor network, PLK1 signaling events, Aurora B signaling and so on in the gene set library of NCI-Nature 2016 and also enriched in fatal ovary in the gene set library of Tissue Protein Expression from Human Proteome Map (Fig. 4a, b). Besides, the negatively correlated genes were mainly enriched in FAS(CD95) signaling pathway, LKB1 signaling events, Nephrin/Neph1 signaling in the kidney podocyte and so on in the gene set library of NCI-Nature 2016 and also enriched in CD8 cells and monocytes of the gene set library of Tissue Protein Expression from Human Proteome Map (Fig. 4c, d). Next, we focused on 30 negatively correlated genes which were enriched in CD8 cells in the gene set library of Tissue Protein Expression from Human Proteome Map. WikiPathways analysis showed that these genes were enriched in TNF alpha Signaling Pathway, TNF-alpha NF-κβ Signaling Pathway and so on which are mainly correlated with T cell apoptosis and survival (Fig. 4e). Especially, MAP3K1, FBXW11 and TAB3 which are negatively correlated with LGALS1 expression and enriched in TNF alpha Signaling Pathway play an important role in T cells survival and proliferation. These suggest LGALS1 could promote tumor progression through affect T cells function.

Discussion

In this study, we had found out 9 candidate genes for potential immune checkpoints (LGALS1, CD276, FCER1G, FCGR2A,

IL1RAP, ALOX5AP, LILRB2, TREM1 and VSIG4) with high expression correlated with a shortened overall survival time in ccRCC patients. We mainly focused on LGALS1, which with high expression level associated with a favorable outcome of anti-PD-1 therapy. At last, genes negatively correlated with LGALS1 expression were enriched in TNF alpha Signaling Pathway in CD8 cells, which play an important role in T cells survival and proliferation. These highly suggest LGALS1 could promote tumor progression through affect $CD8^+$ T cells survival and might be a potential immune checkpoint for ccRCC patients.

Immunotherapy such as high dose IL2 had already proved to be an effective weapon against ccRCC long ago [17]. Recently, treatment base on immune checkpoint inhibitors such as PD-1/PD-L1 and CTLA4 had obtained a significant progress in extending the survival of ccRCC patients [8]. In addition, immunotherapy combined different immune checkpoint inhibitor together gained a more significant improvement in OS and ORR in ccRCC patients than using one immune checkpoint inhibitor alone [2]. As limited immune checkpoint inhibitor had been found in ccRCC patients, novel immune checkpoint inhibitors are urgently needed to be further developed. In the current study, 747 upregulated genes and 1110 downregulated genes had been found in ccRCC tissue compared with normal kidney tissue. For overexpressed genes, the most enriched GO terms were in immune response of biological process, which suggest immune dysregulation play a pivotal role in ccRCC development. This explain why immunotherapy could achieve a preferable outcome in the treatment of ccRCC.

As overexpressed immune checkpoints displayed in the cell membrane or acting through receptors on cell membranes is easy to be blocked, we mainly focus on 47 genes which were enriched both in immune response and plasma membrane. Our results showed 9 genes (LGALS1, CD276, FCER1G, FCGR2A, IL1RAP, ALOX5AP, LILRB2, TREM1 and VSIG4) out of 47 genes with higher expression significantly associated with shorter overall survival. LGALS1 and CD276 are mainly involved in adaptive immune response such as regulation of T cell mediated immune response and inhibition of T cell survival [18, 19]. As known, FCER1G, FCGR2A, IL1RAP, ALOX5AP, TREM1, VSIG4 and LILRB2 are mainly involve in innate immune response, such as the promotion of phagocytosis of opsonized antigens, stimulation of neutrophil and monocyte-mediated inflammatory responses, regulation of allergic reaction and so on [20, 21]. These highly suggest these 9 genes might be the potential immune checkpoints targets for future treatment of ccRCC patients.

Our research was mainly focused on LGALS1. Only a few ccRCC patients have a favorable outcome to immunotherapy. It is well known that immunotherapy is effective because it can disturb the immunosuppressive microenvironment in the tumor tissues and thereby the immune cells can better recognize and kill tumor cells [22, 23]. Our results show that, out of nine genes, only a high expression level of LGALS1 correlated with a preferable outcome of anti-PD-1 therapy. These highly suggest LGALS1 is an immune checkpoint in ccRCC and tumor immunosuppressive microenvironment induced by LGALS1 could be recovered by anti-PD1 treatment in ccRCC patients.

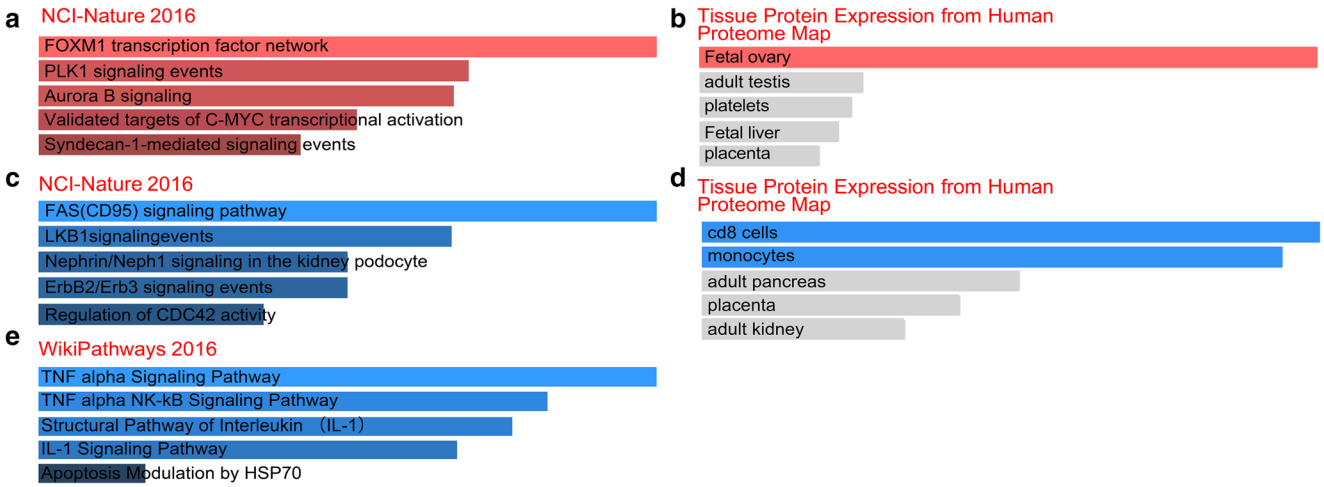

Fig. 4 Gene set enrichment analysis of LGALS1 correlated genes in ccRCC. a Gene set enrichment analysis by EnrichR showed the enriched positively correlated genes of LGALS1 in the library of NCI-Nature 2016. **b** Gene set enrichment analysis by EnrichR showed the enriched positively correlated genes of LGALS1 in the library of Tissue Protein Expression from Human Proteome Map. **c** Gene set enrichment analysis by EnrichR showed the enriched negatively correlated genes of LGALS1 in the library of NCI-Nature 2016. **d** Gene set enrichment analysis by EnrichR showed the enriched negatively correlated genes of LGALS1 in the library of Tissue Protein Expression from Human Proteome Map. **e** 30 negatively correlated genes which were enriched in CD8 cells in the gene set library of Tissue Protein Expression from Human Proteome Map were further studied by WikiPathways analysis. The gray bars represent $p > 0.05$; the red bars or blue bars represent $p < 0.05$

LGALS1 which gene product is galectin-1 is widely expressed by different kinds of immune cells and tumor cells [24]. In addition, it selectively induces apoptosis on pro-inflammatory Th1 and Th17 cell subsets, but not on naive, Th2 or regulatory $F_{oxP}3^+$ T cells [25]. The function of LGALS1 is similar to the immunosuppressive function of PD-L1 [19, 26], thus we speculate LGALS1 and PD-L1 might have synergistic effects on T cell immune tolerance. Our results showed that patients with high expression of LGALS1 and PD-L1 had a favorable outcome to anti-PD1 treatment and low expressed LGALS1 and PD-L1 had a resistance outcome to anti-PD1 treatment**.** This suggests that when the expression of these two genes is both increased, the immune function of T cells will be significantly inhibited, leading to impaired elimination of tumor cell carrying tumor antigens by T cells. However, the immune function of T cells in the tumor environment may be not significantly weakened when the expression levels of both genes are reduced. Tumor growth may be due to the lack of identifiable tumor antigens. However, when only one gene expression is elevated, especially when only LGALS1 expression is elevated, treatment with PD-1 alone may not improve T cell function. This explains why by using PD-L1 alone could not effectively predict the outcome of anti-PD1 therapy in ccRCC. It also suggests that when we give PD1 treatment, combined with LGALS1 treatment may achieve a better outcome in some patients.

At last, our results show that 30 genes which negatively correlated LGALS1 and enriched in CD8 cells were enriched in TNF alpha Signaling Pathway. MAP3K1, FBXW11 and TAB3 which are enriched in TNF alpha Signaling Pathway and negatively correlated with LGALS1 expression play an important role in T cells survival and proliferation [27, 28]. These suggest LGALS1 could promote tumor progress through affect $CD8^+$ T cells function.

Conclusions

In conclusion, we have identified LGALS1 as a potential immune checkpoint. In addition, we found that combined the PD-L1 expression level with LGALS1 together could more efficiently predict the outcome of anti-PD1 treatment than each gene expression alone. At last, we found that LGALS1 could promote tumor progression through affect $CD8^+$ T cells survival.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest. The founding sponsors had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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