As a prognostic biomarker of clear cell renal cell carcinoma RUFY4 predicts immunotherapy responsiveness in a PDL1-related manner

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Abstract

Background: Clear cell renal cell carcinoma (ccRCC) is one of the most lethal malignancies in the urinary system and the existing immunotherapy has not achieved satisfactory outcomes. Therefore, this study aims at establishing a novel gene signature for immune infiltration and clinical outcome (overall survival and immunotherapy responsiveness) in ccRCC patients.

Methods: Based on RNA sequencing data and clinical information in The Cancer Genome Atlas (TCGA) database, we calculated proportions of immune cells in 611 samples using an online tool CIBERSORTx. Multivariate survival analysis was conducted to determine crucial survival-associated immune cells and immune-infiltration-related genes (IIRGs). Next, the clinical specimens and common renal cancer cell lines were applied to confirm IIRGs expression at protein and RNA levels. Finally, functional enrichment analyses and siRNA technology targeted to RUFY4 were implemented to verify its function of predicting immunotherapy response.

Results: Follicular helper T cells (TFHs) and Regulatory T cells (Tregs) were highly infiltrated in the tumor microenvironment (TME) and their relative proportions were independent prognostic factors for patients. Among IIRGs of TFHs and TREGs, RUFY4 was found to be highly activated in tumor microenvironment and its co-expression network was enriched in PDL1/PD1 checkpoint pathway in cancer. Additionally, knockdown of RUFY4 led to the decline of PDL1 and proliferation ability in ccRCC cell lines.

Conclusion: TFHs and Tregs were considered as prognostic biomarkers and RUFY4 was an immunotherapeutic predictor of ccRCC patients in a PDL1-Related manner.

Keywords: Clear cell renal cell carcinoma, Immune infiltration, Immunotherapy, PD-1 blockade, Prognostic signatures

Background

Global statistics indicate that kidney cancer accounts for approximately 2.2% of all cancers, with 1.8 million new cases and 175,100 deaths recorded in 2018 [1]. Clear cell renal cell carcinoma (ccRCC) is the predominant subtype among all kidney cancer subtypes, representing over 70% of all RCC cases [2]. In the past decade, antiangiogenic drugs targeting vascular endothelial growth factor (VEGF), mechanistic target of rapamycin (mTOR) inhibitors, and immune checkpoint inhibitors (ICI) have rapidly expanded treatment options for metastatic ccRCC [3]. Though great improvements in managing ccRCC have been achieved via specific cellular and molecular targets, the prognosis of patients is still unsatisfactory.
The interaction between cancer and immune cells in the tumor microenvironment (TME) has been a new hallmark of cancer since 2011 [4]. As an indispensable part of the TME, immune infiltration cells have been shown to play crucial roles in the occurrence and development of tumors [5]. In previous studies, ccRCC has been considered as a highly immune-infiltrated tumor and infiltration of different immune cells was correlated with distinct clinicopathologic features [6, 7]. Studies by Ueda [8] and Mikami [9] have suggested that immune-infiltrated-related genes (IIRGs) are associated not only with the response to immunotherapy but also with the clinical outcome of ccRCC patients. However, their accuracy and specificity in predicting sensitiveness to immunotherapy and prognosis of ccRCC remain problematic. Therefore, to improve the survival time and life quality of ccRCC patients and provide worthy information to conduct precise individual treatment, novel immune predictors and prognostic indicators are urgently needed.

RUN and FYVE domain-containing proteins (RUFYs), consisting of an N-terminal RUN domain and a phosphatidylinositol 3-phosphate (PI3P)-interacting C-terminal FYVE domain, encompass four genes named \( RUFY1-4 \) [10]. Briefly, \( RUFY1-2 \) contain a RUN domain and a FYVE domain, separated by two coiled coil domain and both of them are identified as downstream effectors of Etk protein kinase [10]. \( RUFY3 \), the smallest of the RUFY proteins with only a RUN domain, is mostly expressed in neurons [11]. \( RUFY4 \) is atypical among the RUFY family members, since it only holds one coiled coil domain [12]. Currently, RUFYs have taken center stage in the field of oncology [11]. \( RUFY1 \) has been shown to interact with podocalyxin-like (PODXL) protein, an ion exchanger regulatory factor in the membrane protein complex associated with poor prognosis of different cancers [13, 14]. Shin [15] suggested \( RUFY2 \) as one of the extremely frequently mutated genes in colorectal cancer. Besides, researches by Zheng [16] and Staubitz [17] showed that \( RUFY2 \) participated in tumorigenesis in lung adenocarcinoma and papillary thyroid carcinoma. \( RUFY3 \) is the final well-known protein in the RUFY family and its dysregulation is implicated in the growth, invasion, and metastasis of lung adenocarcinoma [18] and colorectal cancer [19]. Although \( RUFY4 \) is reported to be the first molecule that affects autophagy and endosome dynamics in a subset of immune cells and that \( RUFY4 \) is an important factor that defines the nature of the response of cells to the direct immune environment [12], there is no strong evidence that \( RUFY4 \) is relevant to any type of cancer.

Based on The Cancer Genome Atlas (TCGA) database, the present study quantified the composition of immune cells in ccRCC and figured out two types of immune cell that possess prognostic ability of ccRCC. Furthermore, we identified \( RUFY4 \), as a novel IIRG, that could simultaneously predict patient prognosis and immunotherapy responsiveness in a \( PD-L1 \)-related manner. Our study also offered a novel method for researchers to creatively tap into immunotherapeutic cells and gene signatures for other cancers.

**Materials and methods**

**Data source and cleaning**

The ccRCC RNA-Seq data were obtained from TCGA database via the Data transfer tool. Samples information and clinical data which consist of gender, age, clinical TNM stage, histopathological grade, survival time, etc. were directly collected from the website. Then, two gene expression matrices of COUNT and FPKM (Fragments Per Kilobase of exon model per Million mapped) and one clinical information table were constructed through R software (version: 64.4.0.3). Meanwhile, Yusenko renal datasets from the Oncomine (https://www.oncomine.org/) database and GSE126864 from GEO (Gene Expression Omnibus) database were also analyzed in this study. The analytical procedures were shown in Additional file 1: Fig. S1.

**Establishment of immune infiltration landscape**

CIBERSORTx is an online tool to accurately infer cell type abundance from RNA profiles of intact tissues [20]. This study conducted CIBERSORTx analysis online (https://cibersortx.stanford.edu/) according to the following parameters:

- **sigmatrix**: LM22.update-gene-symbols.txt, **perm**: 500, **verbose**: TRUE, **rbatchBmode**: TRUE, **QN**: FALSE

From calculation results of CIBERSORTx, 609 samples were selected with \( p \leq 0.05 \) and each sample was equipped with the relative proportion matrix of 22 immune cell types. Then R packages “pheatmap”, “barplot” and “vioplot” were installed in R software to establish the immune infiltration landscape.

**Identification of survival-associated immune cells**

On basis of the proportions of 22 immune cells, Kaplan–Meier (K-M) analysis of overall survival rate was performed. Among K-M survival analysis, the cut-off was set as the upper and lower quartiles and statistically significant means that \( p \)-values were less than 0.05. Univariate survival analysis was used to verify the prognostic predictive effect of known factors, such as age, gender, T stage, M stage, etc. Additionally, this study created a multivariate model which was adjusted for those factors whose hazard ratio (HR) > 1. By setting the cut-off as the median of relative proportion, 537 samples were distributed into high and low groups. Then the proportion of
each immune cell was added as a new binary variable for survival analysis.

**Identification of key genes related to immune infiltration**
Among genes that are highly expressed in tumors, this study searched for genes that are closely related to the high degree of immune-infiltration of immune cells. Also, the same method was used to identify the low expressed key genes. R package “VennDiagram [21]” was installed on R software to visualize these results. The receiver operating characteristic (ROC) curve of each key gene was used to assess the capability of distinguishing from patients for healthy individuals according to areas under this curve (AUC).

**Construction of RUFY4 co-expression network**
The cBioPortal offers researchers online assistance for studying multidimensional cancer genomics data [22]. To construct the co-expression network of RUFY4, genes with a Spearman correlation index > 0.55 were added to co-expression network.

**Functional enrichment analysis**
This study carried out functional classification and annotation of immune-associated genes on the website DAVID [23] by the methods of GO and KEGG analysis. The cut-off of p-value was 0.05. R packages “ggplot2” were used to visualize the top 10 terms of GO analysis and KEGG pathway analysis. Then, Cytoscape [24] was also applied to construct the pathway cross-talk network via a “Cluego” plug-in. Gene set enrichment analysis (GSEA) was performed by Windows desktop program v4.1.0. The GSEA result of RUFY4 was therefore conducted on all known genes ranked by enrichment scores from most positive and most negative. 1000 random sample permutations were carried out.

**Prediction of immunotherapy response**
ImmuCellAI was used to predict the response of Immune checkpoint blockade (ICB) therapy with the ICB response prediction being checked based on gene expression matrix [25]. This study conducted ICB prediction analysis online (http://bioinfo.life.hust.edu.cn/ImmuCellAI#!/analysis) based on the compositions and proportions of immune cells of patients in TCGA.

**Cell culture and reagents**
The human renal cell carcinoma cell line A498, 786O, Caki, OsRC and control cell line HK2 were obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in with Dulbecco's modified eagle medium (DMEM, Gibco, USA) supplemented with 10 percent fetal bovine serum (FBS, Gibco, USA) and were cultured in the incubator at 37 °C, 5% carbon dioxide.

**RNA interference**
Small interfering RNA for RUFY4 was transfected in 786O and CAKI cell lines using Lipofectamine 6000 (Beyotime, China), respectively. The siRNA sequences for RUFY4 (GenePharma, China) were followings:

siRNA#1: 5′- CAA GGU CAC CAA AGA CCU AAG -3′
siRNA#2: 5′- GGA GAA UCC ACA AGU GCA AAC -3′
siRNA#3: 5′- GCA GAG GGU CAG AGA ACA ACA -3′

Cell lysates and total RNA were collected 72 h after the transfection to verify knockdown efficiency by western blot and qPCR.

**Tissue samples**
16 pairs of human ccRCC tissues and adjacent normal tissues were collected from Department of Urology, Union Hospital, Tongji Medical College (Wuhan, China) in 2020. This process had fully informed consent of the patients. And this study was approved by the Institutional Review Board of Huazhong University of Science and Technology. The license number of the ethical review for the study was S1892.

**RNA isolation and real-time PCR analysis**
The Magzol reagent (Thermo, Massachusetts, USA) was used to extract total RNA of tissues and cell lysates. 500 ng of total RNA from tissue and cell were applied for reverse transcription. qPCR analysis was conducted (LightCycler 480II; Roche, Basel, Switzerland) with the Hieff® qPCR SYBR Green Master Mix (11201ES03, Yeasen, China). Samples were normalized by Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH).

| GAPDH Forward 5′-CCAGAACACCATCCCTGCGT-3′ | Reverse 5′-CCTGCTTACCACCTTCTTG-3′ |
| RUFY4 Forward 5′-ACGCCAAGAAGACATCTCGG-3′ | Reverse 5′-CTCTGACCCTCTGCAACCAG-3′ |

**Western blotting assays**
The protein of cells and tissues was extracted by radio-immunoprecipitation assay (RIPA) protein lysis buffer (Beyotime, China) with protease inhibitor cocktail (Beyotime, China) and Phenylmethanesulfonylfluoride (PMSF, Beyotime, China). 30 µg of protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel. The proteins were then separated by gel electrophoresis and transferred to polyvinylidene
fluoride (PVDF, Roche, Basel, Switzerland) membranes. 5% nonfat dried skimmed milk was used to block the membranes for 1.5 h at room temperature. Then, the membranes were incubated overnight with primary antibodies.

RUFIY4, LS-C307570 LSBio, USA, dilution 1:2000
PDL1, A1645 ABclonal, China, dilution 1:1000
β-actin, AC026 ABclonal, China, dilution 1:100,000

Finally, the membranes were washed and incubated in blocking buffer with secondary antibodies (Anti-Rabbit AS014 ABclonal, China; Anti-Mouse AS003 ABclonal, China) for 2 h before detection.

Cell viability assays
Each 96-well plate was plated with 2000 cells. The proliferation rate of cells was detected using the cell counting kit (CCK-8, Yeasen, China). 110 μL CCK8 solution (10 μL CCK8:100 μL medium) were added to each well and the 96-well plate was incubated in dark for 1 h. Cell viability was assessed at 0, 24, 48, 72 and 96 h upon treatments by NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA) at 450 nm.

Transwell assays
For migration and invasion assays, cells were cultured in serum-free medium for 24 h. Then, cells were plated in the top chamber of transwell chamber (REF3422, Corning, USA) and cells were allowed to invade through the Matrigel (Corning, USA, dilution 1:8) or not. With or without Matrigel were used for invasion and migration assay. After 24 h, cells invading the lower surface of the chamber membrane were fixed in 100% methanol. Then, cells were stained with 0.05% crystal violet and 10 fields were randomly photographed for counting. More details were described in previous study [26].

Results
A unique immune infiltration landscape was established for ccRCC
When the RNA-seq matrix derived from 611 ccRCC samples were screened by the CIBERSORTx algorithm, the differences of immune infiltration between ccRCC and adjacent normal tissues were comprehensively tracked out. The proportions of 22 immune cells in ccRCC and adjacent normal tissues were shown in Fig. 1A. The heatmap of the 22 immune cells was shown in Fig. 1B. To further examine the difference of immune infiltration cells, we generated a violin plot to quantitatively describe the differences based on tumor tissues (537 samples) and adjacent normal tissues (72 samples). According to the violin plot (Fig. 1C), naïve B cells, plasma cells, CD8 T cells, naïve CD4 T cells, resting memory CD4 T cells, activated memory CD4 T cells, follicular helper T cells (TFHs), regulatory T cells (Tregs), gamma delta T cells, monocytes, M0 macrophages, M1 macrophages, resting dendritic cells, activated dendritic cells, resting mast cells, eosinophils and neutrophils presented different relative proportions and the differences were statistically significant. Immune cell types, such as CD8 T cells, were markedly highly infiltrated in tumor tissues. These results showed that the proportions of immune-infiltrated cells might help researchers to distinguish ccRCC from normal individuals.

TFHs and Tregs were identified as survival-associated immune cell
In the previous step, 17 immune cell types were obtained based on their differences in immune infiltration between tumor and adjacent normal tissues. Here, the correlation among the proportions of the 17 immune cell types and overall survival (OS) rates were analyzed via Kaplan–Meier curves. The results in Additional file 2: Fig. S2A–H showed that the proportions of 8 immune cells were related to the OS rates, including activated memory CD4 T cells, resting memory CD4 T cells, CD8 T cells, M0 macrophages, M1 macrophages, Tregs, TFHs, and neutrophils. Notably, TFHs and Tregs had the highest hazard ratio (HR) values of 1.59 and 1.62, respectively, while resting memory CD4 T cells and neutrophils were negatively correlated with the OS rate. To further understand the clinical predictive value of survival-associated cells, the spearman correlation analysis was conducted among the relative proportion of immune cells and clinical attributes. As shown in Fig. 2C–K and Additional file 3: Fig. S3A–C, the proportions of TFHs, Tregs and CD8 T cells elevated with the increase of histopathological grade and TNM stage. The results of other immune cells were shown in Additional file 3: Fig. S3D–G. Next, multivariate survival analysis was conducted to assess

(See figure on next page.)

Fig. 1 Establishment of immune-infiltration landscape. A The relative proportions of immune cells in the samples from TCGA. Each column represents a sample, and each column with a different color and height indicates the relative proportions of immune cells in this sample. B The heatmap of 22 immune cells in tumor and adjacent normal tissues. Each row represents one type of immune cell and the color of each small square represents the content of immune cells. C The difference of 22 immune cell relative proportions between the ccRCC and control groups in TCGA. The blue violins represent adjacent normal tissues while the red violins represent tumor tissues. Wilcoxon-test were used in statistical analysis. p values were displayed in Figures.
Fig. 1 (See legend on previous page.)
the prognostic value of the 8 immune cells and a clinicopathologic parameter was chosen as OS rates for ccRCC patients (Table 1). Relative proportions of TFHs and Tregs were independent prognostic factors for OS, after adjustment for known risk factors such as age, clinical stage, T stage, M stage, and histopathological grade (Fig. 2A, B). Survival analyses indicated that a higher immune infiltration of TFHs or Tregs was associated with inferior OS. OS curves based on TFHs or Tregs proportions in ccRCC tumor tissues were distinctly disjointed. Therefore, TFHs and Tregs held much appeal for this study.

IIRGs of Tregs and TFHs participated in several overlapped biological processes

This study obtained 680 genes that had significantly different expression levels between high and low proportions of TFHs in the TME, 620 of which presented much higher expressions (Fig. 3A). As for Tregs, 676 differentially expressed genes were identified, where 353 genes were related to higher immune infiltration (Fig. 3B). After uploading these genes to the DAVID website, the top 10 items of TFHs in Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were obtained (Fig. 3D–G). The biological process of the GO analysis demonstrated that TFHs might be involved in the cellular protein metabolic process and immune response (Fig. 3D). The cellular component showed that these genes were enriched in extracellular exosome and plasma membrane (Fig. 3G). The molecular function of these genes was gathered in serine-type endopeptidase activity, antigen binding and sequence-specific DNA binding (Fig. 3E). The KEGG analysis suggested that cytokine-cytokine receptor interaction was the dominant pathway and PD1/PD1 pathway was also enriched (Fig. 3F). As for Tregs, cellular protein metabolic process at the biological process level, extracellular exosome at the cellular component level and serine-type endopeptidase activity at molecular function level were the most crucial enrichment items (Additional file 4: Fig. S4A–D). KEGG pathway analysis results were largely similar to TFHs results. Collectively, these results showed that the two crucial immune-associated cells had a considerable overlapped function, implying that the two cells might participate in the same biological process and there might be cross-talks within the TME.

Upregulated-RUFY4 was a prognostic biomarker of ccRCC

Three gene sets, including genes related to low immune infiltration of Tregs, low immune infiltration of TFHs, and genes low-expressed in tumor tissues (shown in the previous Fig. 3C), were submitted to the “VennDiagram” Package on R software. As shown in Fig. 4A, SLC12A1 was closely related to the low degree of immune infiltration of the two crucial immune cells and it was silenced in tumor tissues. Remarkably, after narrowing the search scope to genes that were highly expressed in tumor tissues, LAG3, PDCD1, and RUFY4 were found to be associated with the high degree of immune infiltration of Tregs and TFHs. From Fig. 4B, it could be observed that 94 genes were not only associated with the increased immune infiltration of Tregs but also with the immune infiltration of TFHs. The ROC results of the aforementioned 4 key genes, including one downregulated gene SLC12A1 and 3 upregulated genes LAG3, PDCD1 and RUFY4, are presented in Fig. 4C–F. RUFY4 had a relatively higher AUC (area under the curve $=0.946$) than the other key genes (0.890, 0.912, 0.008), suggesting that it had a more accurate diagnostic value for ccRCC. Though LAG3 also had a significant AUC, it had little prognosis prediction value (Additional file 5: Fig. S5A). Therefore, RUFY4 was considered as the key gene in the TME related to the high immune infiltration of TFHs and Tregs (Table 2). Besides, RUFY4 had an activated expression in tumor tissues, hence could accurately and sensitively distinguish between normal and ccRCC patients. To verify these results, the high throughput sequencing data in GSE126964 were downloaded from GEO database. From Fig. 4G, it could be demonstrated that RUFY4 was also upregulated in ccRCC. The same results were obtained from the collected clinical samples (Fig. 4H). Correspondingly, changes of the protein level were consistent with the RNA level of RUFY4 (Fig. 4I). Additionally, common RCC cell lines, such as 786O [27] and CAKI [27], presented higher protein and RNA levels than HK2 that is an immortalized proximal tubule epithelial cell line from normal adult human kidney (Fig. 4J–K). Moreover, the immunohistochemical results from the Human

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Fig. 2 TFHs and Tregs were identified as survival-associated immune cell. A, B The multivariate survival analysis for the relative proportions of immune cells. The red line indicates a high proportion group of immune cells, and the blue line indicates a low proportion group of immune cells. HR means hazard ratio and CI means confidence interval. p-value were on Figures. C–K The relationship between the relative proportions of immune cell and G grade, clinical stage and T stage. The ordinate represents the proportion of immune cells and the horizontal line inside the box represents the median value of immune cell proportions. Kruskal-test and Wilcoxon-test were used in statistical analysis and p-values were on Figures.
Fig. 2 (See legend on previous page.)
Protein Atlas [28] (HPA is a resource for many areas of biomedical research, including protein science and biomarker discovery) also showed that RUFY4 was highly expressed in renal cancer (Additional file 5: Fig. S5B).

Next, this study determined the correlation between RUFY4 and ccRCC clinical attributes. RUFY4 expression levels were consistently correlated with ccRCC clinical stage (Fig. 4L), histopathological grade (Fig. 4M), T stage (Fig. 4N), M stage (Fig. 4O), N stage (Fig. 4P). Survival analysis revealed that the increased expression of RUFY4 was associated with inferior OS (Fig. 4Q). In summary, these results confirmed that RUFY4 was a new prognostic biomarker of ccRCC.

RUFY4 depletion impaired proliferation rather than migration or invasion of cancer cells
To ascertain the function of RUFY4 in TME, small interfering RNAs targeted RUFY4 were applied to knockdown RUFY4 in RCC cells, 786O and CAKI. As was shown in Fig. 5A and B, RUFY4 was silenced in RCC cell lines both in RNA and protein levels. And Fig. 5D, E demonstrated RUFY4 depletion impaired cell proliferation. Our previous results showed that RUFY4 is related to the distant metastasis and lymphatic metastasis in cancer patients (shown in the previous Fig. 4O, P). Here, we found that down-regulated RUFY4 had no influence on cell migration and invasion (Fig. 5C). These results hinted that RUFY4 itself has no significant role in the progress of ccRCC. More in-depth research needs to be carried out to explain these contradictory results.

RUFY4 predicted immunotherapy in a PDL1-related manner
We hypothesized that the immune cells in TME might be involved in the tumor-promotive function of RUFY4 based on the findings that RUFY4 functioned little on migration and invasion of ccRCC. Table 2 demonstrated that there were correlations between 27 types of immune cells and RUFY4, 6 types of which were positively correlated with RUFY4 while 11 were negatively correlated. Next, a co-expression network of RUFY4 was constructed (Additional file 5: Fig. S5C). As expected, the network of RUFY4 was shown to be involved in several crucial immunomodulation processes, including cytokine-cytokine receptor interaction, PD-L1 (programmed cell death 1 ligand 1) expression and PD-1 (programmed death-1) checkpoint pathway in cancer, T helper cells differentiation, T cell receptor signaling pathway, and primary immunodeficiency (Fig. 6E). These results emphasized that the RUFY4 might have an inseparable connection with immunomodulation in TME.

| Variable | HR | 95% CI | p-value |
|----------|----|--------|---------|
| Age (years) | | | |
| ≤ 60 (n = 267) | 1.71 | 1.231 | 2.374 | 0.001 |
| > 60 (n = 270) | | | |
| Gender | | | |
| Female (n = 186) | | | |
| Male (n = 351) | | | |
| Clinical stage | | | |
| C1–C3 (n = 452) | | | |
| C4 (n = 82) | 14.832 | 3.417 | 64.394 | <0.001 |
| CX (n = 3) | | | |
| T stage | | | |
| T1 and T2 (n = 348) | 1.689 | 1.150 | 2.482 | 0.008 |
| T3 and T4 (n = 189) | | | |
| M stage | | | |
| M0(n = 447) | 0.189 | 0.044 | 0.811 | 0.025 |
| M1 (n = 80) | | | |
| MX (n = 10) | | | |
| G stage | | | |
| G1 and G2 (n = 249) | 1.797 | 1.212 | 2.665 | 0.004 |
| G3 and G4 (n = 281) | | | |
| GX (n = 7) | | | |
| Tregs | | | |
| Low (n = 268) | 1.494 | 1.073 | 2.080 | 0.018 |
| High (n = 269) | | | |
| TFHs | | | |
| Low (n = 268) | 1.589 | 1.141 | 2.212 | 0.006 |
| High (n = 269) | | | |

Multivariate models were adjusted for T, M classification, age, and gender
Hazard ratio, estimated from Cox proportional hazard regression model
Confidence interval of the estimated HR

(See figure on next page.)

**Fig. 3** IIRGs of Tregs and TFHs participated in several overlapped biological processes in TME. A DEGs between high TFHs-infiltration group and low TFHs-infiltration group. B DEGs between high Tregs-infiltration group and low Tregs-infiltration group. C DEGs between ccRCC and adjacent normal tissues. Each red dot shows an upregulated gene and each blue dot shows a downregulated gene. DEGs means different expression genes. D–G the enrichment analysis results of IIRGs at biological processes, cellular components, molecular functions, and KEGG levels. The top 10 results of each term are shown, and the color indicates the statistical significance and the size indicates the number of genes enriched for each result. IIRGs is the abbreviation of immune-infiltration-related genes.
Fig. 3 (See legend on previous page.)
of ccRCC. After choosing canonical pathways gene sets derived from the KEGG pathway database as the molecular signatures database (Fig. 6C, D), GSEA indicated that RUFY4 was highly associated with T cell receptor signaling pathway and cytokine–cytokine receptor interaction. Moreover, as presented in Fig. 6A, B, it was found that when compared with gene sets derived from the WikiPathways pathway database, cancer-immunotherapy with PD1-blockade and costimulatory signaling of T cell receptor were the two most significant enrichment pathways, whose q-FDR (false discovery rate) values both equaled to zero. These pieces of evidence indicated that the cancer-promoting effect of RUFY4 was inseparable from the assistance of immunomodulation in TME. Therefore, PD-1 (programmed death-1) checkpoint pathway in cancer that repeatedly appeared in the functional enrichment results appealed to this study. Moreover, The RNA-Seq data from Yusenko Renal study showed that PDL1 was upregulated in ccRCC (Fig. 6F). To explore how RUFY4 contributed to PD-1/PDL1 in TME, the correlation analysis between PDL1 and RUFY4 was conducted on the basis of the ccRCC RNA-Seq data. There was a strong relationship between them and the spearman R index equaled 0.5142 (Fig. 6G). Also, small interfering RNA targeted RUFY4 were applied to degrade the expression level of RUFY4. As was shown in previous Fig. 5B, the depletion of RUFY4 caused the decrease of PDL1 in RCC cell lines, 786O and Caki1. Furthermore, the expression level of RUFY4 could predict the responsiveness of tumor patients to ICB immunotherapy (Fig. 6H). Once PDL1 and RUFY4 were united together, the prediction model was more convincing and valuable (Fig. 6I). And as for immune infiltration prediction, the joint prediction ability of PDL1 and RUFY4 was stronger than the single one (Fig. 6I). Taken together, these observations suggested that RUFY4 might be an indicator for immune infiltration and immunotherapy in a PDL1-related manner.

Discussion

Clear cell RCC is one of the most lethal types of urogenital tumors with an increasing mortality rate over several years [29]. Since Galon [30] first proposed the concept of immune contexture in 2007, researches associated with the location, density, and functional orientation of different immune cells in the TME have put immunotherapy into the cutting-edge frontier. Among the current therapeutic strategies, ICI has become a key option for various cancer types [31]. Ample clinical trials have shown that PD-1 blockade is a vital method in the management of ccRCC and therapies based on anti-PD-1 are the first-line choices for refractory patients [32, 33]. Hence, we aimed at identifying immune cells and genes closely associated with both immune infiltration and immunotherapy responsiveness in the TME.

In this study, TFHs and Tregs were related to patients’ OS after adjusting for known risk factors. TFHs are specialized T helper (TH) cells and different from other subgroups such as TH1, TH2, and TH17. TFHs predicted improved survival in breast cancer [34] and were associated with a positive prognosis in colorectal cancer [35]. Contrarily, TFHs predicted a negative clinical prognosis in lung squamous cell carcinoma [36]. These results put TFHs in a controversial position and thus further exploration should be performed to determine whether it is a protective factor or a risk factor. Treg is another crucial survival-associated immune cell, which is a widely known restrainer of the immune regulatory network. Tregs are involved in balancing immune responses [37] and act as a double-edged sword. On the one hand, they can restrain unnecessary immune activations, such as autoimmunity. On the other hand, useless immune responses are utilized by Tregs for pathogens or cancer cells, resting in the progression of cancer [38]. In renal cancers, there has been evidence for the association between Tregs and poor prognosis, as well as immunotherapy resistance. In summary, these cells play important roles in the construction of the TME.
Fig. 4 (See legend on previous page.)
Among IIRGs in this study, RUFY4 is closely related to the dense infiltration of TFHs and Tregs. Although the importance of IIRGs in tumor progression and immunotherapy has been recognized, there are few studies dedicated to genes related to the immune infiltration of TFHs and Tregs. Here, this research innovatively proposes a method of a targeted selection of IIRGs. Additionally, our results noted that the depletion of RUFY4 caused the decrease of PDL1. Previous studies [39–41] revealed that JAK-STAT axis primarily regulates PDL1 expression in tumor cells through the activation of transcription pathway. Angel [39] considered that the JAK-STAT3 regulated PDL1 expression by activation of its promoter. The role and function of the NF-kB signaling pathway cannot be overlooked. Peng [42] found that chemotherapy induces local immune suppression in ovarian cancer through NF-kappa B-mediated PDL1 upregulation. Fabrizio proposed that NF-kappa B directly induces PDL1 gene transcription by binding to its promoter, and it can also regulate PDL1 post-transcriptionally through indirect pathways [43]. Together with our finding that RUFY4 was also closely linked to JAK-STAT and NF-kappa B pathways, we speculate that RUFY4 regulated the expression level of PDL1 by participating in JAK-STAT and/or NF-kappa B signaling pathways.

Another finding in this study is that RUFY4 depletion has no effect on migration or invasion of cancer cell in vitro while RUFY4 is related to the distant metastasis and lymphatic metastasis in cancer patients. To explain this conflict phenomenon, we make a hypothesis that the immune system might be involved in the tumor-promotive function of RUFY4 based on the following evidence: (a) Metastasis is under the control of complex and redundant pathways involving the tumor cell and the microenvironment [44, 45]. More and more evidence confirms that a large array of genes is known to facilitate or regulate cancer metastasis, not just a single molecular effect [44, 46–50]; (b) TME represents the necessary prerequisite for cancer progression and metastasis [51–53]; (c) Tregs [54] and TFHs [55] control cancer invasion and migration because they might establish an immunosuppressive microenvironment within primary lesions; and (d) RUFY4 is considered an immunological biomarker of THFs and Tregs, and its depletion contributes to the reduction of PDL1. Thus, we guess that the depletion of PDL1 could be recognized by THFs or Tregs, which might be responsible for RUFY4-mediated cancer metastasis. There is no doubt that more in-depth mechanism research needs to be carried out in the future.

### Conclusion

The present study analyzed and verified a unique gene RUFY4 closely related to immune infiltration of TFHs and Tregs. These cells and the gene were separately recognized as independent prognostic biomarkers of ccRCC patients. Moreover, RUFY4 could predict the responsiveness of ICI therapy for ccRCC patients in a PDL1-related manner. Our findings also provide strategies to explore effective signatures of immunotherapy and clinical outcome in cancer research.

### Table 2 Spearman correlation analyses of immune cell fraction and RUFY4&LAG3

| Immune cells                  | RUFY4 Spearman r | p-value | LAG3 Spearman r | p-value |
|-------------------------------|------------------|---------|------------------|---------|
| Naive B cells                 | -0.505           | ****    | -0.596           | ****    |
| Memory B cells                | 0.024            | ns      | 0.003            | ns      |
| Plasma cells                  | -0.071           | ns      | -0.027           | ns      |
| CD8 T cells                   | 0.328            | ****    | 0.821            | ****    |
| Naive CD4 T cells             | -0.212           | ****    | -0.207           | ****    |
| Resting memory CD4 T cells    | -0.383           | ****    | -0.590           | ****    |
| Activated memory CD4 T cells  | 0.052            | ns      | 0.137            | ***     |
| Follicular helper T cells     | 0.471            | ****    | 0.454            | ****    |
| Regulatory T cells (Tregs)    | 0.362            | ****    | 0.315            | ****    |
| Gamma delta T cells           | 0.278            | ****    | 0.562            | ****    |
| Resting NK cells              | -0.255           | ****    | -0.503           | ****    |
| Activated NK cells            | 0.135            | ***     | 0.245            | ****    |
| Monocytes                     | -0.171           | ****    | -0.350           | ****    |
| Macrophages M0                | 0.049            | ns      | 0.069            | ns      |
| Macrophages M1                | 0.183            | ****    | 0.222            | ****    |
| Macrophages M2                | -0.289           | ****    | -0.375           | ****    |
| Resting Dendritic cells       | -0.211           | ****    | -0.264           | ****    |
| Activated Dendritic cells     | -0.390           | ****    | -0.375           | ****    |
| Resting Mast cells            | -0.363           | ****    | -0.480           | ****    |
| Activated Mast cells           | -0.015           | ns      | -0.060           | ns      |
| Eosinophils                   | -0.348           | ****    | -0.380           | ****    |
| Neutrophils                   | -0.271           | ****    | -0.284           | ****    |

**Note:** p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05, ns = no significance
Fig. 5  RUFY4 depletion impaired proliferation rather than migration or invasion of cancer cells. **A** Relative mRNA levels of RUFY4 in the control cells and RUFY4-knockdown cells. t-test, ****p < 0.0001. **B** Western blot assay for the protein levels of RUFY4 in indicated cells. **C** Migration and invasion assay for indicated RCC cells (Magnification: 200×). t-test, p = ns (no significance). **D** Cell growth curves of CCK8 assays for indicated cells. t-test, ****p < 0.0001.

(See figure on next page.)

Fig. 6  RUFY4 predicted immune infiltration and immunotherapy in a PD1 related manner. **A-D** GSEA for the correlation between RUFY4 and TCR signaling pathway, cancer immunotherapy by PD-1 blockades, TCR and costimulatory signaling, and TCR costimulatory signaling. FDR < 25%, p < 0.05 was considered statistically significant. **E** Pathway network diagram established by clueGO based on the co-expressed genes of RUFY4. The color of each circle represents one type of signal pathway and its size represents the degree of statistical significance. Genes involved in each signal pathway are marked with red labels. **F** The mRNA levels of PDL1 in 22 ccRCC tissues and 5 adjacent normal tissues in ccRCC based on data from the Oncomine database. t-test, *p < 0.05. **G** The relationship between the expression level of RUFY4 and PDL1 based on the data from TCGA database. Spearman-test, p < 0.0001. **H** The relationship between the expression level of RUFY4 and the sensitivity of ICB immunotherapy. Chi-square tests were used to calculate the p-value. The cut-off for classifying a patient into High/Low expression is the median of expression level. **I** The relationship between the expression level of RUFY4-PDL1 and the sensitivity of ICB immunotherapy. Low PDL1-RUFY4 means RUFY4 and PDL1 were both lower than the median expression level. Chi-square tests were used to calculate the p-value. **J** The difference of 8 immune cell proportions between low and high PDL1/RUFY4 groups. Low PDL1 + low RUFY4 means RUFY4 and PDL1 were both lower than the median of expression level. Chi-square tests were used to calculate the p-value. ** *p < 0.01, ***p < 0.001, ****p < 0.0001. p = ns (no significance).
Fig. 6 (See legend on previous page.)
Abbreviations
IIRG: Immune-infiltrated-related gene; TFHs: Follicular helper T cells; Tregs: Regulatory T cells; RUFY: RUN and FYVE domain-containing protein; PI3P: Phosphatidylinositol 3-phosphate; PODXL: Podocalyxin-like; TCGA: The Cancer Genome Atlas Project; KM: Kaplan–Meier; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; PD-L1: Programmed cell death 1 ligand 1; PD-1: Programmed death-1; ccRCC: Clear cell Renal cell carcinoma; ICi: Immune checkpoint inhibitors; TME: Tumor microenvironment; DMEM: Dulbecco’s modified eagle medium.

Supplementary Information
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Additional file 1: Figure S1. Flowchart of data processing.
Additional file 2: Figure S2. A–I The univariate survival analysis for the relative proportions of the nine immune cells. The red line indicates a high proportion group of immune cells, and the blue line indicates a low proportion group of immune cells. The cut-off for classifying a patient into High/Low is the median of relative proportion. Long-rank tests were used for statistics. P-values were on Figures.
Additional file 3: Figure S3. A–G The relationship between the relative proportions of immune cells and pathological stage and clinical stage. The ordinate represents the proportions of immune cells and the horizontal line inside the box represents the median value of immune cell proportions. Kruskal-test and Wilcoxon-test were used in statistical analysis. P-values were on Figures.
Additional file 4: Figure S4. A–D The enrichment analysis results of IIRGs at biological processes, cellular components, molecular functions, and KEGG levels. The top 10 results of each term are shown and IIRGs is the abbreviation of immune-infiltration-related genes.
Additional file 5: Figure S5. A The multivariate survival analysis for the expression level of LAG3. The red line indicates a high expressing group of LAG3, and the blue line indicates a low expressing group. HR means hazard ratio and CI means confidence interval. P-value was on the Figure. B The histological expression of RUFY4 from Human Protein Atlas. C Verifying the correlations between genes in the co-expression network of RUFY4. The size of each dot represents the statistical significance and its color means the correlation index.

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Authors’ contributions
YHM and ZXP designed and directed data processing procedures. SJ, XZY, XW, MGX, LQY and XKR analyzed the data. MDJ wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets analyzed during the current study are available in the TCGA repository (https://portal.gdc.cancer.gov/), Gene Expression Omnibus (GEO) at GSE126864 and Oncomine repository. Other data generated in this study are available upon request from the corresponding author.

Declarations
Competing interests
The authors declare no potential conflicts of interest.

Consent for publication
All participants agreed to publish this article.

Ethics approval and consent to participate
This study was approved by the Institutional Review Board of Huazhong University of Science and Technology. And the license number of the ethical review for the study is S1892.

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References
1. Bray F, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA. 2018;68(6):394–424.
2. Hsieh JJ, et al. Renal cell carcinoma. Nat Rev Dis Primers. 2017;3:17009.
3. Choueiri TK, Motzer RJ. Systemic therapy for metastatic renal-cell carcinoma. N Engl J Med. 2017;376(4):354–66.
4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74.
5. Fridman WH, et al. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer. 2012;12(4):298–306.
6. Yoshihara K, et al. Inferred tumour purity and stromal and immune cell admixture from expression data. Nat Commun. 2013;4:2612.
7. Şenbabaoğlu Y, et al. Tumor immune microenvironment characterization in clear cell renal cell carcinoma identifies prognostic and immuno-therapeutically relevant messenger RNA signatures. Genome Biol. 2016;17(1):231.
8. Ueda K, et al. Prognostic value of PD-1 and PD-L1 expression in patients with metastatic clear cell renal cell carcinoma. Urol Oncol. 2018. https://doi.org/10.1016/j.urolonc.2018.07.003.
9. Mikami S, et al. Clinical significance of programmed death-1 and programmed death-ligand 1 expression in the tumor microenvironment of clear cell renal cell carcinoma. Cancer Sci. 2019;110(6):1820–8.
10. Kitaeghi Y, Matsuda S. RUFY, Rab and Rap family proteins involved in a regulation of cell polarity and membrane trafficking. Int J Mol Sci. 2013;14(3):6487–98.
11. Char R, Pierre P. The RUFYS, a family of effector proteins involved in intracellular trafficking and cytoskeleton dynamics. Front Cell Dev Biol. 2020;8:779.
12. Terawaki S, et al. RUFY and FYVE domain-containing protein 4 enhances autophagy and lysosome tethering in response to Interleukin-4. J Cell Biol. 2015;210(7):1133–52.
13. Taniuchi K, et al. Measurement of serum PODXL concentration for detection of pancreatic cancer. Onco Targets Ther. 2018;11:1433–43.
14. He S, et al. PODXL might be a new prognostic biomarker in various cancers: a meta-analysis and sequential verification with TCGA datasets. BMC Cancer. 2020;20(1):620.
15. Shin N, et al. Identification of frequently mutated genes with relevance to nonsense mediated mRNA decay in the high microsatellite instability cancers. Int J Cancer. 2011;128(12):2872–80.
16. Guseva NV, et al. Anchored multiplex PCR for targeted next-generation sequencing reveals recurrent and novel USP6 fusions and upregulation of USP6 expression in aneurysmal bone cyst. Genes Chromosom Cancer. 2017;56(4):266–77.
17. Staubitz JL, et al. Novel rearrangements involving the RET gene in papillary thyroid carcinoma. Cancer Genet. 2019;230:13–20.
