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Rho GTPase-activating protein 4 is upregulated in Kidney Renal Clear Cell Carcinoma and associated with poor prognosis and immune infiltration

Xuesong Xiao[a,1], Xiaofei Lv[b,1], Tianyu Lin[a,1], Jianqiao Li[a], Rui Wang[c], Shaoping Tian[c], Xinyu Liu[c], Shiming Liu[c], Huamao Jiang[d], Dan Yue[c,*] and Yong Wang[a,*]

[a]*Department of Urology, Tianjin Institute of Urology, The Second Hospital of Tianjin Medical University, Tianjin Medical University, Tianjin, China*

[b]*Department of Stomatology, The Second Hospital of Tianjin Medical University, Tianjin Medical University, Tianjin, China*

[c]*School of Medical Technology, Tianjin Medical University, Tianjin, China*

[d]*Department of Urology, The First Affiliated Hospital of Jinzhou Medical University, Jinzhou, Liaoning, China*

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Abstract.
BACKGROUND: Kidney Renal Clear Cell Carcinoma (KIRC) is a malignant tumor that seriously threatens human health. Rho GTPase-activating protein 4 (ARHGAP4) plays an important role in the occurrence and development of tumors.
OBJECTIVE: The purpose of this study was to explore the role of *ARHGAP4* in the progression of KIRC and its diagnostic and prognostic value.
METHODS: Multiple analytical methods and in vitro cell assays were used to explore the expression of *ARHGAP4* and its value in the progression, diagnosis and prognosis of KIRC. The biological function of *ARHGAP4* was studied by GO analysis and KEGG pathway analysis, and then the relationship between *ARHGAP4* and immune infiltration was analyzed.
RESULTS: The expression of *ARHGAP4* was significantly up-regulated in KIRC. We found that the high expression of *ARHGAP4* was related to the progression of KIRC and suggested a poor prognosis. Compared with normal tissues, *ARHGAP4* had a better diagnostic value in KIRC. The biological function of *ARHGAP4* was related to immunity, and its expression was also closely related to tumor immune infiltration and immune checkpoints.
CONCLUSIONS: Our study demonstrated that *ARHGAP4* may be a biomarker, which is related to the progression, diagnosis and prognosis of KIRC. Its biological functions are related to tumor immune infiltration.

Keywords: Rho GTPase-activating protein 4, KIRC, clinical prognosis, immune cell infiltration, biomarker

1. Introduction

Renal cell carcinoma (RCC) is one of the most common malignant tumors of the urinary system, with the highest incidence in North America, Australia and Europe and the lowest in India, Japan, Africa and China [1]. RCC is also a serious threat to human health.

[1]Equal contribution and first authorship: These authors contributed equally to this work and share first authorship.

*Corresponding authors: Yong Wang, Department of Urology, Tianjin Institute of Urology, The Second Hospital of Tianjin Medical University, Tianjin 300211, China. E-mail: wy@tmu.edu.cn; Dan Yue, School of Medical Technology, Tianjin Medical University, Tianjin 300203, China. E-mail: yuedan@tmu.edu.cn.

It is reported that the 5-year disease-specific survival rate of patients with RCC stage IV is less than 10% [2]. KIRC is the main subtype of RCC, with the highest incidence, accounting for about 80% of all RCCs. The symptoms of KIRC are hidden and appear late, about 25% of patients have metastases at the time of initial diagnosis [3,4]. Nowadays, more and more treatments are used for RCC, including surgery, chemotherapy, radiotherapy and targeted therapy, which show a certain effect on the survival of RCC. However, compared with other types of RCC, KIRC has a poorer therapeutic effect and prognosis [5,6]. In addition, the median survival time of metastatic KIRC was only about 10 months [7].

Immune response is closely related to the clinical outcome and prognosis of cancer. Different from most cancers, the increase of $CD8^+$ T cells and neutrophils in KIRC are predictors of poor prognosis [8]. Tumor infiltrating immune cells (TIIC) play an important role in tumor microenvironment. In many studies, it has been proved that KIRC is significantly related to immune infiltration [9]. In recent years, immunotherapy, as a new treatment for RCC, is an effective supplement to surgery, radiotherapy and chemotherapy, especially in the treatment of metastatic RCC. However, it is estimated that only about 20% of RCC patients could achieve better results from immunotherapy. It is necessary to look for some predictive biomarkers of RCC to evaluate the potential response and prognosis of patients receiving immunotherapy [10,11].

ARHGAP4 is a small GTP enzyme that can degrade activated GTP into inactivated GDP and negatively regulate Rho A protein. It not only plays an important role in inhibiting the movement of cells and axons, but also closely related to the occurrence and development of many kinds of tumors [12,13]. It has been reported that *ARHGAP4* can regulate the migration and invasion of pancreatic cancer cells, and its mechanism may be related to HDAC2/β-Catenin signal pathway. Furthermore, *ARHGAP4* may regulate mTOR and HIF-1α signaling pathways and affect the Warburg effect of pancreatic cancer [13,14,15,16]. Meanwhile, Sanger sequencing confirmed that *ARHGAP4* mutations might be associated with mental retardation [17]. It is worth noting that several studies have shown that continuous deletions of *AVPR2* and *ARHGAP4* genes may lead to congenital nephrogenic diabetes insipidus and may be associated with immunodeficiency [18,19,20,21]. Similarly, in patients with HPV infection, the *ARHGAP4* gene is associated with the immune microenvironment, showing an increase in immune cells, but a higher degree of immune dysfunction [22]. However, the expression and role of ARHGAP4 in KIRC have not been studied.

We analyzed the mRNA expression level and protein expression level of *ARHGAP4* in KIRC in TCGA, GEO and UALCAN databases. Then, the data of KIRC in TCGA were used to analyze the relationship between the expression of *ARHGAP4* and clinical features and prognosis. In addition, we further studied the biological functions and pathways of differentially expressed genes related to *ARHGAP4* by GO and KEGG analysis. Finally, we systematically evaluated the state of infiltrating immune cells and clarified the relationship between *ARHGAP4* and KIRC immunity. Our study suggested that *ARHGAP4* is associated with the progression and clinical outcome of KIRC. Furthermore, it could be a new prognostic marker related to immune infiltration in KIRC.

2. Materials and methods

2.1. Data collection

The RNA sequencing data of 539 cases of KIRC and 72 cases of para-carcinoma tissues were obtained from TCGA database, as well as the clinicopathological data of the patients (https://portal.gdc.cancer.gov/). We converted the RNA sequencing data in FPKM format into transcript per million readings (TPM) format for further analysis. And when we carried on the classified statistical analysis, we discarded the data without clinical information.

In addition, we also obtained the RNA sequencing data of three data sets (GSE66270, GSE66271, GSE71963) from the GEO database (https://www.ncbi.nlm.nih.gov/geo/).

2.2. Analysis of ARHGAP4 expression level in cancer and normal tissues

The mRNA expression of *ARHGAP4* was analyzed by RNA sequencing data of 33 kinds of tumors and corresponding para-carcinoma tissues in TCGA database. Similarly, RNA sequencing data of GSE66270, GSE66271 and GSE71963 datasets were extracted from GEO database to verify the mRNA expression of *ARHGAP4* in KIRC.

UALCAN database (http://ualcan.path.uab.edu/) is a free and open data analysis website, which has three main modules: TCGA analysis module, CPTAC analysis module and children's brain tumor (CBTTC) analysis module. We used CPTAC analysis module to analyze the difference of ARHGAP4 expression at the protein level.

2.3. Analysis of correlation between ARHGAP4 expression and clinicopathological characteristics in KIRC

According to the median of *ARHGAP4* mRNA expression, 539 patients with KIRC were divided into high expression group and low expression group. The differences of clinicopathological parameters such as TNM stage, pathological stage, histological grade, overall survival time (OS), disease specific survival time (DSS) and disease progression-free interval (PFI) between *ARHGAP4* high and low expression group were compared with R packages. Dunn's multiple hypothesis test, Kruskal-Wallis Test and Shapiro-Wilk normality test were used to analyze the differences of pathological parameters between the two groups. We used Logistic regression analysis to study the correlation between the expression level of *ARHGAP4* and the clinicopathological features of KIRC.

2.4. Analysis of ARHGAP4 expression and its diagnostic and prognostic value in KIRC

The survival data were statistically analyzed with "survival" package (Version 3.2-10), and the data visualization was carried out with "survminer" package (Version 0.4.9). Univariate and multivariate COX regression analysis were used to evaluate the relationship between *ARHGAP4* expression and prognosis of KIRC patients. The diagnostic ROC curve and time-dependent survival ROC curve were performed with "pROC" (Version 1.17.0.1) and "timeROC" (Version 0.4) packages to evaluate the value of *ARHGAP4* expression in the diagnosis and prognosis of KIRC.

2.5. Functional analysis of ARHGAP4-related differentially expressed genes in KIRC

The differentially expressed genes (DEGs) between *ARHGAP4* low expression group and high expression group were analyzed by "DESeq2" package (Version 1.26.0), and genes screening was carried out with the absolute value of logarithmic variation > 1.5 and $p < 0.05$ as threshold parameters. Subsequently, functional annotation and pathway enrichment analysis of these differential genes were carried out by GO analysis and KEGG pathway analysis.

2.6. Analysis of correlation between the expression of ARHGAP4 and immune cell infiltration in KIRC

TIMER (https://cistrome.shinyapps.io/timer/) is an open and comprehensive database that can be used to analyze immune infiltration of different types of cancer. We used this database to analyze the correlation between *ARHGAP4* and immune cell infiltration. The "GSVA" package (Version 1.34.0) and Spearman correlation analysis were used to evaluate the correlation between *ARHGAP4* expression level and 24 kinds of tumor immune cells in KIRC.

2.7. Analysis of correlation between the expression of ARHGAP4 and immune checkpoint genes in KIRC

The correlation between the level of *ARHGAP4* in KIRC and immune checkpoint genes was explored by Spearman correlation analysis.

2.8. Tissue specimens and Immunohistochemical (IHC) staining

In this study, 50 pairs of tumor tissues and matched para-carcinoma tissues were collected from the second Hospital of Tianjin Medical University. In addition, the clinicopathological data of these patients were obtained, including age, sex, tumor stage and Fuhrman grade. These patients had the following inclusion criteria: (1) all of these patients were diagnosed with KIRC; (2) none of the patients had other tumors; (3) none of the patients received radiotherapy or chemotherapy; (4) the patients underwent radical nephrectomy or partial nephrectomy. The study was approved by the informed consent of the patients and the Ethics Committee of the second Hospital of Tianjin Medical University.

We embedded these tissues in paraffin and sliced them, and then dewaxed and dehydrated the paraffin sections. Then, these sections were treated with citrate buffer for antigen repair, endogenous peroxidase activity was inactivated with 3% hydrogen peroxide, followed by blocking of tissue sections with 5% bovine serum albumin (BSA). Next, they were incubated overnight with antibody ARHGAP4 (1:60,16697-1-AP, Proteintech) at 4°C. The secondary antibody (ZSGB-BIO, China) was incubated at room temperature for 1 h, then they were colored with HRP DAB detection kit (ZSGB-BIO, China) and re-stained with hematoxylin, and finally sealed with neutral resin. The staining of different tissue sections was observed under an optical microscope. It was evaluated by two independent pathologists based on staining intensity and the proportion of positively stained tumor cells. The evaluation criteria are as follows: (1) staining intensity: 0, negative; 1, moderate (yellowish brown); 2, strong

(brown), (2) the proportion of tumor cells with positive staining: 0, 0–1%; 1, 1%–5%; 2, 6%–10%; 3, 11%–20%; 4, 21%–50% and 5, 51%–100%. The final score is the sum of intensity and percentage, which is divided into negative (0–3) and positive (4–7).

2.9. Cell lines and culture methods

All the cell lines used were purchased from the American Type Culture Collection Library (ATCC), including human embryonic kidney cells HEK-293T and human renal cancer cells ACHN and 786-O.

ACHN used MEM medium (Viva Cell), 786-O and HEK-293T cells used DMEM medium (Viva Cell), and all cell media were supplemented with 10% fetal bovine serum (FBS, Viva Cell) and 1% penicillin-streptomycin (PS, Bioind). The cells were cultured in an incubator containing 5% CO_2 at 37°C.

2.10. Construction of plasmid and stable cell lines

We designed the sgRNA of ARHGAP4 using CRISPOR website (http://crispor.tefor.net/) and ligated sgRNA (F-CACCGTTCATGCGGCGCCGCGCTG, R-AAACCAGCGCGGCGCCGCATGAAC) into lenti-CRISPRv2 plasmid. Then the empty plasmid (WT) and ARHGAP4-KO plasmid were transfected into 786-O and ACHN cell lines by lentivirus infection. Puromycin was used for screening, and Western blotting was used to verify the gene knockout efficiency.

2.11. Western blotting

We added SDS protein lysate and protease inhibitor to the cell, shake on the ice for 10 minutes, ultrasonicate at 30% power for 20 seconds, then heat at 97°C for 10 minutes, and finally use BCA kit (Thermo) to determine the protein concentration. The proteins were fractionated in 10% acrylamide gel and then transferred to the PVDF membrane. After 5% skimmed milk powder was sealed at room temperature for 2 hours, the corresponding primary antibodies were incubated overnight at 4°C, and the second antibodies labeled by HRP were incubated at room temperature for 1 hour, then the chemiluminescence instrument was used to detect the target band.

2.12. Colony formation assay

The cells in logarithmic growth phase were digested and resuscitated, and the cell density was adjusted to 1000 cells/ml. The cells were inoculated in 12-well plate with 100 ul in each well. After 7 days of cell culture, the cells were fixed with 4% paraformaldehyde for 20 minutes, stained with Giemsa's dye for 10 minutes, washed in distilled water until the background was clean, dried and photographed under the microscope.

2.13. Cell proliferation assay

The cells in logarithmic growth phase were inoculated in 96-well plate with 1000 cells per well. After the cells were adhered to the wall (4–8 hours), 100 ul CCK8 (Biosharp) mixed reagent was added to each well and incubated at 37°C for 2 hours, the OD value (450 nm) was measured by enzyme labeling instrument for 0 h, and then the OD value was measured every 24 hours to calculate the cell proliferation rate.

2.14. Scratch healing assay

Draw horizontal lines on the back of the six-well plate, inoculate the cells into the 6-well plate. After the cells were covered with the bottom of the wells, the cells were scratched perpendicular to the plate and washed with PBS for 3 times before adding the serum-free medium. The photo field was determined according to the intersection of the scratch and the horizontal line on the back, and the photos were taken at 0, 6 and 18 hours.

2.15. Cell migration and invasion assay

The cells in logarithmic growth phase were digested with trypsin and re-suspended with serum-free culture medium, and the cell density was adjusted to 2×10^5 cells/ml. For the invasion assay, Matrigel glue (BD) was prepared with serum-free medium dilution in advance, the ratio was 1:4, and then 40 ul base glue was applied in the upper chambers of Transwell (Biosharp). 200 ul cell suspension was added to the upper chambers, and the upper chambers were placed in the wells of a 24-well plate, 600 ul 20% FBS medium was added to each well, and then incubated in a 37°C incubator. The migration experiment incubated for 18 hours, and the invasion experiment incubated for 24 hours. Then fixed with 4% paraformaldehyde for 20 minutes, stained with Giemsa for 5 minutes, washed with distilled water, wiped the cells without membrane in the room with a cotton swab, observed under a positive microscope after air-drying, and randomly selected 5 visual fields for photo counting.

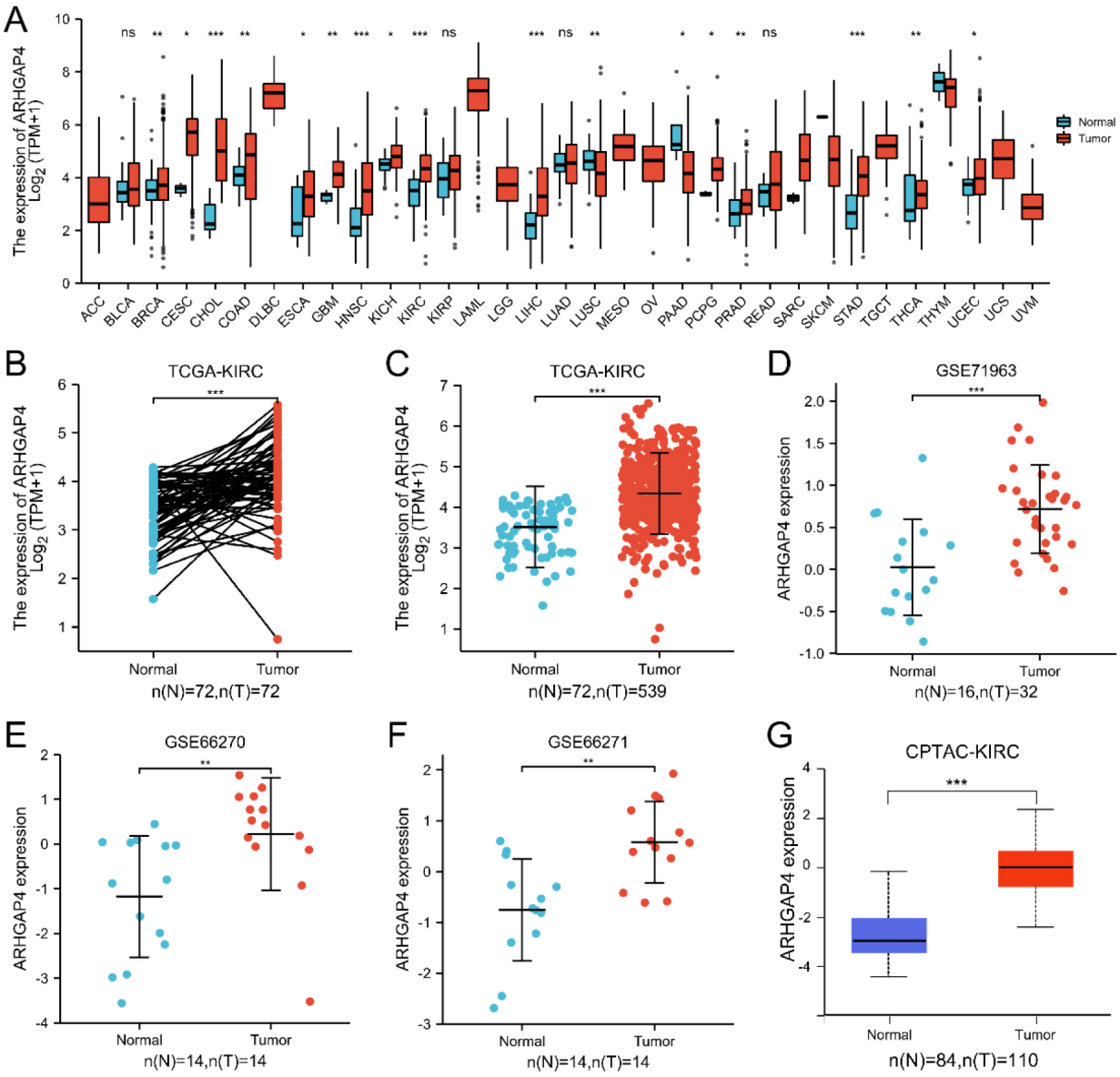

Fig. 1. *ARHGAP4* is highly expressed in KIRC. (A) The mRNA expression level of *ARHGAP4* in 33 kinds of cancer tissues and their corresponding normal tissues in TCGA database. (B) The mRNA expression level of *ARHGAP4* in paired KIRC carcinoma tissues and adjacent renal tissues. (C) The mRNA expression level of *ARHGAP4* in unmatched KIRC carcinoma tissues and normal renal tissues in the TCGA-KIRC dataset. (D–F) The mRNA expression level of *ARHGAP4* in KIRC carcinoma tissues and normal renal tissues in (D) GSE71963, (E) GSE66270, (F) GSE66271 dataset of GEO database. (G) The protein expression level of ARHGAP4 in KIRC tissues and para-carcinoma tissues from CPTAC samples. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

2.16. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 25 and GraphPad Prism software 8.0. The relationship between *ARHGAP4* protein expression and clinicopathological features was analyzed by Chi-square test. Significance was considered when $p < 0.05$.

3. Results

3.1. ARHGAP4 was upregulated in KIRC

We analyzed the expression of *ARHGAP4* using 33 cancer datasets in TCGA database. The results showed that compared with the para-carcinoma tissues, the mRNA expression of *ARHGAP4* was significantly up-

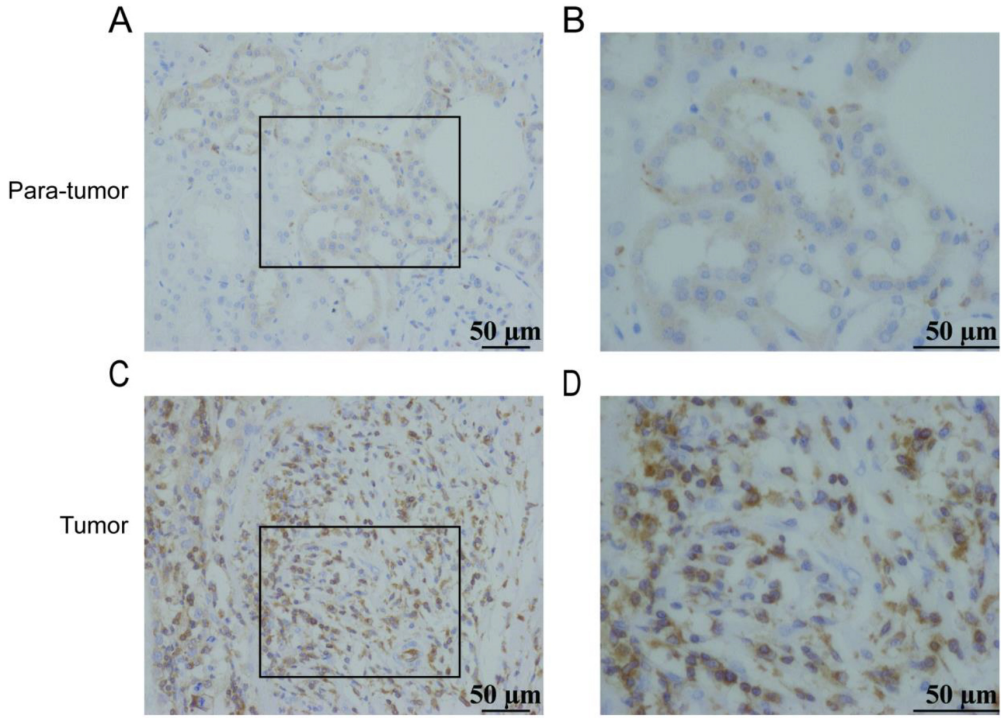

Fig. 2. Expression of ARHGAP4 in KIRC tissues. Low expression of ARHGAP4 in para-carcinoma tissues of KIRC (A, B). High expression of ARHGAP4 in KIRC tissues (C, D).

regulated in BRCA, CESC, CHOL, COAD, ESCA, GBM, HNSC, KIRH, KIRC, LIHC, PRAD, PCPG, STAD, THCA and UCEC tissues. Conversely, we found that there was significant decrease of *ARHGAP4* expression in LUSC and PAAD tissues (Fig. 1A).

Particularly, we downloaded the RNA-seq data of 539 cases of KIRC and 72 cases of para-carcinoma tissues from TCGA and analyzed the mRNA expression of *ARHGAP4*. The results of paired test showed that the mRNA expression of *ARHGAP4* in KIRC tissues was significantly higher than that in para-carcinoma tissues ($n = 72$, $p < 0.001$) (Fig. 1B). In addition, the mRNA expression of *ARHGAP4* in unmatched samples was consistent with this result ($p < 0.001$) (Fig. 1C). Next, we verified the upregulation of mRNA expression level of *ARHGAP4* in KIRC tissues through GSE71963 (Fig. 1D), GSE66270 (Fig. 1E) and GSE66271 (Fig. 1F) datasets. Furthermore, the analysis of KIRC protein dataset by UALCAN showed that the protein expression level of ARHGAP4 in KIRC tissues was up-regulated ($p < 0.001$) (Fig. 1G).

Considering the specificity of KIRC with high expression of ARHGAP4, this marker was verified in the KIRC tissue via IHC. The IHC staining image was randomly selected as shown in the figures (Figs 2A–D), which showed that the expression of ARHGAP4 in KIRC tissues was significantly higher than that in para-carcinoma tissues. Then we analyzed the relationship between ARHGAP4 expression and clinicopathological parameters of KIRC patients, including gender, age, T stage and Fuhrman grade. The results showed that the expression level of ARHGAP4 in KIRC patients was related to T stage (Table 1).

Table 1
Expression of *ARHGAP4* and clinical characteristics in clinical tissue samples

Characteristic	ARHGAP4			
	N	Negative	Positive	p
Gender				
Man	26	6	20	0.571
Woman	24	4	20	
Age				
< 60	19	3	16	0.56
$\geqslant 60$	31	7	24	
T stage				
T1–2	28	9	19	0.015
T3–4	22	1	21	
Fuhrman grade				
I, II	28	6	22	0.776
III, IV	22	4	18	
Tissue				
Tumor	50	10	40	$< 0.001^{***}$
Para-tumor	50	42	8	

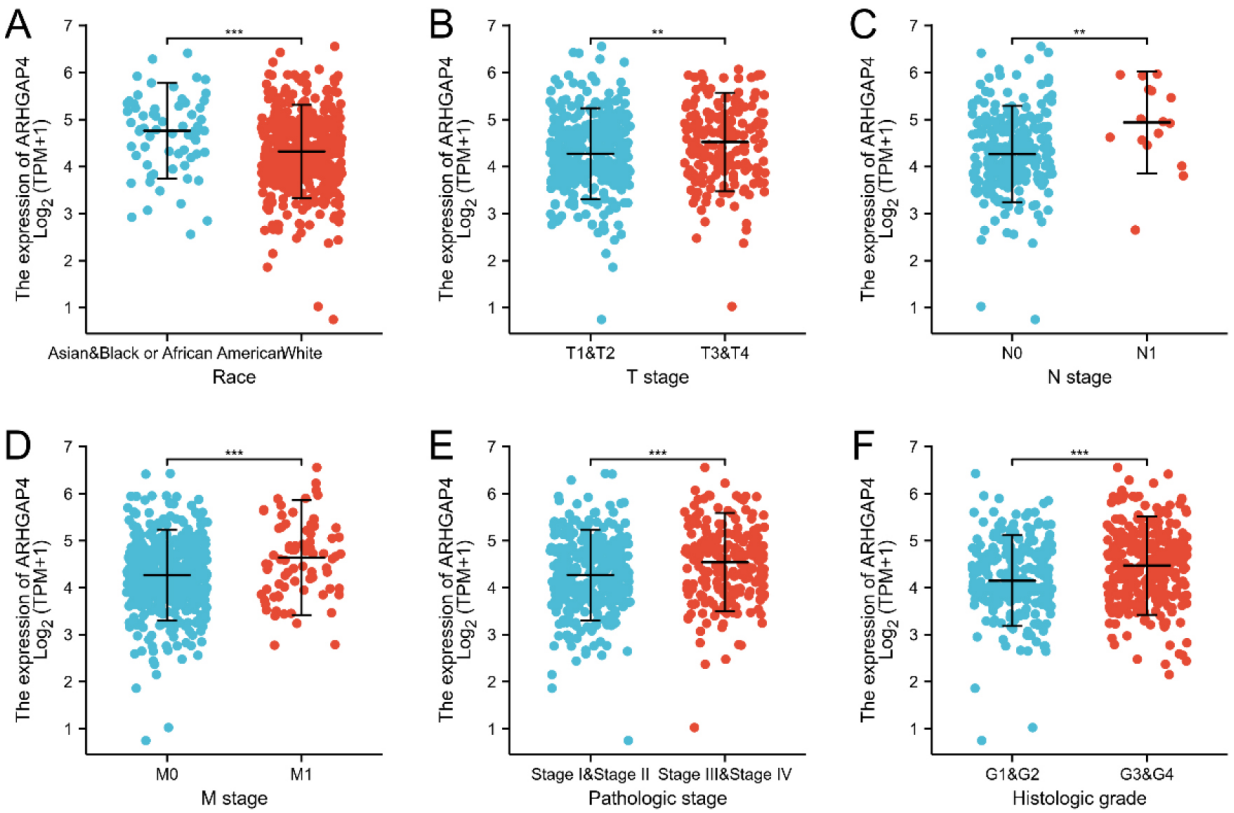

Fig. 3. The relation between *ARHGAP4* expression and clinicopathological characteristics of KIRC. The correlation between *ARHGAP4* and race (A), TNM stage (B-D), pathological stage (E) and (F) histological grade in the TCGA-KIRC dataset. $^{**}p < 0.01$; $^{***}p < 0.001$.

3.2. *Relationship between the expression of ARHGAP4 and clinicopathological characteristics in KIRC*

Then, we evaluated the relationship between *ARHGAP4* expression and various clinicopathological parameters in KIRC patients. 539 samples of KIRC were downloaded from TCGA, including *ARHGAP4* expression data obtained from patients with different clinical characteristics (Table S1). According to the median level of *ARHGAP4* expression, we divided 539 KIRC patients into high expression group and low expression group. Table 2 showed the relationship between the level of *ARHGAP4* expression and the clinicopathological characteristics of KIRC patients. We found that there was significant difference in race, TNM stage, pathological stage, histological grade, overall survival time (OS), disease-specific survival time (DSS) and disease progression-free interval (PFI) between KIRC patients with high and low expression of *ARHGAP4*. The expression level of *ARHGAP4* was significantly correlated with race (Fig. 3A), TNM stage (Figs 3B–D), pathological stage (Fig. 3E) and histological grade (Fig. 3F). In KIRC patients, the expression of *ARHGAP4* in Caucasians was significantly higher than that in other races. Moreover, patients with advanced cancer stages and high histological grades had higher levels of *ARHGAP4* expression. In addition, we used Logistics regression analysis to evaluate the relationship between *ARHGAP4* and different clinical characteristics. From the results, it can be known that the expression of *ARHGAP4* was positively correlated with TNM stage, pathological stage and histological grade (Table 3).

3.3. *Predictive effect of ARHGAP4 on the diagnosis and prognosis of KIRC*

We analyzed the effect of *ARHGAP4* expression on the survival rate of KIRC patients by K-M survival curve. From the results, it can be found that the overall survival time (OS) of *ARHGAP4* low expression group is significantly higher than that of high expression group. Similarly, the disease-specific survival time (DSS) and progression-free interval (PFI) of patients showed the same trend (Figs 4A–C). Next, we evaluated

Table 2
Clinical and pathological characteristics of patients with high and low *ARHGAP4* expression levels in the TCGA-KIRC dataset

Characteristic	Low expression of *ARHGAP4*	High expression of *ARHGAP4*	p
n	269	270	
Age, n (%)			0.83
$\leqslant 60$	136 (25.2%)	133 (24.7%)	
> 60	133 (24.7%)	137 (25.4%)	
Gender, n (%)			0.903
Female	94 (17.4%)	92 (17.1%)	
Male	175 (32.5%)	178 (33%)	
Race, n (%)			0.012*
Asian	2 (0.4%)	6 (1.1%)	
Black or African American	19 (3.6%)	38 (7.1%)	
White	242 (45.5%)	225 (42.3%)	
T stage, n (%)			0.005**
T1	157 (29.1%)	121 (22.4%)	
T2	33 (6.1%)	38 (7.1%)	
T3	77 (14.3%)	102 (18.9%)	
T4	2 (0.4%)	9 (1.7%)	
N stage, n (%)			0.018*
N0	127 (49.4%)	114 (44.4%)	
N1	3 (1.2%)	13 (5.1%)	
M stage, n (%)			0.012*
M0	234 (46.2%)	194 (38.3%)	
M1	30 (5.9%)	48 (9.5%)	
Pathologic stage, n (%)			0.009**
Stage I	155 (28.9%)	117 (21.8%)	
Stage II	27 (5%)	32 (6%)	
Stage III	54 (10.1%)	69 (12.9%)	
Stage IV	32 (6%)	50 (9.3%)	
Histologic grade, n (%)			0.004**
G1	7 (1.3%)	7 (1.3%)	
G2	137 (25.8%)	98 (18.5%)	
G3	91 (17.1%)	116 (21.8%)	
G4	29 (5.5%)	46 (8.7%)	
OS event, n (%)			0.011*
Alive	197 (36.5%)	169 (31.4%)	
Dead	72 (13.4%)	101 (18.7%)	
DSS event, n (%)			< 0.001***
Alive	228 (43.2%)	192 (36.4%)	
Dead	35 (6.6%)	73 (13.8%)	
PFI event, n (%)			0.026*
Alive	201 (37.3%)	177 (32.8%)	
Dead	68 (12.6%)	93 (17.3%)	

Table 3
Logistic regression analysis of the relationship between the expression level of *ARHGAP4* and clinical pathological characteristics in the TCGA-KIRC dataset

Characteristics	Total (N)	Odds ratio (OR)	p
Race (White vs. Asian & Black or African American)	532	0.444 (0.252–0.761)	0.004**
T stage (T3 & T4 vs. T1 & T2)	539	1.679 (1.176–2.405)	0.004**
N stage (N1 vs. N0)	257	4.827 (1.510–21.444)	0.016*
M stage (M1 vs. M0)	506	1.930 (1.184–3.191)	0.009**
Pathologic stage (Stage III & Stage IV vs. Stage I & Stage II)	536	1.690 (1.190–2.408)	0.003**
Histologic grade (G3 & G4 vs. G1 & G2)	531	1.851 (1.313–2.619)	< 0.001***

the diagnostic value of *ARHGAP4* expression level in KIRC by ROC curve. As shown in the figure (Fig. 4D), the AUC value is 0.829, indicating that the expression level of *ARHGAP4* has a high accuracy in the differential diagnosis of tumor and normal tissue. In addition, compared with tumors in early stage, *ARHGAP4* has higher differential ability (Figs 4E–H) for tumors in late stage. Then, univariate and multivariate COX regression

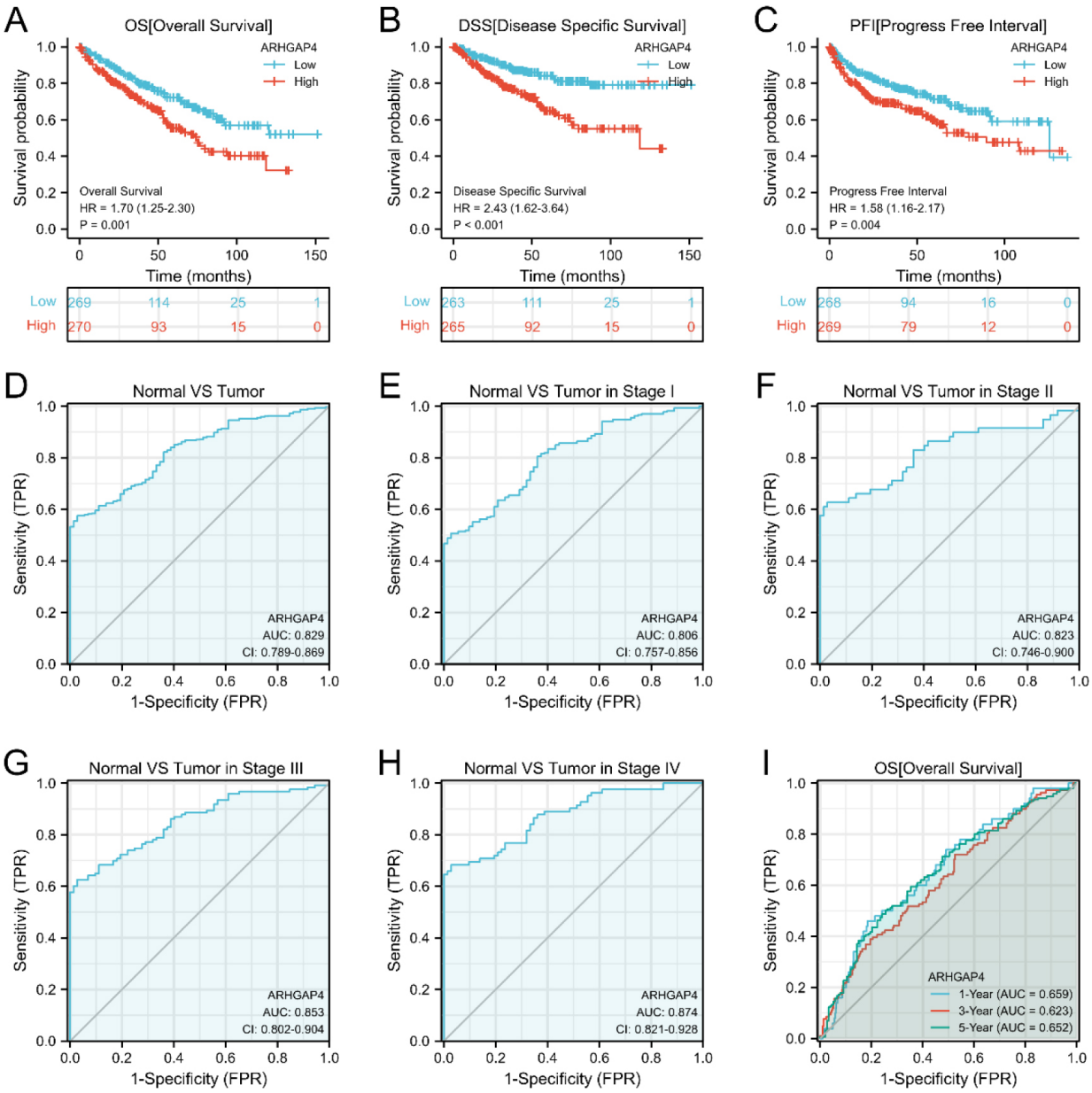

Fig. 4. The value of *ARHGAP4* in the diagnosis and prognosis of patients with KIRC. (A–C) The effects of *ARHGAP4* expression on (A) overall survival time (OS), (B) disease-specific survival time (DSS) and (C) progression-free interval (PFI) of patients in the TCGA-KIRC dataset were analyzed by survival curve. (D) The diagnostic ROC curve of differentiating KIRC tissue from normal tissue by *ARHGAP4* in the TCGA-KIRC dataset. (E–H) The ROC curves of *ARHGAP4* in the TCGA-KIRC dataset differentiate normal tissues from KIRC tissues of different stages. (I) The time-dependent ROC curves of 1-, 3-and 5-year overall survival (OS) predicted by *ARHGAP4* in the TCGA-KIRC dataset.

analysis was used to evaluate the effects of *ARHGAP4* expression and common clinicopathological features on OS, DSS and PFI in KIRC patients (Table 4 and Tables S2, S3). It can be seen from these results that the high expression of *ARHGAP4*, like age, TNM stage, pathological stage and histological grade, is also a factor affecting the prognosis of KIRC patients. Furthermore, the expression of *ARHGAP4* could be an independent risk factor affecting OS in KIRC patients. The results of time-dependent ROC curve (Fig. 4I) analysis showed that according to the expression level of *ARHGAP4*, the AUC values of OS in patients with KIRC at 1, 3 and 5 years were all above 0.6. In addition, the results of the ROC curve (Fig. S1) of DSS and PFI suggested that the

Table 4
Univariate and multivariate COX regression analysis showed the impact of *ARHGAP4* expression levels and different clinical pathological characteristics on overall survival (OS) in the TCGA-KIRC dataset

Characteristics	Total (N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	p	Hazard ratio (95% CI)	p
Age	539				
$\leqslant 60$	269	Reference			
> 60	270	1.765 (1.298–2.398)	$< 0.001^{***}$	1.659 (1.083–2.543)	0.020^{*}
Race	532				
Asian & Black or African American	65	Reference			
White	467	1.222 (0.678–2.201)	0.505		
T stage	539				
T1 & T2	349	Reference			
T3 & T4	190	3.228 (2.382–4.374)	$< 0.001^{***}$	1.504 (0.664–3.410)	0.328
N stage	257				
N0	241	Reference			
N1	16	3.453 (1.832–6.508)	$< 0.001^{***}$	1.540 (0.764–3.102)	0.227
M stage	506				
M0	428	Reference			
M1	78	4.389 (3.212–5.999)	$< 0.001^{***}$	2.764 (1.635–4.672)	$< 0.001^{***}$
Pathologic stage	536				
Stage I & Stage II	331	Reference			
Stage III & Stage IV	205	3.946 (2.872–5.423)	$< 0.001^{***}$	1.240 (0.490–3.139)	0.65
Histologic grade	531				
G1 & G2	249	Reference			
G3 & G4	282	2.702 (1.918–3.807)	$< 0.001^{***}$	1.563 (0.941–2.598)	0.085
ARHGAP4	539				
Low	269	Reference			
High	270	1.681 (1.240–2.279)	$< 0.001^{***}$	1.627 (1.034–2.562)	0.035^{*}

expression of *ARHGAP4* could also predict DSS and PFI in KIRC patients.

3.4. Verification of the effect of ARHGAP4 on the progression of KIRC in vitro

In order to further explore and verify the role of *ARHGAP4* in KIRC, we used CRISPR technique to down-regulate the expression of *ARHGAP4* in 786-O and ACHN cell lines, and verified the down-regulation efficiency of *ARHGAP4* in cells by Western blotting analysis (Fig. 5A). Subsequently, the cell proliferation assays showed that down-regulation of *ARHGAP4* significantly inhibited the proliferation and growth of these cells (Figs 5B, C). Similarly, the results of colony formation assays showed that both the number of colonies and the colony size in the *ARHGAP4*-KO groups were lower than those in the control groups suggesting that the knockout of *ARHGAP4* significantly reduced the colony formation ability of the two cell lines (Figs 5D, E). Then we carried out the scratch healing assays to study the effect of *ARHGAP4* on the migration ability of renal carcinoma cells (Figs 6A, B). The migration ability of *ARHGAP4* knockout cells was significantly weaker than that of control cells, indicating that the down-regulation of *ARHGAP4* could inhibit the migration ability of renal carcinoma cells. In order to further verify the effect of *ARHGAP4* on the migration and invasion of renal carcinoma cells, we carried out Transwell migration and invasion assays. The number of cells passing through the chambers in the *ARHGAP4* knockout groups was significantly lower than that in the control groups, which were consistent with the results of scratch assays (Figs 6C, D). Similarly, the down-regulation of *ARHGAP4* also inhibited the ability of cells to pass through Matrigel (Figs 6E, F).

3.5. Biological function Analysis of ARHGAP4-related differentially expressed genes in KIRC

Using absolute logarithmic variation > 1.5 and $p < 0.05$ as threshold parameters, we found that there were 599 differentially expressed genes (DEGs) between *ARHGAP4* high and low expression groups (Table S4), including 232 up-regulated genes and 367 down-regulated genes (Fig. 7A). Then, we analyzed the functional annotation and pathway enrichment of differentially expressed genes related to *ARHGAP4* in KIRC patients using the "cluster Profiler" R package. The results of KEGG pathway enrichment analysis (Fig. 7B) showed that the differentially expressed genes related to *ARHGAP4* were mainly concentrated in collecting tubular acid secretion, synaptic vesicle cycle, Vibrio cholerae infection, rheumatoid arthritis and α-linolenic

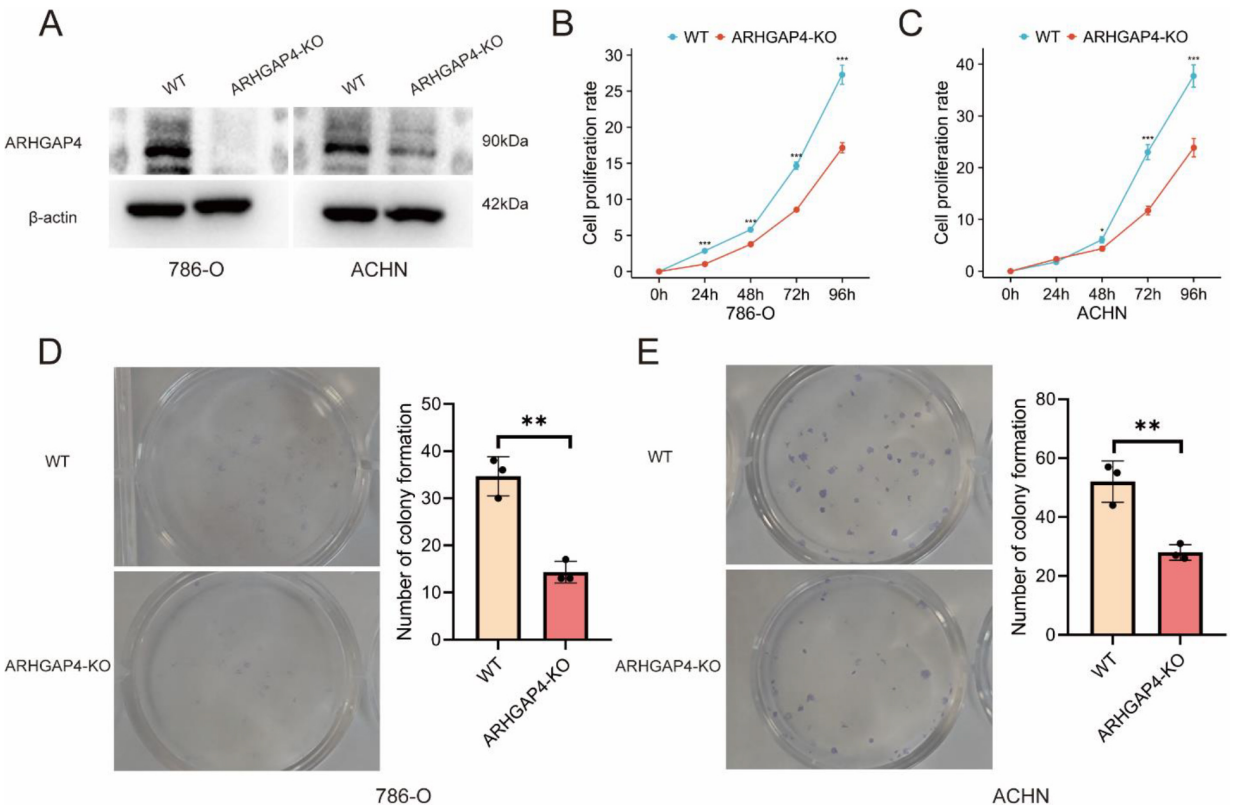

Fig. 5. Verification of the effect of *ARHGAP4* on growth and proliferation *in vitro*. (A) Expression of ARHGAP4 protein in 786-O and ACHN cells in control group and *ARHGAP4*-KO group. (B, C) Cell proliferation rate of control group and *ARHGAP4*-KO group in 786-O and ACHN cells. (D, E) Number of colonies in control group and *ARHGAP4*-KO group in 786-O and ACHN cells. $^{**}p < 0.01$; $^{***}p < 0.001$.

acid metabolism. In addition, the results of GO enrichment analysis mainly showed three aspects, including biological process (BP) (Fig. 7C), cellular component (CC) (Fig. 7D) and molecular function (MF) (Fig. 7E). BPs were mainly enriched in B cell receptor signaling pathway, complement activation, classical pathway and humoral immune response. The most enriched CCs were vacuolar proton-transporting V-type ATPase complexes, blood particles, immunoglobulin complexes, and the extemal side of the plasma membrane. The main MFs were antigen binding, immunoglobulin receptor binding, anion transmembrane transporter activity, active ion transmembrane transporter activity and serine-type endopeptidase activity.

3.6. *Relationship between ARHGAP4 expression and immune infiltration in KIRC*

Tumor immunity plays an important role in the overall survival rate and prognosis of patients. Previous biological functional analysis showed that *ARHGAP4* was related to tumor immune cells. Therefore, we evaluated the relationship between the expression level of *ARHGAP4* in KIRC and immune cell infiltration using the Timer database. As shown in the following figures, *ARHGAP4* was positively correlated with $CD4^+$ T cells, DC cells, B cells, Macrophages, $CD8^+$ T cells and neutrophils, especially with $CD4^+$ T cells (Figs 8A, B). Next, we evaluated the difference in tumor immunity between patients with high and low levels of *ARHGAP4* expression. We used ssGSEA algorithm to evaluate the difference of expression of 24 kinds of immune cells between the two groups (Fig. 8C). We also evaluated the correlation between *ARHGAP4* and 24 kinds of immune cells in KIRC by Spearman correlation analysis. The results showed that the expression of *ARHGAP4* was positively correlated with some kinds of immune cells in KIRC, including cytotoxic cells ($r = 0.357$), T cells ($r = 0.348$), T helper cells ($r = 0.341$), Treg ($r = 0.339$), NK CD56 bright cells ($r = 0.328$), Tem ($r = 0.307$), $CD8^+$T cells ($r = 0.283$), TFH ($r = 0.276$), aDC ($r = 0.266$), Th1 ($r = 0.260$) and B cells ($r = 0.247$), but negatively correlated with Tgd ($r = -0.157$) (Fig. 8D and Table S4).

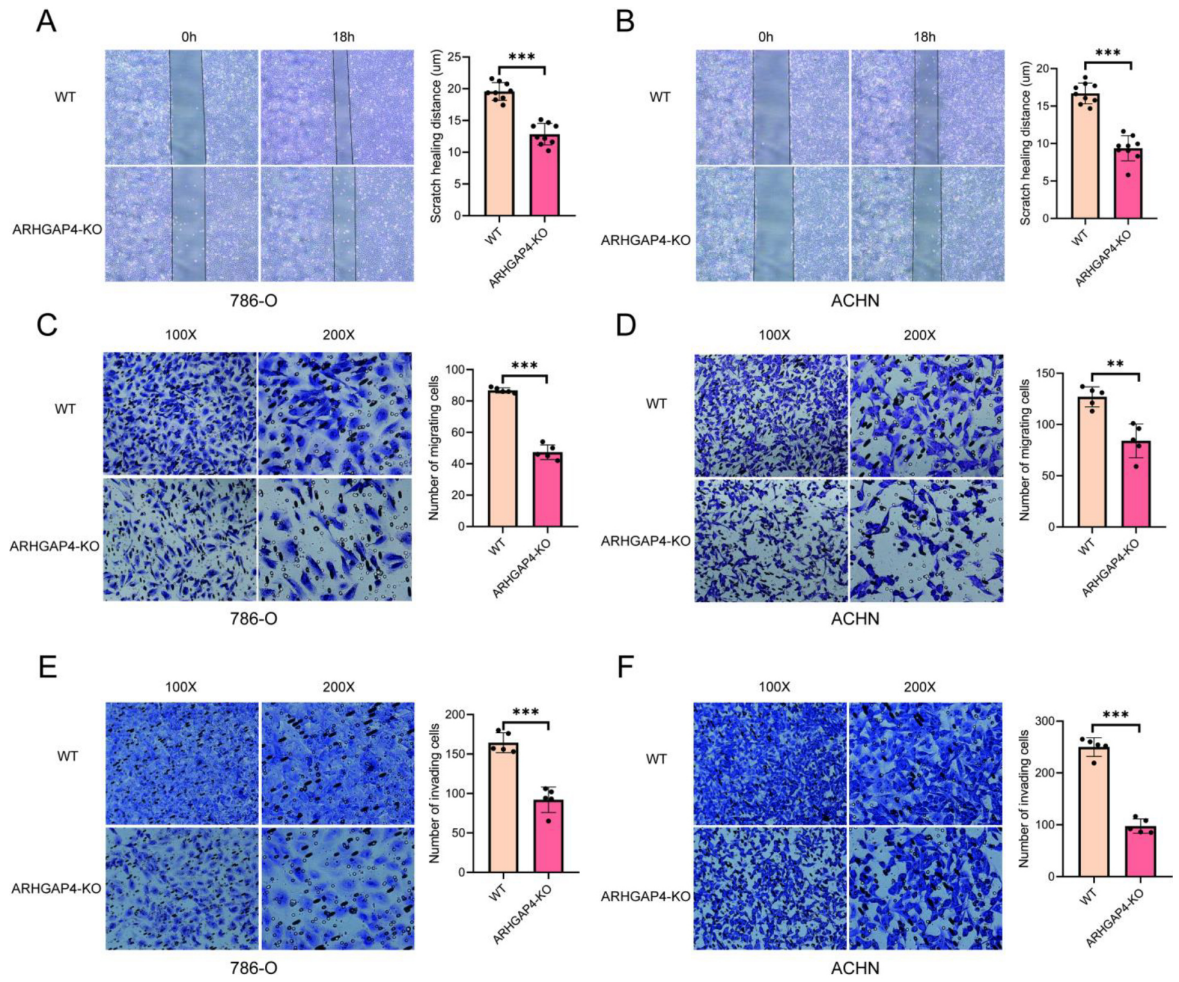

Fig. 6. Verification of the effect of *ARHGAP4* on migration and invasion in vitro. (A, B) Scratch healing distance between control group and *ARHGAP4*-KO group in 786-O and ACHN cells. (C, D) Number of cell migration in control group and *ARHGAP4*-KO group in 786-O and ACHN cells. (E, F) Number of cell invasion in control group and *ARHGAP4*-KO group in 786-O and ACHN cells. $^{**}p < 0.01$; $^{***}p < 0.001$.

3.7. The relationship between ARHGAP4 and immune checkpoint genes expression in KIRC

Tumor immunotherapy is a new treatment with broad prospects, which is often used as an adjuvant therapy, combined with conventional treatments such as surgery, chemotherapy and radiotherapy, playing an increasingly important role in tumor treatment. However, the tumor immune escape mechanism will significantly reduce the effectiveness of immunotherapy, and immune checkpoint proteins are closely related to tumor immune escape [23]. Programmed cell death 1 (*PDCD1*), programmed cell death ligand-1 (*PDCD-L1*, *CD274*) and cytotoxic T lymphocyte antigen 4 (*CTLA-4*) are the most widely studied and recognized immune checkpoint proteins [24]. In addition, Lymphocyte activation gene-3 (*LAG3*), B and T cell lymphocyte attenuator (*BTLA*), OX40 (*TNFSF4*), B7 homolog 3 (*B7-H3*, *CD276*), T cell immunoglobulin and ITIM domain (TIGIT) and tumor necrosis factor receptor superfamily member 9 (*TNFRSF9*) are promising genes of new immune checkpoint pathway. Our results proved that in the KIRC samples of TCGA, the expression level of *ARHGAP4* was positively correlated with the expression level of these immune checkpoint genes (Figs 9A–I), especially with the expression of *PDCD1* ($r = 0.516$) and *CTLA-4* ($r = 0.542$) genes.

4. Discussion

Renal cell carcinoma (RCC) is a malignant tumor that seriously affects human health, and its morbid-

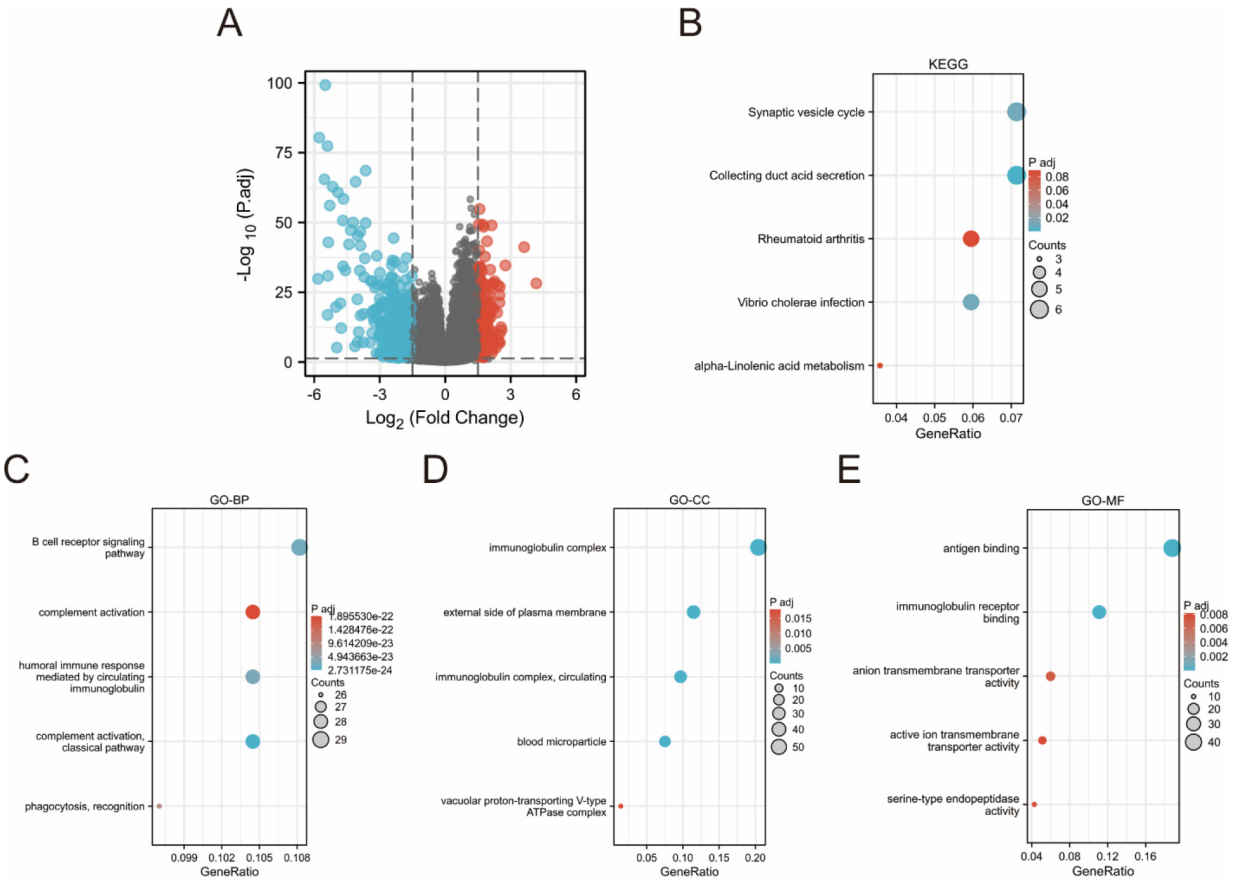

Fig. 7. Biological function analysis of *ARHGAP4*-related differentially expressed genes (DEGs) in KIRC. (A) Volcanic map of *ARHGAP4*-related DEGs in the TCGA-KIRC dataset. (B) KEGG pathway analysis showed the functions and pathways of *ARHGAP4*-related DEGs in the TCGA-KIRC dataset. (C-E) GO analysis showed the (C) biological processes (BP), (D) cellular components (CC) and (E) molecular functions (MF) of *ARHGAP4*-related DEGs in the TCGA-KIRC dataset.

ity and mortality remain high [25]. KIRC is the main subtype of RCC. Surgical treatment is mainly used in patients with early KIRC, but the effect of surgical treatment for advanced and metastatic tumors is limited. With the development of medical technology, although great progress has been made in drug treatment, chemotherapy and radiotherapy, the clinical results of patients with advanced KIRC are still not satisfactory due to the influence of tumor drug resistance and radiation resistance [26]. As a new treatment, immunotherapy plays an increasingly important role in tumor treatment, which is often used in combination with radiotherapy, chemotherapy and other treatment methods to improve the therapeutic effect of patients. In addition, immune checkpoint inhibitors (ICIs), such as CTLA-4 inhibitors, PDCD1 inhibitors and PDCD-L1 inhibitors, have been used in the clinical treatment of various malignant tumors, such as lung cancer, breast cancer, gastric cancer, renal cell carcinoma and Hodgkin's lymphoma [23,27,28,29]. However, the specific biomarkers for diagnosis, individualized treatment and predicting prognosis of KIRC are still needed to be evaluated.

Some investigations have shown that *ARHGAP4* is highly expressed in colorectal cancer, gastric cancer and cervical cancer, and is related to tumor progression and prognosis [30,31,32]. Similarly, the expression of *ARHGAP4* has a certain effect on the effect of chemotherapy in ovarian tumors [33]. However, the role of *ARHGAP4* in KIRC is not clear. In this research, we proved the different *ARHGAP4* expression in human cancer tissues. It was significantly high expression in 14 cancers, including KIRC, and low expression in 2 cancers, indicating that *ARHGAP4* has cancer specificity. According to the RNA sequencing data of KIRC patient samples from TCGA and GEO databases, we observed that *ARHGAP4* was upregulated in cancer tissues compared with the corresponding normal tissues. In addition, immunohistochemical staining showed that

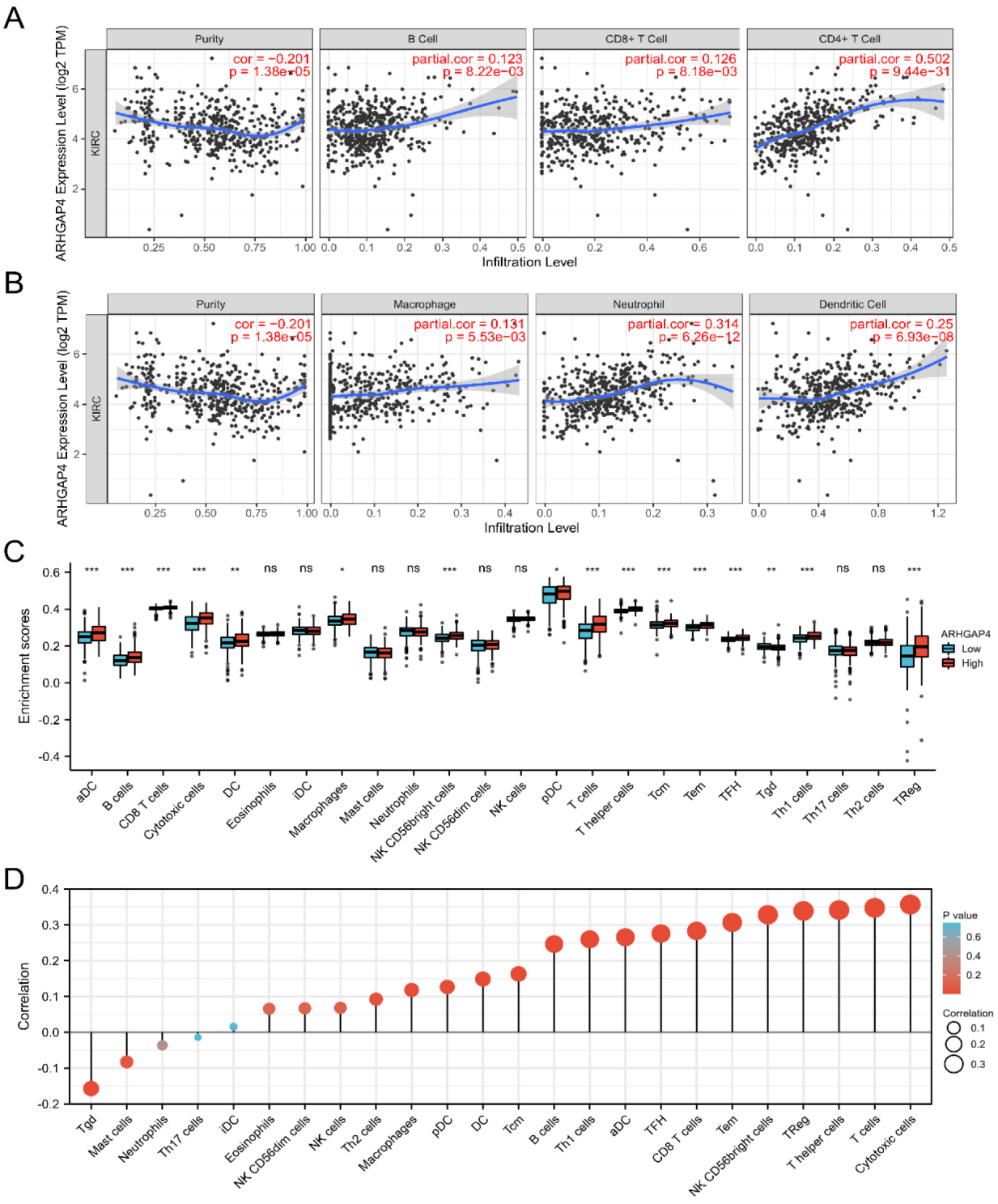

Fig. 8. The expression level of *ARHGAP4* in KIRC was related to immune infiltration. (A, B) The correlation between the expression level of *ARHGAP4* in KIRC tissues and the level of immune cell infiltration in TIMER database. (C) The infiltration level of 24 kinds of immune cells in the TCGA-KIRC dataset with high expression group and low expression group of *ARHGAP4*. (D) The correlation between the expression level of *ARHGAP4* and the infiltration level of 24 kinds of immune cells in the TCGA-KIRC dataset. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

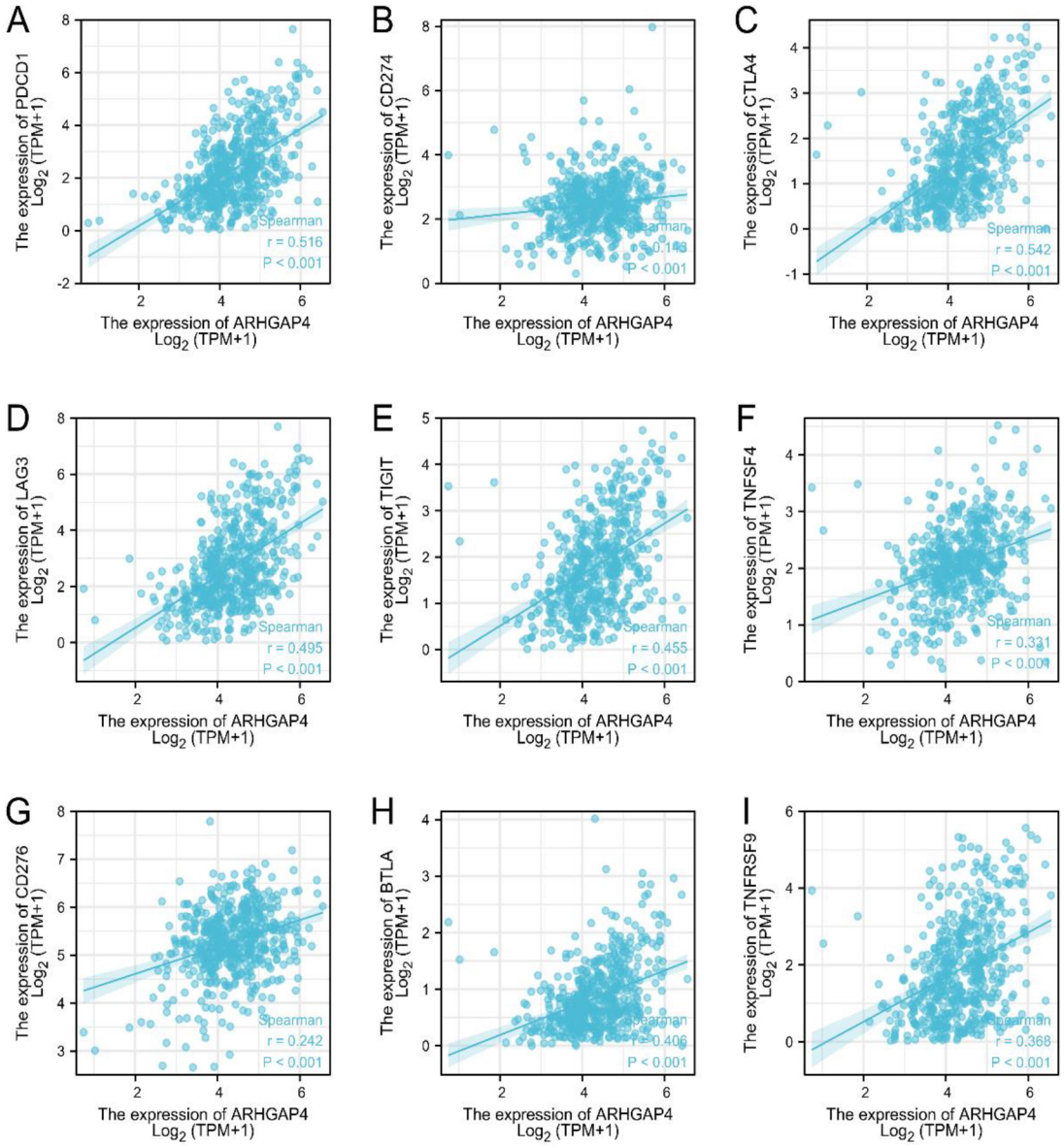

Fig. 9. *ARHGAP4* is associated with immune checkpoint genes in KIRC. (A-I) Correlation analysis between the expression levels of *ARHGAP4* and *PDCD1*, *PDCD-L1* (*CD274*), *CTLA-4LAG3*, *TIGIT*, *OX40* (TNFSF4), *B7-H3* (*CD276*), *BTLA*, and *TNFRSF9* in the TCGA-KIRC dataset.

ARHGAP4 was significantly upregulated in KIRC tissues and correlated with tumor malignancy.

From our results, we can find that the expression level of *ARHGAP4* was significantly correlated with race, TNM stage, clinical stage, pathological stage, OS, DSS and PFI in KIRC patients. In addition, univariate and multivariate COX regression analysis results showed that *ARHGAP4* was an independent prognostic factor in patients with KIRC, and the higher *ARHGAP4* expression meant the worse prognosis. The larger the AUC value of the ROC curve, the stronger the diagnostic and predictive ability. The AUC value of *ARHGAP4* differential diagnosis KIRC was 0.829, which indicated that it had great diagnostic value. Meanwhile, the 1-, 3-and 5-year AUC values of predicted overall survival were all higher than 0.6. As we know, the proliferation, migration and invasion of tumor cells are closely related to the progression of tumor, and also affect the survival and prognosis of patients. Next, we carried out a series of cell assays in vitro to further verify the role of *ARHGAP4* in the progression of KIRC. The results of colony formation assay and cell proliferation

assay suggested that knockout of *ARHGAP4* can significantly inhibit the growth and proliferation of renal carcinoma cells. Scratch healing assay, Transwell migration and invasion assays showed that down-regulation of *ARHGAP4* expression decreased the migration and invasion ability of renal carcinoma cells. These data suggested that *ARHGAP4* may be a diagnostic and prognostic biomarker for KIRC.

Next, we functionally enriched *ARHGAP4*-related DEGs by GO analysis and KEGG pathway enrichment analysis to further study the biological functions of *ARHGAP4*. The results suggested that these differentially expressed genes are enriched in immune and transport-related functions. High expression of *ARHGAP4* in KIRC patients may affect tumor progression and prognosis by regulating tumor immune response.

It is precisely because tumor immunity is closely related to tumor progression, immunotherapy strategy is also considered as a very promising method of tumor therapy. In recent years, immunotherapy for RCC has developed rapidly, from non-specific immunotherapy to targeted immunotherapy, and targeted immunotherapy is also considered to be a more accurate and effective new treatment strategy, which will greatly improve the therapeutic effect of patients [29]. Therefore, exploring the relationship between *ARHGAP4* and tumor immunity in KIRC is of great significance for the prognosis and treatment of KIRC.

Subsequently, our research showed that *ARHGAP4* is positively correlated with $CD4^+$T cells, DC cells, B cells, Macrophages, $CD8^+$T cells and neutrophils. In addition, the expression of *ARHGAP4* was positively correlated with the immune infiltration level of some immune cells in KIRC, including cytotoxic cells, T cells, T helper cells, Tregs, NK CD56 bright cells, Tems, $CD8^+$T cells, TFHs, aDCs, Th1s, B cells and so on. The main function of $CD8^+$T cells is to provide protective immunity and play a key role in the immunotherapy of infections and tumors [34]. In addition, $CD4^+$T cells contribute to the formation of protective memory $CD8^+$T cells after infection or immunization [35]. Mass spectrometry and digital cell analysis have found that the most common types of immune cells in KIRC tumors are $CD4^+$T cells, $CD8^+$T cells, and macrophages. Interestingly, the percentage of $CD8^+$T cells is highly positively correlated with the expression level of *PDCD1* [36]. In addition, studies have shown that high levels of $CD4^+$T cells and $CD8^+$T cell abundance in KIRC patients may indicate shorter survival and poorer surgical outcomes, and their underlying mechanisms may be related to tumor immune escape [37,38]. DCs are special antigen presenting cells, which are powerful and play a key role in initiating, regulating and maintaining immune responses [39]. B cell-mediated humoral immunity can effectively supplement T cell-mediated anti-tumor immunity [40]. In general, macrophages not only have the function of maintaining balance in the body, but also play an important role in immunity. However, tumor-associated macrophages (TAM) in tumor microenvironment (TME) can promote tumor cell proliferation and angiogenesis, participate in tumor immunosuppression, and thus promote tumor progression [41]. Neutrophils have powerful antibacterial functions, however, improper activation or imbalance of neutrophils can also damage the host and lead to autoimmune and inflammatory diseases [42]. Therefore, our data show that the overexpression of *ARHGAP4* may be related to immune cell infiltration in KIRC, which can promote the progression of KIRC.

Renal cell carcinoma has high immune infiltration and low mutation load, and it is highly sensitive to immunotherapy. Therefore, it is of great significance to explore the therapeutic targets and biomarkers that affect the efficacy of renal cell carcinoma. From the initial therapeutic drugs that regulate the immune system, such as interleukin-2 (IL-2) and interferon (IFN), to immune checkpoint inhibitors, such as PDCD1 inhibitors, PDCD-L1 inhibitors and CTLA-4 inhibitors, these drugs have achieved good clinical efficacy [43, 44]. The immune checkpoint pathway is related to tumor immune escape. after being activated, this pathway can make T cells fail and can't kill tumor cells normally, thus suppressing tumor immunity. Our research revealed that there was a positive correlation between *ARHGAP4* and tumor immune checkpoints such as *PDCD1*, *PDCD-L1* (*CD274*), *CTLA-4*, *LAG3*, *TIGIT*, *OX40* (*TNFSF4*), *B7-H3* (*CD276*), *BTLA* and *TNFRSF9*, especially with the expression of *PDCD1* and *CTLA-4* genes. Immunotherapy, based on *PDCD1* and *CTLA-4* pathways, has achieved excellent results in the treatment of a variety of cancers, including KIRC [45, 46,47]. In addition, studies have shown that the survival time of patients can be significantly prolonged when immune checkpoint inhibitors (Navulizumab) and anti-vascular endothelial growth factor drugs (Carbotinib) are used together [48]. Beyond that, some good immunotherapeutic potential drugs including *LAG3*, *TIGIT*, *OX40* (*TNFSF4*), *B7-H3* (*CD276*), *BTLA* and *TNFRSF9* are under research and development [25]. The correlation between *ARHGAP4* and these immune checkpoints genes suggested that it may affect the im-

mune escape pathway of tumors and may be a therapeutic target to improve the efficacy of KIRC tumor immunotherapy.

However, there are some limitations in our research. First of all, it is necessary to verify the clinical value of *ARHGAP4* in KIRC through a larger cohort and more detailed clinical data, because our database-based analysis can't determine whether other diseases affect the expression of *ARHGAP4*. Secondly, we need to further verify the role of *ARHGAP4* in KIRC in vivo. Although our results suggest that *ARHGAP4* may play a role in the immune infiltration of KIRC, the pathway and mechanism of its effect are not clear, and we will verify it through more experiments in the future.

5. Conclusion

In summary, our investigation explored the progress, diagnostic and prognostic value of *ARHGAP4* in KIRC patients for the first time. *ARHGAP4* was upregulated in KIRC patients and indicates a poor prognosis. *ARHGAP4* was mainly related to immune and transport-related functions in KIRC. *ARHGAP4* was significantly correlated with some kinds of immune cells, suggesting that it may affect the immune infiltration of KIRC. In addition, *ARHGAP4* was positively correlated with a variety of immune checkpoint genes, which may affect the prognosis and treatment of KIRC. Therefore, *ARHGAP4* may be a biomarker of KIRC, which is beneficial to the diagnosis of tumors. At the same time, it was related to the immune cell infiltration of tumors and may be a therapeutic target to improve the efficacy of immunotherapy.

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Author contributions

Conception: Xuesong Xiao.

Interpretation or analysis of data: Xuesong Xiao, Xiaofei Lv, Tianyu Lin.

Preparation of the manuscript: Xuesong Xiao.

Revision for important intellectual content: Jianqiao Li, Rui Wang, Shaoping Tian, Xinyu Liu, Shiming Liu, Huamao Jiang.

Supervision: Yong Wang, Dan Yue.

Supplementary data

The supplementary files are available to download from http://dx.doi.org/10.3233/CBM-230388.

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