Research Paper

FCGR2A as one novel potential target for poor survival prognosis of clear cell renal cell carcinoma

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ABSTRACT

Background: Clear cell renal cell carcinoma (ccRCC) is the most common type of renal cell carcinoma (RCC). Immunoglobulin Fc receptor FCGR2A has been implicated in various cancers, however, its role on ccRCC is not well studied.
Methods: A total of 151 patients with ccRCC were recruited for the study. Cox proportional hazards regression analysis was performed to calculate the hazard radios of FCGR2A expression and tumor characteristics. Pathological changes associated with ccRCC in tumor tissue sections were analyzed by hematoxylin-eosin staining. Immunohistochemical and immunofluorescence staining were used to detect the protein expression of FCGR2A in the tissue sections. Correlation between the expression of FCGR2A and the overall survival (OS) of ccRCC patients was analyzed by BP neural network and support vector machine (SVM).
Results: The expression of FCGR2A was significantly correlated with the pathological grade of tumor, family history of ccRCC and Enneking stage of ccRCC. Patients with high FCGR2A expression in the tumor tissue, had poorer OS than the patients with low and moderate FCGR2A expression. The ROC curve showed that FCGR2A can be used as a sensitive and specific biomarker for the diagnosis of ccRCC. Western blotting revealed that the FCGR2A was expressed at higher levels in the ccRCC tissues. BP neural network and SVM fitting showed that the R^2 between FCGR2A and survival time of ccRCC patients was 0.8429 and 0.7669, respectively.
Conclusions: FCGR2A is highly expressed in ccRCC, higher expression of FCGR2A is associated with poorer OS of ccRCC.

INTRODUCTION

Clear cell renal cell carcinoma (ccRCC) is the most aggressive type of renal cell carcinoma (RCC) with a high degree of malignancy and mortality [1, 2]. The patients generally have a poor prognosis [3, 4], due to the absence of specific clinical symptoms in early stages of the disease, which causes delay in the diagnosis and treatment [5, 6]. The etiology of ccRCC is not well-understood [7], and the exploration of the molecular targets for diagnosis and treatment of ccRCC is important to fight this most prevalent cancer of the uro-genital system.

Proteomics is an integral part of systems biology that uses high-resolution protein separation and protein identification technologies to study life phenomena in an integrated, dynamic and quantitative manner [8, 9]. Proteomics and phosphoproteomics have been successfully used to decipher the hub targets of various diseases [10, 11]. PRIDE (Proteomics Identification archive database) has played an important role in this endeavor [12]. Protein phosphorylation plays crucial roles in many biological processes such as cell cycle, signal transduction, differentiation and development, metabolism, apoptosis and carcinogenesis. Hence, phosphoproteomics has always been on the forefront of

biological research [13]. In our previous studies, we have used the sequencing techniques (including transcriptomics, proteomics and phosphoproteomics) to identify the core molecular players of ccRCC, and the results indicate that FCGR2A might be a hub gene involved in the development and progression of ccRCC [14].

The FCGR2A gene encodes a member of the immunoglobulin Fc receptor gene family [15]. Single nucleotide polymorphisms (SNPs) that can affect the expression levels and function of FCGR2A have been reported [16]. The FCGR2A SNPs have been found to be associated with a shorter allograft survival [17, 18] and increase the risk for a variety of diseases [19]. Studies have also indicated the role of FCGR2A in the activation of inflammatory cells involved in chronic allograft rejection [20]. Nevertheless, the relationship between FCGR2A expression and ccRCC development and progression remains unclear and the clinical significance of FCGR2A expression in ccRCC tumor tissues remain unknown.

Therefore, in this study we evaluated the expression of FCGR2A in ccRCC tissues, and investigated the clinical significance of FCGR2A expression in ccRCC patients.

RESULTS

Expression of FCGR2A

There were no differences in the level of expression of FCGR2A between male and female patients (Figure 1A), patients <60 years and ≥60 years (Figure 1B), and the tumor size <3 cm and ≥3 cm (Figure 1C). Compared with the pathologic grade I, the expression of FCGR2A in the pathologic grade III tumors was higher ($P<0.05$). The expression of FCGR2A in the pathologic grade II tumors was lower than that of pathologic grade III tumors (Figure 1D). The expression of FCGR2A was higher in the Enneking stage III and IV tumors than in Enneking stage I tumors (Figure 1E).

Associations between FCGR2A expression and demographic and clinical parameters

Pearson's chi-squared test revealed that pathological grade of tumor ($p<0.001$), family history of ccRCC ($p=0.001$) and the Enneking stage of tumor ($p<0.001$) were significantly associated with FCGR2A expression. However, FCGR2A expression was not correlated with sex, age, and tumor size ($p>0.05$). (Table 1). Similarly, Spearman's test showed that FCGR2A expression was significantly related to pathological grade of tumor ($\rho = 0.406$, $p<0.001$), family history of ccRCC ($\rho = 1.000$, $p=0.001$) and the Enneking stage of tumor ($\rho =0.577$, $p<0.001$). FCGR2A expression was not related with sex ($\rho = -0.091$, $p = 0.266$), age ($\rho = -0.106$, $p = 0.193$), and tumor size ($\rho = 0.057$, $p = 0.484$) (Table 2).

HRs of FCGR2A overexpression in ccRCC by univariate cox regression

Patients with family history of ccRCC had higher risk (HR=1.740, 95% CI, 1.197-2.530, p=0.004) of FCGR2A overexpression. Compared with type I

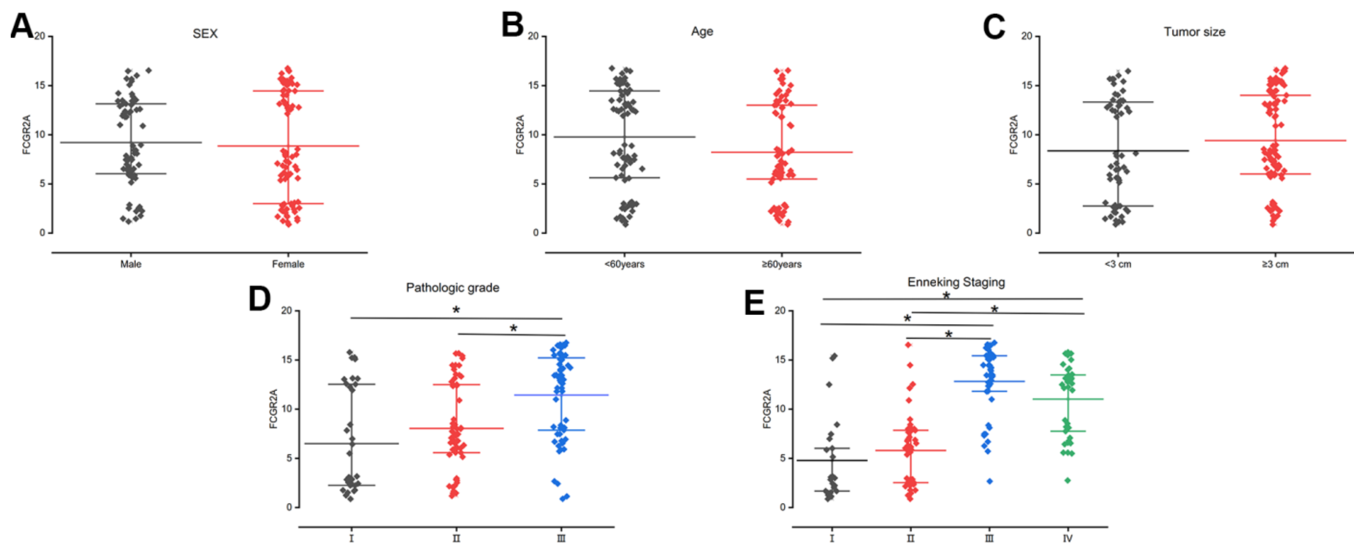


Figure 1. FCGR2A expression by different scatter plots in different groups. (**A**) Sex; (**B**) Age; (**C**) Tumor size; (**D**) Pathologic grade; (**E**) Enneking Staging. * represents that the P <0.05.

Table 1. Clinicopathological variables and the expression status of FCGR2A.

			FCGR2A			P
			-/+(%)	++(%)	+++(%)	
Sex	Male	66	10(6.6 %)	25(16.6%)	31(20.5%)	0.136
	Female	85	24(15.9%)	24(15.9%)	37(24.5%)	
Age	<60years	77	17(11.3 %)	20(13.2%)	40(26.5%)	0.156
	≥60years	74	17(11.3%)	29(19.2%)	28(18.5%)	
Tumor size	<3 cm	58	17(11.3 %)	15(9.9%)	26(17.2%)	0.203
	≥3 cm	93	17(11.3%)	34 (22.5%)	42(27.8%)	
Pathologic grade*	I	35	19(12.6%)	5 (3.3 %)	11(7.3 %)	<0.001*
	II	59	11(7.3%)	30(19.9%)	18(11.9%)	
	III	57	4(2.6%)	14(9.3%)	39(25.8%)	
Family history of ccRCC*	No	84	28(18.5 %)	26(17.2 %)	30(19.9%)	0.001*
	Yes	67	6(4.0 %)	23(15.2 %)	38(25.2 %)	
Enneking stage*	I	25	15(9.9%)	7(4.6 %)	3(2.0%)	<0.001*
	II	44	17(11.3 %)	22(14.6%)	5(3.3 %)	
	III	45	1(0.7 %)	7(4.6 %)	37(24.5%)	
	IV	37	1(0.7 %)	13(8.6 %)	23(15.2 %)	

Pearson's chi-squared test was used. *P<0.05.

Table 2. The corelationship between characteristics of patients and FCGR2A.

Characteristics	FCGR2A	
	ρ	p(spearman)
Sex	-0.091	0.266
Age	-0.106	0.193
Tumor size	0.057	0.484
Pathologic grade*	0.406	<0.001*
Family history of ccRCC *	1.000	0.001*
Enneking stage*	0.577	<0.001*

Spearman-rho test was used. *P<0.05.

pathological grade of tumor, the HR was 2.415 (95% CI, 1.347-4.331, p=0.003) for type II, and 5.475 (95% CI, 3.064-9.784, p<0.001) for type III grade tumors. The HR was 2.831 (95% CI, 1.437-5.574, p=0.003) for Enneking stage II, 14.555 (95% CI, 7.108-29.807, p<0.001) for Enneking stage III and 12.646 (95% CI, 5.950-26.876, p<0.001 for Enneking stage IV tumors. Compared with the ccRCC patients with low FCGR2A expression, those with high FCGR2A had poorer OS (HR=66.901, 95% CI, 28.251-159.428, p<0.001). There was no effect of sex, age and tumor size on the OS of ccRCC (P >0.05) (Table 3).

Effect of demographic and clinical parameters on OS of ccRCC patients by multivariate Cox regression

ccRCC patients with high Enneking stage tumors (HR =1.995, 95% CI: 1.539-2.585, P <0.001) and overexpression of FCGR2A (HR = 7.612, 95% CI: 4.718-12.283, P <0.001) have poorer OS, whereas sex (HR = 0.925, 95% CI: 0.626-1.366, P = 0.694), age (HR = 0.937, 95% CI: 0.638-1.378, P = 0.742), tumor size (HR = 0.796, 95% CI: 0.536-1.183, P = 0.259), pathological grade of tumor (HR = 1.208, 95% CI: 0.902-1.618, P =0.205), family history of ccRCC (HR = 0.792, 95% CI: 0.515-1.218, P = 0.288) showed no significant effect on OS of ccRCC (Table 4).

ROC curve analysis

The area under the curve (AUC) for various ROC curves were calculated. The expression of FCGR2A could be used to predict the parameters associated with the disease with good sensitivity and specificity: tumor size (AUC = 0.57258, P=0.0472), pathological grade (AUC=0.73806, P=0.04145), family history (AUC= 0.62873, P=0.04596), Enneking stage (AUC= 0.64391, P= 0.06109), and survival time (AUC= 0.87944, P= 0.0278). Thus,

Table 3. Characteristics and their effect on OS based on univariate Cox proportional regression analysis.

Characteristics			OS		p
			HR	95% CI	
Sex	Male	66	1		0.193
	Female	85	0.782	0.540-1.133	
Age	<60years	77	1		0.582
	≥60years	74	0.904	0.630-1.296	
Tumor size	<3 cm	58	1		0.981
	≥3 cm	93	1.004	0.695-1.452	
Pathologic grade*	I	35	1		
	II	59	2.415	1.347-4.331	0.003*
	III	57	5.475	3.064-9.784	<0.001*
Family history of ccRCC *	No	84	1		0.004*
	Yes	67	1.740	1.197-2.530	
Enneking stage *	I	25	1		
	II	44	2.831	1.437-5.574	0.003*
	III	45	14.555	7.108-29.807	<0.001*
	IV	37	12.646	5.950-26.876	<0.001*
FCGR2A *	Low(-/+)	34	1		
	Moderate(++)	49	5.362	2.842-10.119	<0.001*
	High(+++)	68	66.901	28.251-159.428	<0.001*

OS, overall survival; HR, hazard ratio; 95% CI, 95% confidence interval. * P < 0.05.

Table 4. Characteristics and their effect on OS based on multivariate Cox regression analysis.

Characteristics	OS		
	HR	95% CI	p
Sex	0.925	0.626-1.366	0.694
Age	0.937	0.638-1.378	0.742
Tumor size	0.796	0.536-1.183	0.259
Pathologic grade	1.208	0.902-1.618	0.205
Family history of ccRCC	0.792	0.515-1.218	0.288
Enneking stage*	1.995	1.539-2.585	<0.001*
FCGR2A *	7.612	4.718-12.283	<0.001*

OS, overall survival; HR, hazard ratio; 95% CI, 95% confidence interval. *P < 0.05.

FCGR2A expression can potentially be used as a diagnostic and prognostic marker for ccRCC (Figure 2).

Effect of tumor and patient characteristics on overall survival of ccRCC patients

There was no effect of sex (HR=0.782, P=0.18166), age (HR=0.904, P=0.57028), and tumor size (HR=1.004, P=0.57028) on OS of ccRCC. The higher the pathologic grade of the tumor, the worse was OS (HR=1.208, P<0.05 had poorer OS (HR=1.740, P<0.05). The higher the Enneking stage of tumor, the worse was OS (HR=1.995, P<0.05) (Figure 3).

Patients with high expression of FCGR2A had poorer OS than patients with low expression of FCGR2A (HR=7.612, P<0.001) (Figure 4).

Pathological changes revealed by HE staining

The number of renal cells were lower in the control tissues as compared with ccRCC tissues (P<0.05). Changes in the cell morphology were noticed in ccRCC tissues and immature cells were more common in the tumor tissues (Figure 5).

The protein expression of FCGR2A in ccRCC tissues

The protein expression of FCGR2A in ccRCC tissues was higher than that in control tissues (P<0.05) in the Immunohistochemical staining. The yellow areas represent the expression of FCGR2A (Figure 6). Immunofluorescence staining also showed that the

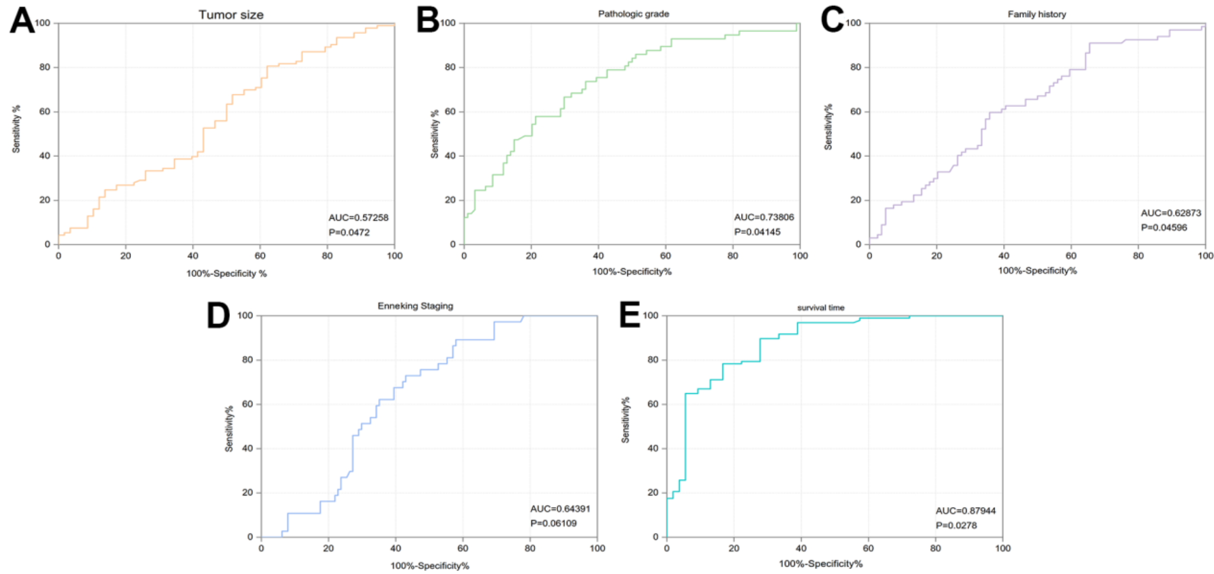


Figure 2. ROC curves to determine the effect of FCGR2A on diagnosing different traits of the ccRCC patients. (**A**) Tumor size. (**B**) Pathologic grade. (**C**) Family history. (**D**) Enneking Staging. (**E**) Survival time.

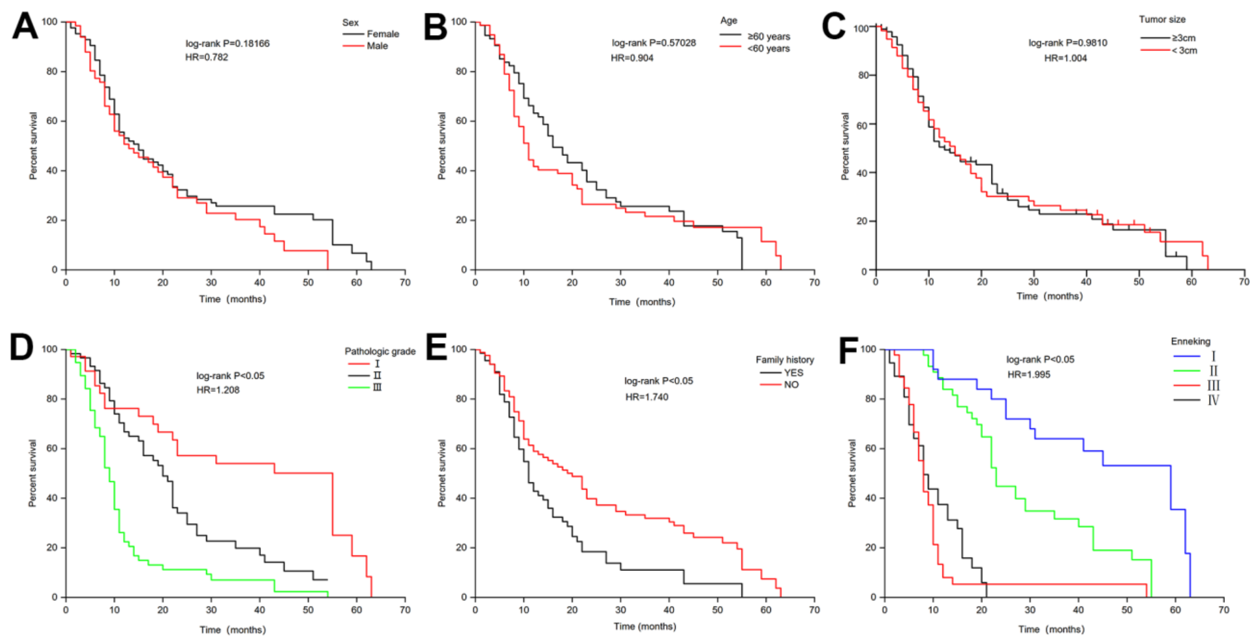


Figure 3. Effect of related characteristics on the overall survival of ccRCC. (**A**) Sex. (**B**) Age. (**C**) Tumor size. (**D**) Pathologic grade. (**E**) Family history. (**F**) Enneking Staging.

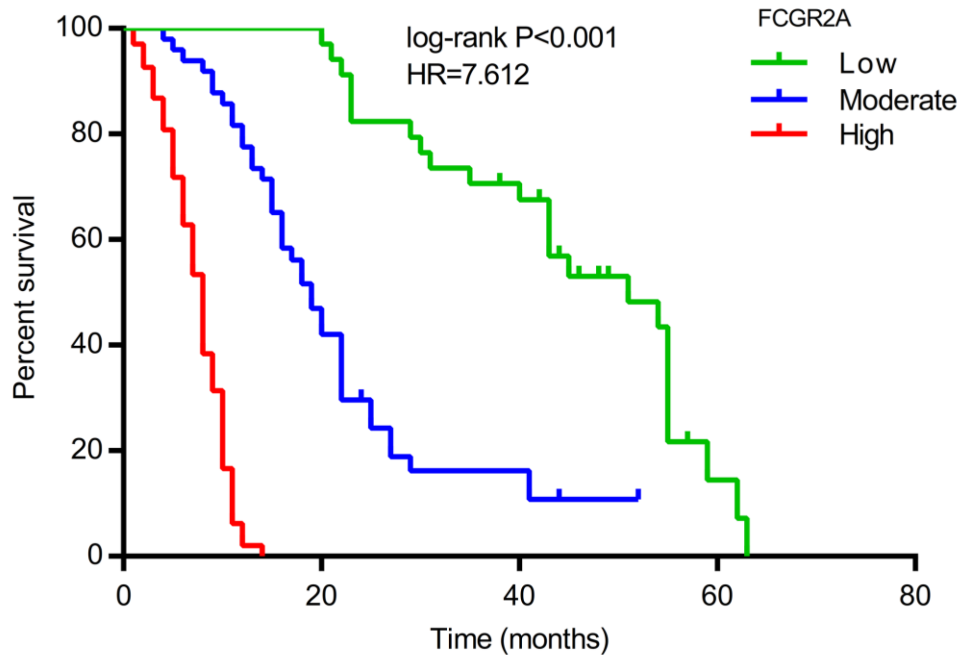


Figure 4. Effect of FCGR2A on the overall survival of ccRCC.

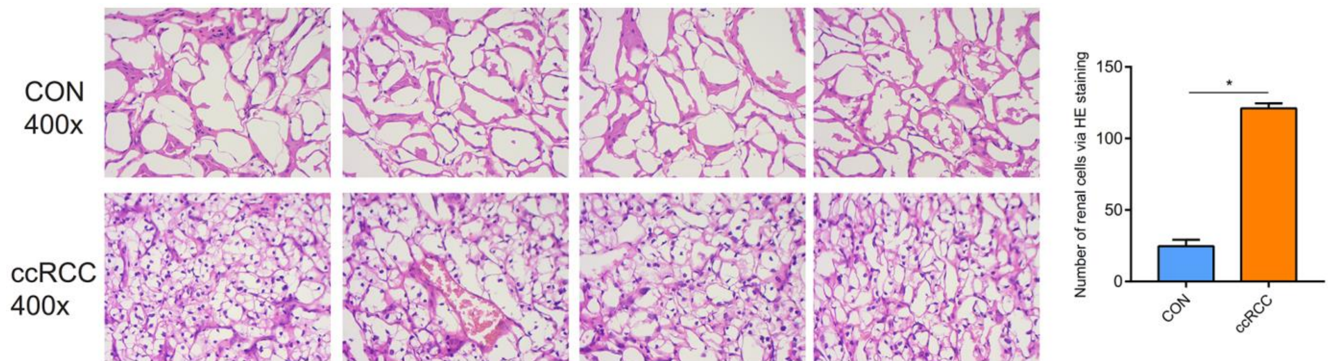


Figure 5. Pathological morphologic changes of ccRCC via the HE staining.

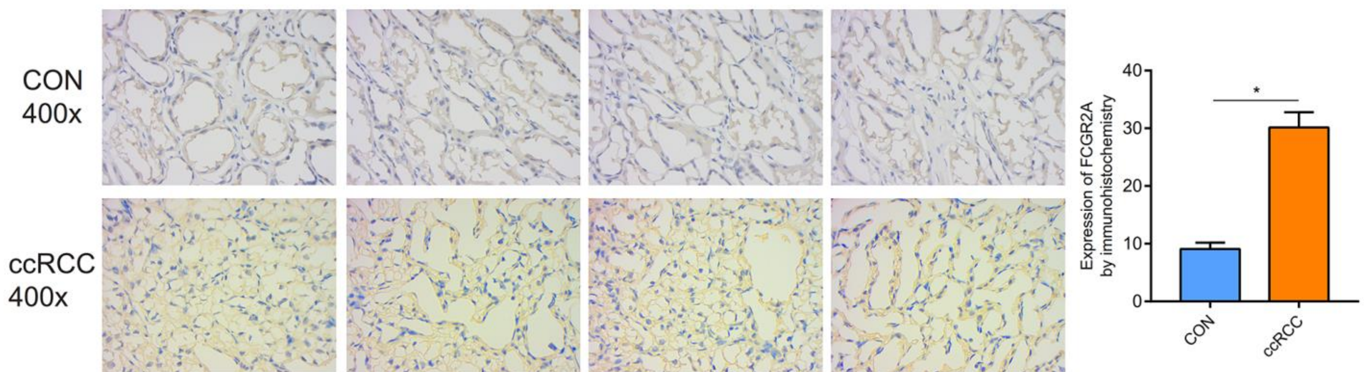


Figure 6. The protein expression of FCGR2A in the ccRCC and control tissues via immunohistochemical assay.

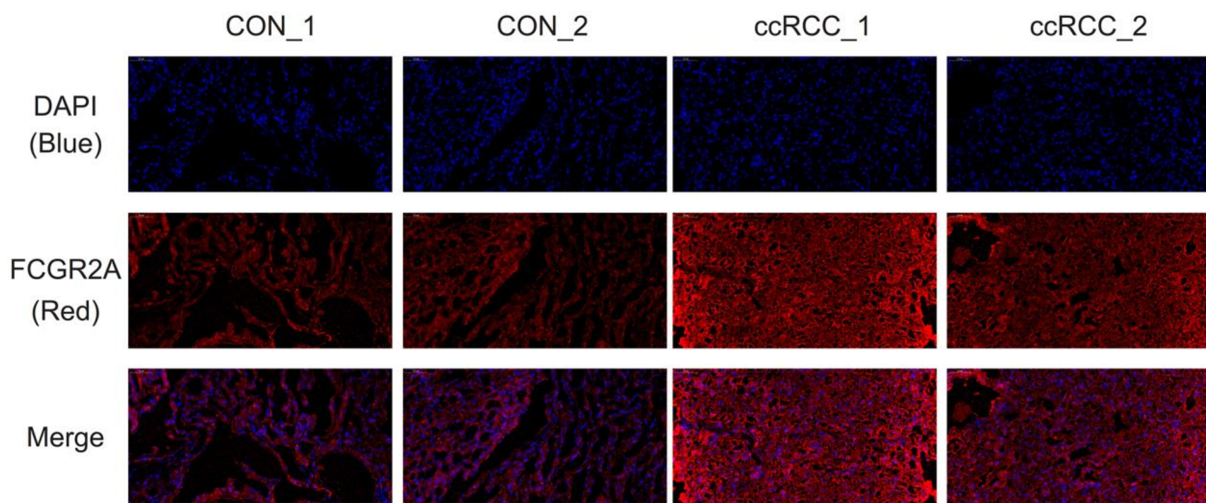
expression of FCGR2A in the control tissue was significantly lower than the ccRCC tissues (Figure 7).

Correlation between the expression of FCGR2A and OS of ccRCC patients based on the BP neural network and SVM fitting

Fitting results of BP neural network showed that the R^2 between FCGR2A expression and OS of ccRCC patients was 0.8429 (Figure 8A), SVM fitting results showed that the R^2 between the FCGR2A expression and OS of ccRCC patients was 0.7669 (Figure 8B). The fitting data of the two prediction methods was more concentrated when OS was small, but the data was scattered when the survival time was large, indicating that the data prediction effect is better when the survival time is small. The R^2 of BP neural network was significantly better than that of SVM, indicating that the prediction capability of BP was better than SVM. Also, the mean square error of SVM was 59.3845, compared with 40.0027 of BP.

Lower protein expression of FCGR2A in the ccRCC compared with control sample via western blotting

Through the western blotting assay, FCGR2A expression was lower in the ccRCC samples than control tissues. And the result was repeated three times, and the same trend was obtained. ($P<0.05$, Figure 9).



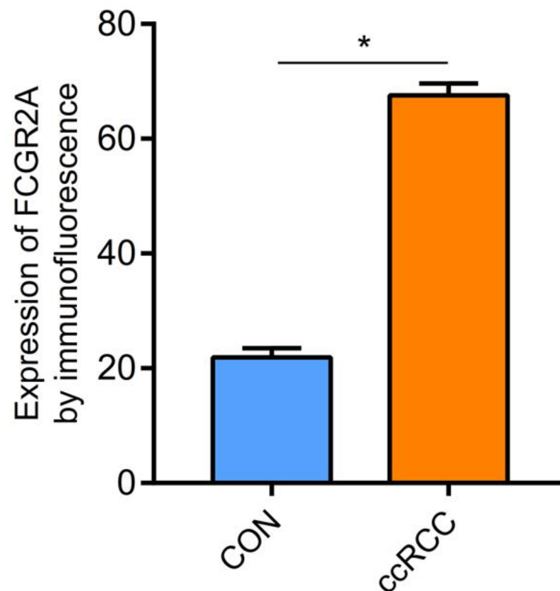


Figure 7. Verification of protein expression of FCGR2A by the immunofluorescence.

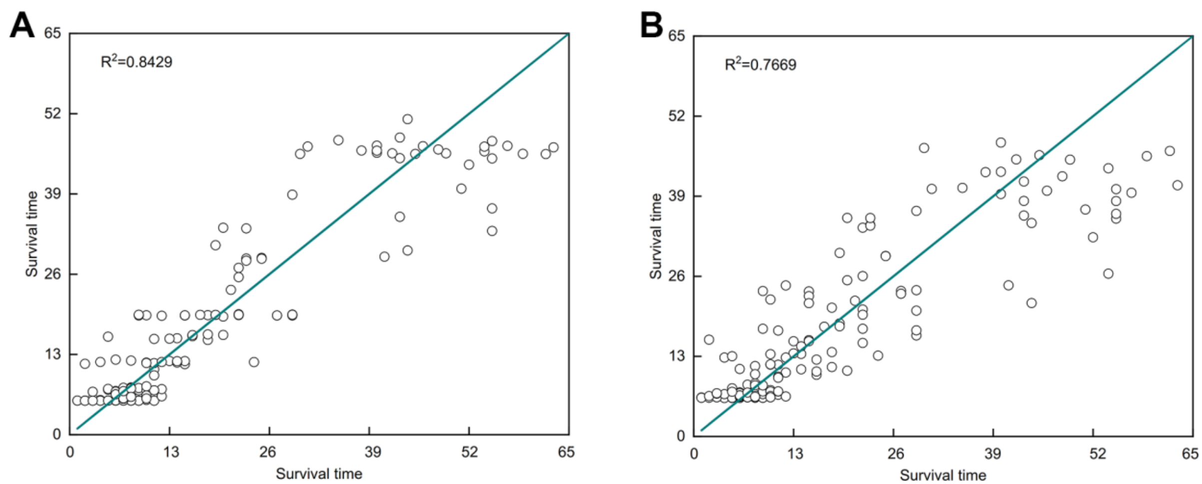


Figure 8. Strong correlation between the expression of FCGR2A and the survival time of ccRCC patients based on the BP neural network and support vector machine (SVM). (**A**) BP neural network. (**B**) SVM model.

Figure 9. Lower protein expression of FCGR2A in the ccRCC compared with control sample via western blotting.

DISCUSSION

Compared with normal renal tissue, FCGR2A was upregulated in patients with ccRCC. The patients with over expression of FCGR2A had poor OS. There was strong correlation between the expression of FCGR2A and OS of ccRCC patients based on the BP neural network and SVM.

Immunoglobulin IgGFc receptor (FcγRs) is a class of transmembrane glycoproteins that can specifically bind to IgGFc fragments and are expressed in a variety of immune cells [21]. The gene for FcγRIIa receptor (FCGR2A), which binds to the Fc fragment of IgG2 antibody is located on chromosome 1q23 [22]. FCGR2A is the only receptor of immunoglobulin G2 (IgG2) antibody, that is expressed in macrophages, lymphocytes and other innate immune cells, and regulates cell recognition, phagocytosis and cytotoxicity [23]. FcγRIIa is the most important immune-activating receptor in its family [24] which are expressed differently on immune cells and link the humoral and cell-mediated immune responses [25, 26]. After binding with IgG and cross-linking, activated FCGRs transmits signals within immune cells and activates the immune system [27–29]. FCGR2A was found to be associated with the clinical response in several clinical trials involving a variety of chimeric or humanized monoclonal antibodies targeting various cancers [30]. Researchers have demonstrated the correlation between FCGR2A genotypes and patients' response to immunotherapy [31, 32]. Therefore, we speculated that FCGR2A might participate in the development and progression of ccRCC by affecting immune function and inflammatory response, and might be used as a target for early diagnosis of ccRCC.

Previous studies have implicated FCGR2A in immune response to tumors. Since FCGR2A has a strong affinity for IgG2, it could play a role in antitumor defense. It could help in mounting immune response to tumors by causing antibody-dependent cell phagocytosis (ADCP) and facilitating antigen processing and presentation [33]. The involvement of FCGR2A in the immune response to ccRCC is indicated by a study that showed significant upregulation of various FCGR proteins, including FCGR2A in ccRCC tissues [34]. FCGRs may also play an *in vivo* antitumor role in ccRCC patients receiving high doses of IL2 [14]. These observations are consistent with our results of differential expression of FCGR2A in ccRCC tissues.

Our results indicate that FCGR2A might serve as an important core target for diagnosis of ccRCC and is closely related to the clinical characteristics of the tumor. In future, the role of FCGR2A in the development and progression of ccRCC can be explored further by siRNA based silencing of FCGR2A gene *in vitro* or in animal models. The molecule can also be used for the developments of kits for diagnosis of ccRCC.

In conclusion, FCGR2A is highly expressed in renal clear cell carcinoma, and when this molecule is highly expressed, the survival prognosis of renal carcinoma is poor. FCGR2A may be a potential target for the diagnosis and treatment of renal clear cell carcinoma.

MATERIALS AND METHODS

Patients

A total of 151 ccRCC patients treated in the Fourth hospital of Hebei medical university hospital, Hubei province, China from March 2015 to June 2020 were recruited for the study. Inclusion criteria for the patients was: age 18-100 years old; ccRCC diagnosis with normal heart function; normal coagulation and fibrinolysis function. Exclusion criteria was: poor pulmonary, cardiac, and liver function and refusal to participate in the study.

Clinical characteristics

Clinical characteristics of ccRCC patients included sex, age (<60 years /≥60 years), tumor size (<3cm/≥3cm), pathologic grade (I/II/III), family history of ccRCC (Yes/No), and the Enneking stage (I/II/III/IV).

HE staining

The Paraffin embedded sections containing renal carcinoma and adjacent tissues were successively washed with solutions containing increasing concentrations of ethyl alcohol. The slices were then dehydrated in ethyl alcohol and treated with xylene. The nuclei were stained with hematoxylin and cytoplasm with eosin. The slides were mounted in the mounting medium and examined under the microscope.

Immunohistochemistry

Paraffin sections were dewaxed and incubated with 3% H_2O_2 for 5 min to eliminate the endogenous peroxidase activity. The sections were then rinsed with distilled water and soaked in PBS for 5 minutes and blocked with 5% goat serum for 10 minutes. Overnight incubation with an anti-FCGR2A primary antibody [FCGR2A monoclonal antibody (15625-1-AP, Proteintech Group, Inc, USA)] was performed at 4° C. The sections were rinsed with PBS and incubated with a biotin-labeled secondary antibody at 37° C for 30 min. Horseradish

enzyme-labeled working solution was added drop wise to the sections and incubated for 5 min. The paraffin sections were washed three times, 5 min each, in PBS (pH 7.4) rotary shaker. After the slices were slightly dried, freshly prepared DAB color development solution was added drop wise and the color development time was controlled by observing the slides under the microscope. Nuclei were counterstained with hematoxylin.

Immunofluorescence staining of FCGR2A

Paraffin sections were dewaxed and rehydrated. After washing with PBS (pH7.4) three times. 5 min each, the sections were immersed in EDTA antigen retrieval buffer (pH 8.0) (Servicebio G1206, Wuhan, China) to retrieve the antigens. The sections were blocked with 3% BSA (Servicebio, G5001, Wuhan, China) in PBS (pH 7.4) for 30min and incubated overnight at 4° C with FCGR2A antibody (1:600, 15625-1-AP, Proteintech Group, Inc, USA). The sections were washed with PBS (pH 7.4) three times, 5 min each, and fluorescently-labeled secondary antibody (1:5000) was added. The slides were incubated in the secondary antibody for 50 min at RT in dark. After washing with PBS (pH 7.4) three times, 5 min each, the slides were incubated with DAPI solution (Servicebio, G1012, Wuhan, China) in dark for 10 min at RT to counterstain the nucleus. Spontaneous fluorescence was quenched with the spontaneous fluorescence quenching reagent (Servicebio, G1221, Wuhan, China) and the slides were sealed after adding the anti-fade mounting medium. Fluorescence microscope (Nikon NIKON ECLIPSE C1) was used to observe the slides. The nuclei were stained blue (excitation wavelength 330-380nm and emission 420nm) and the positive expression of FCGR2A exhibited red fluorescence.

RT-qPCR

T7 RNA polymerase, LA Taq polymerase, Ex Taq polymerase and DNA size markers were purchased from Takara (Tokyo, Japan). RNeasy Mini kit, and QLA quick Gel Extraction Kit were obtained from QIAGEN (Germany). The RT-PCR was performed on an ABI 7500 RT-PCR System (USA). RNA was extracted using RNeasy Mini Kit as per manufacturer's instructions. The thermocycler was programmed as follows: 55° C (2min), 72° C (3min), 94° C (30s), 60° C (30s), and 72° C (30s). A total of 72 cycles of amplification was performed. The final extension was performed at 72° C for 10 min. The PCR products were separated on agarose gels and the PCR fragments were purified by QLA Quick Gel Extraction Kit. Relative expressions of the hub genes were calculated by the $2^{-\Delta\Delta Ct}$. GAPDH gene was used as an endogenous control. The following primer pairs were used for the amplification of FCGR2A gene: Forward: TCAGG GGGTGAGAGAAGAGACTAG; Reverse: CTAGTCT CTTCTCTCACCCCCTGAA.

Western blotting

Total protein was extracted from the tissue blocks frozen at -80° C. Tissue block was washed 3 times with pre-cooled PBS (pH 7.4) and homogenized in the lysis buffer. The lysate was incubated on ice for 30 min and centrifuged at 12000 x g for 10 min at 4° C. The supernatant was collected and protein concentration was estimated using Bradford's reagent. The proteins were separated on a 10% SDS-PAGE gel and transferred on to a PVDF membrane using a semi-dry electroblotter. The membrane was blocked with 5% skimmed milk in PBS (pH 7.4) for 30 min and incubated overnight at 4° C with the primary antibody (anti-FCGR2A, 15625-1-AP, Proteintech, Wuhan, China). The blot was washed with PBS 3 times, 10 min each, and incubated with the secondary antibody for 1 h. After washing 3 times, 10 min each, with PBS (pH 7.4), the blot was placed in the chemiluminescence reagent and sealed in a transparent plastic wrap. The blot was exposed to the X-ray film in a cassette for 1-2 min and the film was developed. Alpha software (version: 12.3. USA) was used to analyze the image.

Statistical methods

Pearson's chi-squared test and Spearman's Rho test were used to explore associations between the FCGR2A expression and demographic and clinical parameters of the patients. Hazard ratios (HRs) of FCGR2A overexpression for different clinical and demographics parameters of the patients were calculated by univariate Cox regression. Correlation between the various demographic and clinical parameters and overall survival (OS) of ccRCC patients was determined by multivariate Cox regression. Receiver operating characteristic (ROC) curves were constructed to explore the role of FCGR2A as a diagnostic marker for ccRCC. Correlation between the various demographic and clinical parameters and overall survival (OS) of ccRCC patients was also performed by the BP neural network and support vector machine (SVM). SPSS 24.0 (IBM Corp., USA), and Matlab (R2017a, MathWorks.Inc, USA) were used for statistical analysis. Statistical significance was achieved at $P < 0.05$.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the fourth hospital of Hebei medical university. Written informed consent was obtained from all patients.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Abbreviations

ccRCC: Clear cell renal cell carcinoma; RCC: Renal Cell Carcinoma; SVM: support vector machine; FcγRs: Immunoglobulin IgGFc receptor; HRs: hazard ratios; 95%CI: 95% confidence intervals; OS: overall survival; AUC: area under the curve; FCGR2A: FcγRIIa receptor; ADCP: antibody dependent cell phagocytosis.

AUTHOR CONTRIBUTIONS

Feng Li performed the experiments, and was a major contributors in writing. Feng Li was also involved in the revision of the manuscript with critical inputs. Changjin Shi made substantial contributions to research conception and provided technical support. Yueyue Wei designed the draft of the research process., analyzed the data and submitted the manuscript for publication. All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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