

RHBG Expression Is Correlated With ccRCC Proliferation and Immune Infiltration

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Abstract Clear cell renal cell carcinoma (ccRCC) is one of the pathological types of renal cell carcinoma with poor prognosis, which poses a serious threat to human life. Therefore, there is a need to develop appropriate tumor markers to predict the development, treatment, and prognosis of ccRCC. As a member of a new protein family involved in ammonia transport in the mammalian kidney, RHBG plays an important role in the kidney. Therefore, the relationship between RHBG and clinical characteristics, prognostic risk, overall survival and immune infiltration in patients with clear cell renal cell carcinoma was explored by bioinformatics. The Cancer Genome Atlas (TCGA) database was used to evaluate the correlation between RHBG and ccRCC. The results showed that the expression of RHBG in renal cancer tissues was lower than that in normal tissues ($P < 0.001$); ROC curve showed that RHBG had a high diagnostic value ($AUC = 0.957$). The low expression of RHBG is associated with the immune status of renal clear cell carcinoma. These data suggest that RHBG is a viable potential biomarker for ccRCC with great potential for future clinical application and translation.

Keywords: TCGA, ccRCC, RHBG

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1. Introduction

Renal cell carcinoma (RCC), a malignant tumor originating from renal tubular epithelial cells, accounts for about 90% of renal malignant tumors. (ccRCC) is the most common type of renal cell carcinoma. [1,2,3] The 5-year survival rate of patients with early clear cell renal cell carcinoma after surgical resection is more than 90%. However, patients with advanced clear cell renal cell carcinoma are prone to relapse after surgery. Even with chemotherapy and radiotherapy, most patients survive for about 3 years on average. [4,5,6,7] In order to effectively control or eradicate tumor cells, there is an urgent need to develop new cancer treatments.

With the development of biomedical technology, targeted therapy and immunotherapy are the main forces for the treatment of advanced clear cell renal cell carcinoma. [8,9,10,11] Compared with traditional tumor therapy, targeted therapy has the characteristics of identifying and analyzing the mechanism of action and key genes of RCC, which lays the foundation for the preparation and development of subsequent targeted drugs. [12,13,14,15] Therefore, in order to provide a solid theoretical basis for the treatment of renal cell carcinoma, we study the genes of renal cell carcinoma in order to

screen out the key genes for the diagnosis and treatment of renal cell carcinoma and achieve targeted therapy. [16,17].

RH blood group proteins have two non-erythroid homologs, RHCG (Rh family C glycoprotein) and RHBG (Rh family B glycoprotein), which share homology with specific ammonia transporters in primitive organisms and plants, are mainly expressed in the kidney, and represent members of a new family of proteins involved in ammonia transport in mammalian kidneys. [18,19,20,21,22] Among them, RHCG is expressed in the apical pole of all connecting tubule (CNT) cells as well as in intercalated cells of collecting duct (CD), whereas relatively few studies have been performed on RHBG. Recently, Regine *et al.* produced a polyclonal antibody against the RHB type glycoprotein to detect the localization of RHBG in the nephron. [23] The presence of RHBG and RHCG at the major sites of renal ammonia secretion, CNT and CD, is consistent with their role in ammonia transport. Lee *et al.* found that RHBG participates in the transport of NH_3/NH_4^+ and methylamine (hydrochloric acid) (MA/MA $^+$) in oocytes in a manner distinct from NH_3/NH_4^+ . [24] Based on this, we screened according to TCGA database that RHBG has potentially important value in the diagnosis and prognosis of patients with renal cell carcinoma.

In this study, we investigated the association of RHBG with renal clear cell carcinoma using clinical data in TCGA, including clinical characteristics, prognosis and survival data.

2. Materials and Methods

2.1. RNA Expression and Data Mining

From the TCGA database (<https://portal.gdc.cancer.gov>) to download and organize TCGA - KIRC (renal clear cell carcinoma) project STAR process RNAseq data and extract the TPM (Transcripts Per Million) format of the data and clinical data. The clinicopathological data included age, height, weight, gender, and race. As this was a bioinformatics study, no ethics committee or institutional review board approval was required. This study was conducted in accordance with the published guidelines provided by TCGA.

2.2. Clinicopathological Analysis

TCGA data were used for clinicopathological analysis. The Kruskal-Wallis test was selected according to the data format characteristics, and the data were visualized using the ggplot2 package. Clinical prognostic information included overall survival (OS) and disease-specific survival (DSS). Kaplan-Meier (K-M) method was used for univariate and multivariate Cox regression analysis.

2.3. Construction and Prediction of Nomograms

Considering the results of the multivariate analysis, nomograms were evaluated in order to personalize the prediction of life expectancy. The survival package was used for proportional hazards hypothesis testing and Cox regression analysis, and the rms package was used to construct and visualize the nomogram correlation model. Calibration and discrimination are the most commonly used methods to evaluate model performance. A calibration chart was constructed to evaluate the predictive accuracy of the nomogram based on the prognostic model.

2.4. Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) was used to establish a series of genes related to RHBG expression. Samples were divided into RHBG high and RHBG low expression groups as training sets to distinguish latent functions and elucidate significant survival differences. Gene set alignments were performed multiple times for each examination. Standardized enrichment scores and adjusted P values were used to rank pathways enriched for each phenotype.

2.5. Immune Infiltration

Spearman correlation method was used to analyze the correlation between RHBG and immune cells, and Wilcoxon rank sum test was used to compare the infiltration of these cells between samples with high and low RHBG expression.

2.6. Statistical Analysis

Wilcoxon signed rank test was used to analyze the

expression level of RHBG gene in unpaired samples of renal cell carcinoma patients. Paired sample t test was used to analyze the expression of RHBG gene in renal cell carcinoma patients. Mann-Whitney U test (Wilcoxon rank sum test) and Wilcoxon signed rank test were used to analyze the difference of RHBG[ENSG00000132677.13] between multiple groups and paired samples. Cox regression was used to compare the prognosis between high and low RHBG groups and between subgroups. The pROC package was used for ROC analysis of the data, and the results were visualized by ggplot2. spearman analysis was used to analyze the correlation between RHBG and immune infiltration and presented in the form of a lollipop chart. GSEA analysis was performed with the clusterProfiler package. Significant markers: ns, $P \geq 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Baseline Characteristics of the Patients

Firstly, we retrieved 541 samples of ccRCC patients from TCGA database, including 269 samples with low RHBG expression and 272 samples with high RHBG expression. Table 1 lists the detailed clinical and genetic characteristics of ccRCC patients. There were significant differences in pathological M stage, pathological stage and hemoglobin between patients with low RHBG expression and patients with high RHBG expression. The low expression of RHBG was correlated with pathological M stage ($P = 0.030$), pathological stage ($P = 0.032$) and hemoglobin ($P = 0.013$).

3.2. RHBG is Highly Expressed in Renal Clear Cell Carcinoma

In TCGA dataset, RHBG was highly expressed in renal clear cell carcinoma (Figure 1A). Paired sample analysis using Wilcoxon rank sum test also showed that RHBG was significantly upregulated in ccRCC ($P < 0.001$) (Figure 1B). These results suggest that RHBG may play a role in the pathogenesis of clear cell renal cell carcinoma. qRT-PCR analysis of RHBG expression in human renal cortical/proximal tubular cell line HK-2, human renal adenocarcinoma cell line (ACHN) and human renal carcinoma cell line (A-498) showed that the expression of RHBG was significantly down-regulated in ACHN ($P < 0.01$) and A-498 ($P < 0.01$) cells (Figure 1C and 1D). In addition, pan-cancer RHBG expression was evaluated, and by Mann-Whitney U test (Wilcoxon rank sum test), RHBG expression was found to be higher in 15 tumors than in their corresponding normal tissues, including: BLCA ($P < 0.001$) BRCA ($P < 0.001$) COAD ($P < 0.001$) HNSC ($P < 0.001$) KICH ($P < 0.001$) KIRC ($P < 0.001$) KIRP ($P < 0.001$) LUAD ($P < 0.001$) LUSC ($P < 0.001$) READ ($P < 0.001$) UCEC ($P < 0.001$) GBR ($P < 0.01$) PRAD ($P < 0.01$) CHOL ($P < 0.05$) PCPG ($P < 0.05$) (Figure 1E). By Wilcoxon signed rank test, RHBG expression was found to be higher in 11 tumors than in their corresponding normal tissues, including BRCA ($P < 0.001$) COAD

($P<0.001$) HNSC ($P<0.001$) KICH ($P<0.001$) KIRC ($P<0.001$) KIRP ($P<0.001$) LUAD ($P<0.001$) LUSC ($P<0.001$) BLCA ($P<0.01$) READ ($P<0.05$) UCEC ($P<0.05$) (Figure 1F).

Based on this, we hypothesized that the low expression of RHBG in renal cell carcinoma may be associated with prognosis. Therefore, the study of Table 2 was performed. The results were consistent with our predictions. Low

RHBG expression was associated with T stage (T3&T4 vs. T1&T2: OR = 1.525 (1.069-2.175), $P=0.020$) and Pathologic M stage (M1 vs. M0: OR = 1.719 (1.051-2.813), $P=0.031$), Pathologic stage (Stage III&Stage IV vs. Stage I&Stage II: OR = 1.652 (1.163-2.348), $P=0.005$) and Hemoglobin (Normal vs. Low&Elevated: OR = 0.598 (0.411-0.869), $P=0.007$).

Table 1. Clinicopathological characteristics of ccRCC patients in TCGA database

Characteristics	Level	Low expression of RHBG	High expression of RHBG	P value
n		269	272	
Pathologic T stage, n (%)	T1	153 (28.3%)	126 (23.3%)	0.081
	T2	34 (6.3%)	37 (6.8%)	
	T3	78 (14.4%)	102 (18.9%)	
	T4	4 (0.7%)	7 (1.3%)	
Pathologic N stage, n (%)	N0	122 (47.3%)	120 (46.5%)	0.606
	N1	7 (2.7%)	9 (3.5%)	
Pathologic M stage, n (%)	M0	220 (43.3%)	209 (41.1%)	0.030
	M1	30 (5.9%)	49 (9.6%)	
Pathologic stage, n (%)	Stage I	150 (27.9%)	123 (22.9%)	0.032
	Stage II	30 (5.6%)	29 (5.4%)	
	Stage III	54 (10%)	69 (12.8%)	
	Stage IV	32 (5.9%)	51 (9.5%)	
Primary therapy outcome, n (%)	PD	3 (2%)	8 (5.4%)	0.368
	SD	4 (2.7%)	2 (1.4%)	
	PR	1 (0.7%)	1 (0.7%)	
	CR	67 (45.6%)	61 (41.5%)	
Gender, n (%)	Female	94 (17.4%)	93 (17.2%)	0.854
	Male	175 (32.3%)	179 (33.1%)	
Race, n (%)	Asian	3 (0.6%)	5 (0.9%)	0.776
	Black or African American	29 (5.4%)	28 (5.2%)	
	White	233 (43.6%)	236 (44.2%)	
Age, n (%)	≤ 60	134 (24.8%)	135 (25%)	0.966
	> 60	135 (25%)	137 (25.3%)	
Histologic grade, n (%)	G1	8 (1.5%)	6 (1.1%)	0.084
	G2	126 (23.6%)	110 (20.6%)	
	G3	104 (19.5%)	103 (19.3%)	
	G4	28 (5.3%)	48 (9%)	
Serum calcium, n (%)	Low	109 (29.7%)	95 (25.9%)	0.265
	Normal	74 (20.2%)	79 (21.5%)	
	Elevated	3 (0.8%)	7 (1.9%)	
Hemoglobin, n (%)	Low	128 (27.8%)	136 (29.5%)	0.013
	Elevated	2 (0.4%)	3 (0.7%)	
	Normal	99 (21.5%)	93 (20.2%)	
Laterality, n (%)	Left	127 (23.5%)	126 (23.3%)	0.804
	Right	141 (26.1%)	146 (27%)	

Table 2. Analyze the correlation between RHBG expression and clinicopathological features using logistic regression

Characteristics	Total (N)	OR (95% CI)	P value
Pathologic T stage (T3&T4 vs. T1&T2)	541	1.525 (1.069 - 2.175)	0.020
Pathologic N stage (N1 vs. N0)	258	1.307 (0.472 - 3.623)	0.607
Pathologic M stage (M1 vs. M0)	508	1.719 (1.051 - 2.813)	0.031
Pathologic stage (Stage III&Stage IV vs. Stage I&Stage II)	538	1.652 (1.163 - 2.348)	0.005
Primary therapy outcome (PR&CR vs. PD&SD)	147	0.638 (0.229 - 1.779)	0.391
Gender (Male vs. Female)	541	1.034 (0.725 - 1.474)	0.854
Race (White vs. Asian&Black or African American)	534	0.982 (0.585 - 1.650)	0.946
Age (> 60 vs. ≤ 60)	541	1.007 (0.719 - 1.411)	0.966
Histologic grade (G3&G4 vs. G1&G2)	533	1.321 (0.940 - 1.859)	0.109
Serum calcium (Normal vs. Low&Elevated)	367	1.172 (0.774 - 1.776)	0.453
Hemoglobin (Normal vs. Low&Elevated)	461	0.598 (0.411 - 0.869)	0.007
Laterality (Right vs. Left)	540	1.044 (0.744 - 1.463)	0.804

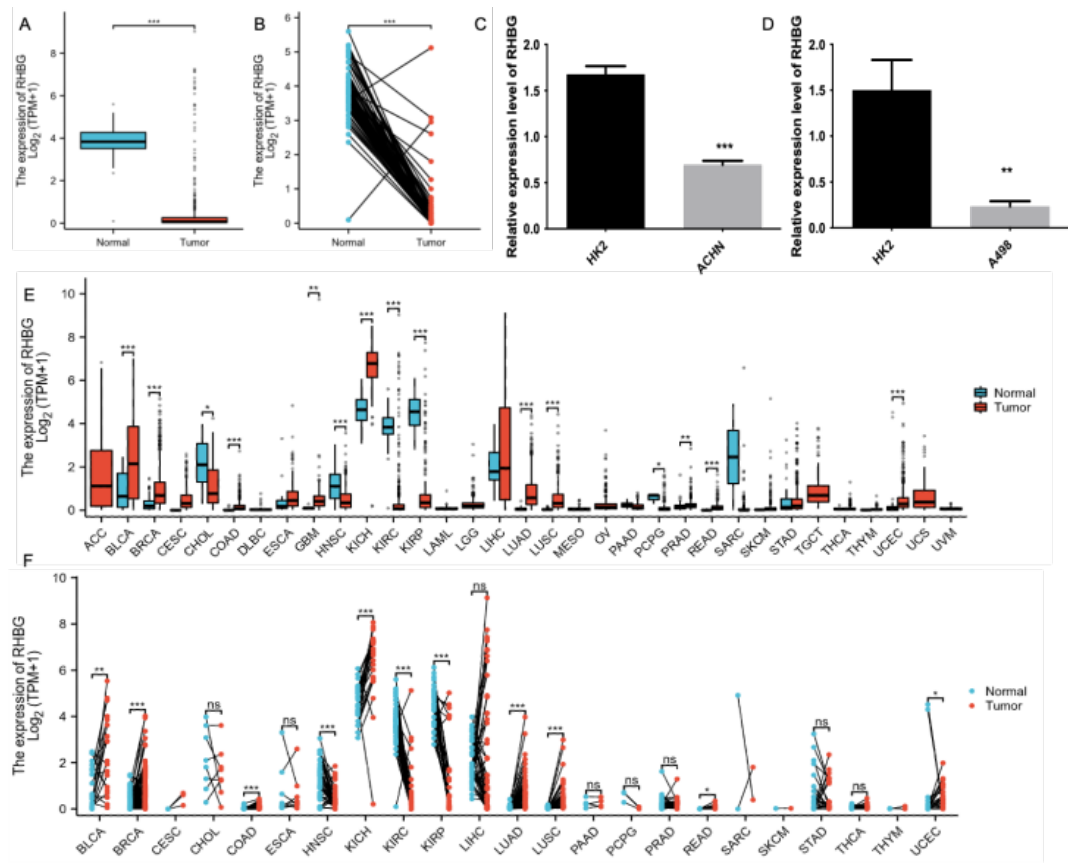


Figure 1. Expression of RHBG in normal renal tissues and renal carcinoma tissues. (A) RHBG expression in unpaired samples. (B) RHBG expression in paired samples. (C-D) The expression of RHBG in ACHN and A498 cells was lower than that in HK2. (E) expression of RHBG in unpaired samples of different tumor samples. (F) expression of RHBG in paired samples of different tumor samples. (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

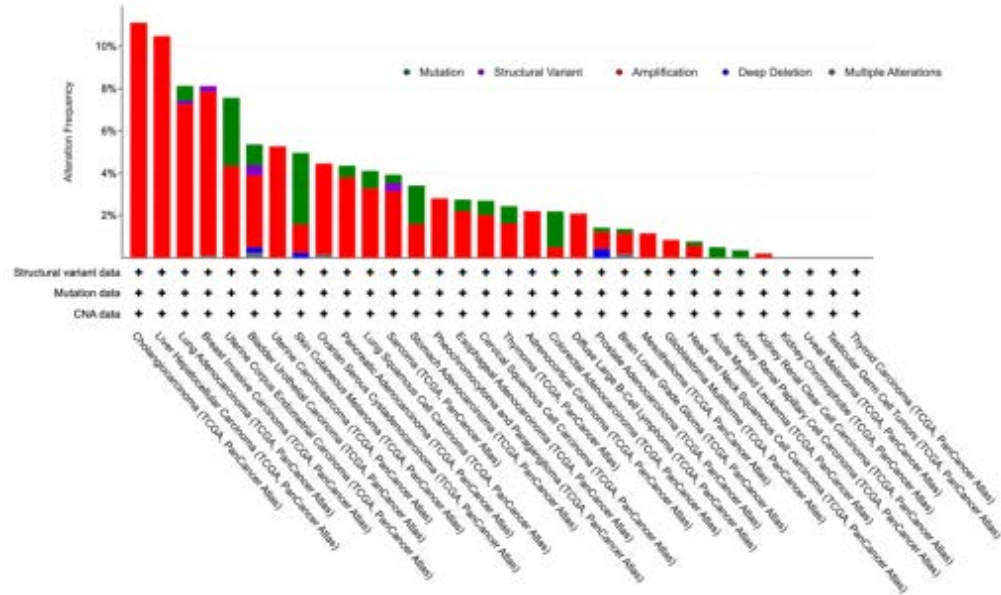


Figure 2. Genetic characteristics of RHBG, including mutation, amplification and deletion, in different tumors from TCGA analyzed by cBioPortal

3.3. Genetic and Epigenetic Alterations of RHBG in ccRCC

The cBioPortal website was used to explore the genetic alterations of RHBG gene in different types of cancers in TCGA cohort. As shown in Figure 2, among CHOL(Cholangiocarcinoma) patients with “amplification” as the main change, the frequency of RHBG changes was

the highest (11.11%). Alterations in the RHBG gene were found in only 0.2% of the “amplification” frequencies of all ccRCC cases.

3.4. Multifaceted Prognostic Value of RHBG Expression in Cancer

The relationship between RHBG expression and OS of cancer patients was studied by basic evaluation of RHBG

expression in different tumors. Kaplan-Meier survival analysis ($P=0.011$, $HR=1.48$) and multiple clinical subgroups (Figure 3A-O), including T3($P=0.005$, $HR=2.00$), M0($P=0.006$, $HR=2.20$), stage III ($P=0.004$, $HR=3.07$), female ($P=0.002$, $HR=3.22$), male ($P=0.005$, $HR=1.77$), white ($P=0.017$, $HR=1.47$), age ≤ 60 years old ($P=0.004$, $HR=2.14$), age > 60 years ($P=0.017$, $HR=1.64$), G3($P=0.006$, $HR=2.03$), G4($P=0.016$, $HR=1.70$), hypocalcemia ($P=0.005$, $HR=2.11$), normal serum calcium ($P=0.003$, $HR=2.58$), left laterally ($P=0.002$, $HR=2.07$) and right laterally ($P=0.019$, $HR=1.81$). Univariate Cox analysis (logrank test) confirmed that the low expression of RHBG was related to the good prognosis of patients with renal cell carcinoma (hazard ratio (HR)=1.483, 95% CI, 1.094-2.010, $P=0.011$). Multivariate Cox analysis confirmed that low RHBG expression was associated with a good prognosis

in patients with renal cell carcinoma [hazard ratio (HR)=1.408, 95% CI, 1.033-1.920, $P=0.030$] (Table 3).

3.5. RHBG Has a High Diagnostic Value in Clear Cell Renal Cell Carcinoma

According to ROC curve analysis, RHBG was not only closely related to the prognosis of renal cell carcinoma, but also had a high diagnostic value ($AUC = 0.957$) (Figure 4A). In addition, a nomogram was constructed to predict patient survival at 1, 3, and 5 years, taking into account RHBG expression and other predictive variables such as age and stage (Figure 4B). We constructed a bias correction line in the calibration plot to approximate the ideal curve (45-degree line), which indicates perfect agreement between prediction and observation (Figure 4C).

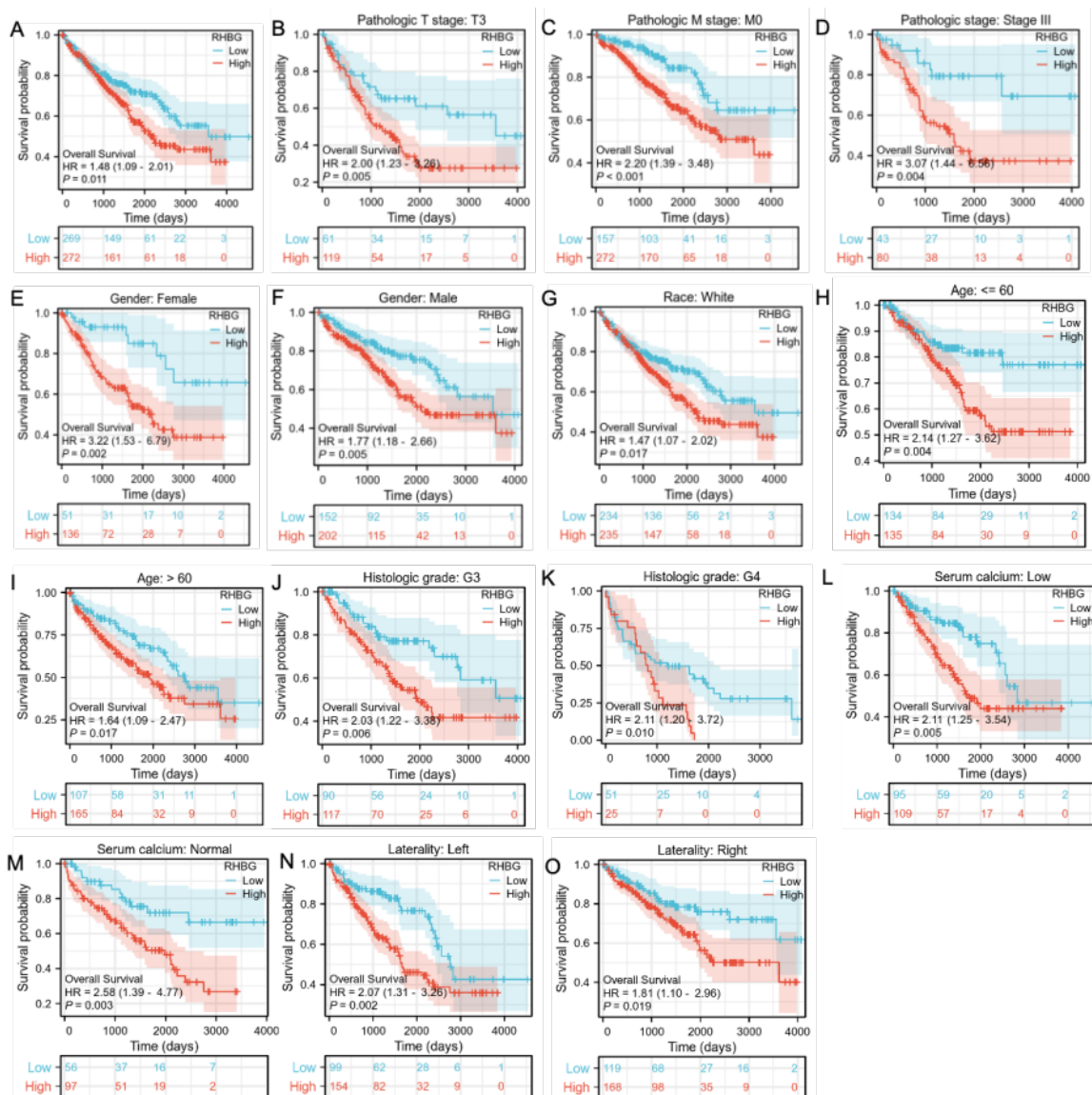


Figure 3. Prognostic value of RHBG expression for overall survival (OS) in different types of tumors was analyzed using Tumor 2.0 database. (A) T3; (B) M0; (C) stage III; (D) Women; (E) Male; (F) White people; (G) age ≤ 60 years old; (H) age > 60 years old; (I) G3; (J) G4; (K) hypocalcemia; (L) normal serum calcium; (M) left laterally; (N) right laterally. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

Table 3. Results of Univariate and Multivariate Cox Regression Analyses

Characteristics	Total(N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
Pathologic T stage	541				
T1&T2	350	Reference		Reference	
T3&T4	191	3.210 (2.373 - 4.342)	< 0.001	0.613 (0.341 - 1.102)	0.102
Pathologic stage	538				
Stage I&Stage II	332	Reference		Reference	
Stage III&Stage IV	206	3.910 (2.852 - 5.360)	< 0.001	4.658 (2.528 - 8.582)	< 0.001
Gender	541				
Female	187	Reference			
Male	354	0.924 (0.679 - 1.257)	0.613		
Age	541				
≤ 60	269	Reference		Reference	
> 60	272	1.791 (1.319 - 2.432)	< 0.001	1.639 (1.203 - 2.234)	0.002
Histologic grade	533				
G1&G2	250	Reference		Reference	
G3&G4	283	2.665 (1.898 - 3.743)	< 0.001	1.822 (1.277 - 2.599)	< 0.001
Laterality	540				
Left	253	Reference		Reference	
Right	287	0.707 (0.525 - 0.952)	0.022	0.697 (0.515 - 0.943)	0.019
RHBG	541				
Low	269	Reference		Reference	
High	272	1.483 (1.094 - 2.010)	0.011	1.408 (1.033 - 1.920)	0.030

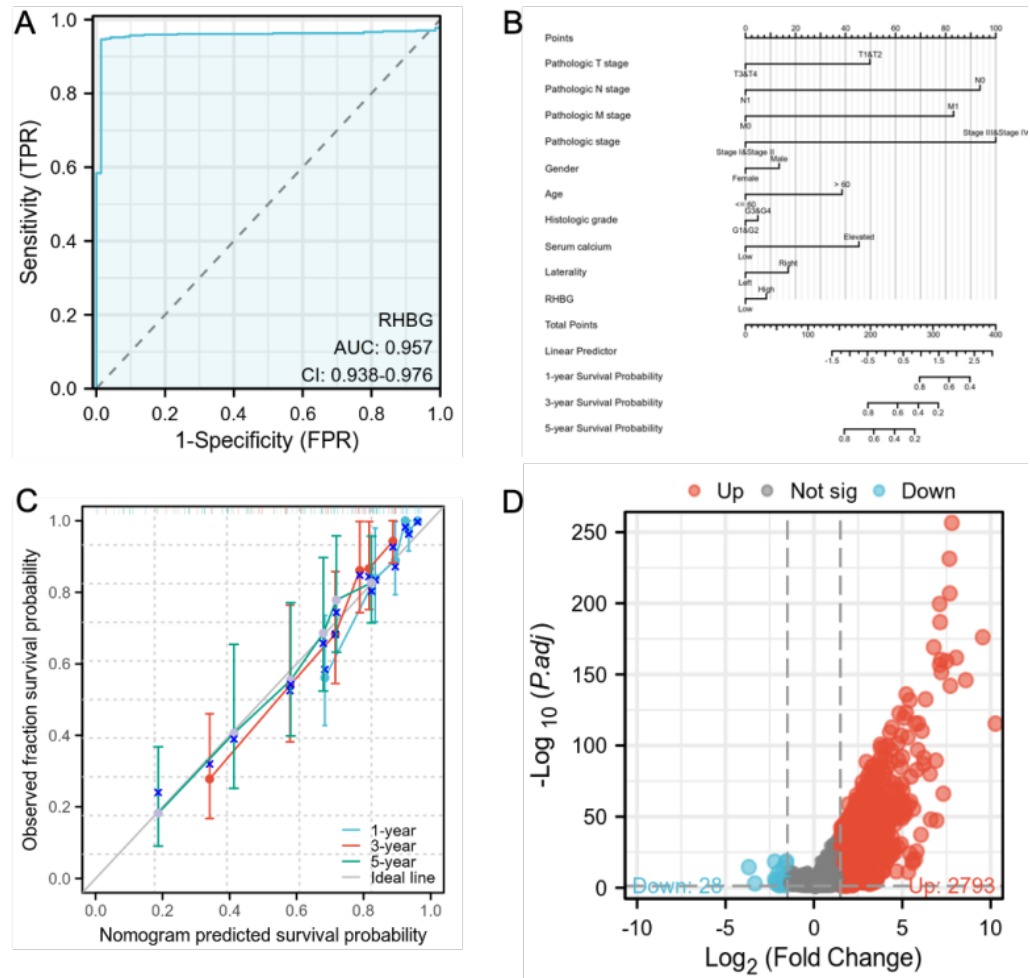


Figure 4. Survival curve results of different expression levels of RHBG on the prognosis of patients with renal clear cell carcinoma. (A) Total ROC curve of RHBG in renal cell carcinoma. (B) Nomogram was used to predict 1-, 3-, and 5-year OS for the entire TCGA cohort. (C) Calibration curve for predicting OS. (D) Differential expression analysis of RHBG high and low expression groups in volcano plot. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

3.6. GSEA Identifies RHBG-related Signaling Pathways

The gene expression profiles of RHBG high and low expression groups were compared by RNAseq gene expression analysis to clarify whether RHBG plays a role in the occurrence and development of ccRCC. We verified RHBG expression in normal and tumor samples using paired plots, and the results showed statistically significant differences. A total of 2793 up-regulated genes were detected in RHBG high expression group ($P > 1.5$ -fold) and 28 down-regulated genes (> -1.5 times). The expression of DEG is shown by the volcano plot (Figure 4D).

3.7. Analysis of Correlation

Spearman correlation coefficient was used to analyze RHBG-related gene expression in the data grouped according to RHBG gene expression, and the ggplot package was used to visualize the co-expression heat map of the analysis results with RHBG as the main variable (Figure 5). RHBG expression was positively correlated with DPM1, and negatively correlated with GCLC, NFYA, FUCA2 and other genes.

3.8. Correlation Between Immune Cell Infiltration and RHBG Expression

We analyzed the immune cell infiltration in patients with renal clear cell carcinoma from the TCGA database. The expression of RHBG correlated with B cells, aDC, CD8T cells, Cytotoxic, Eosinophils, Macrophages, Mast cells, Neutrophils, NK CD56dim cells, NK cells, p DC and T A negative correlation was observed in cells (Figure 6A-L). The results of the chord chart were consistent with the results of the immune infiltration scatter plot. The expression of RHBG was significantly negatively correlated with T cell infiltration, B cell infiltration, NK cell infiltration, CD8 cell infiltration, Neutrophils cell infiltration and Macrophages (Figure 6M). The results indicate that low expression of RHBG inhibits T cells, B cells, NK cells, CD8 cells, Neutrophils and Macrophages, suggesting that low expression of RHBG is related to the immune status of renal clear cell carcinoma. Spearman correlation analysis between RHBG expression and immune cell enrichment (ssGSEA generation) showed a positive correlation between RHBG expression and NK CD56 bright cell abundance ($P = 0.0249$) (Figure 6N).

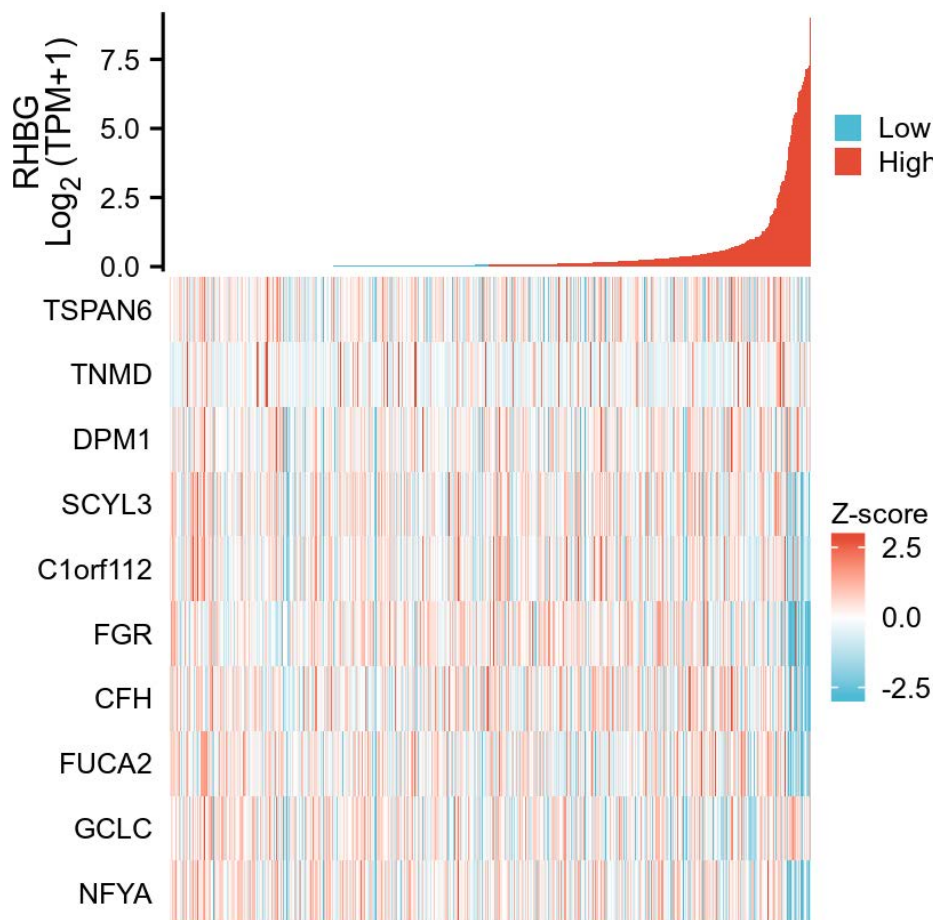


Figure 5. Heat map of RHBG single gene co-expression

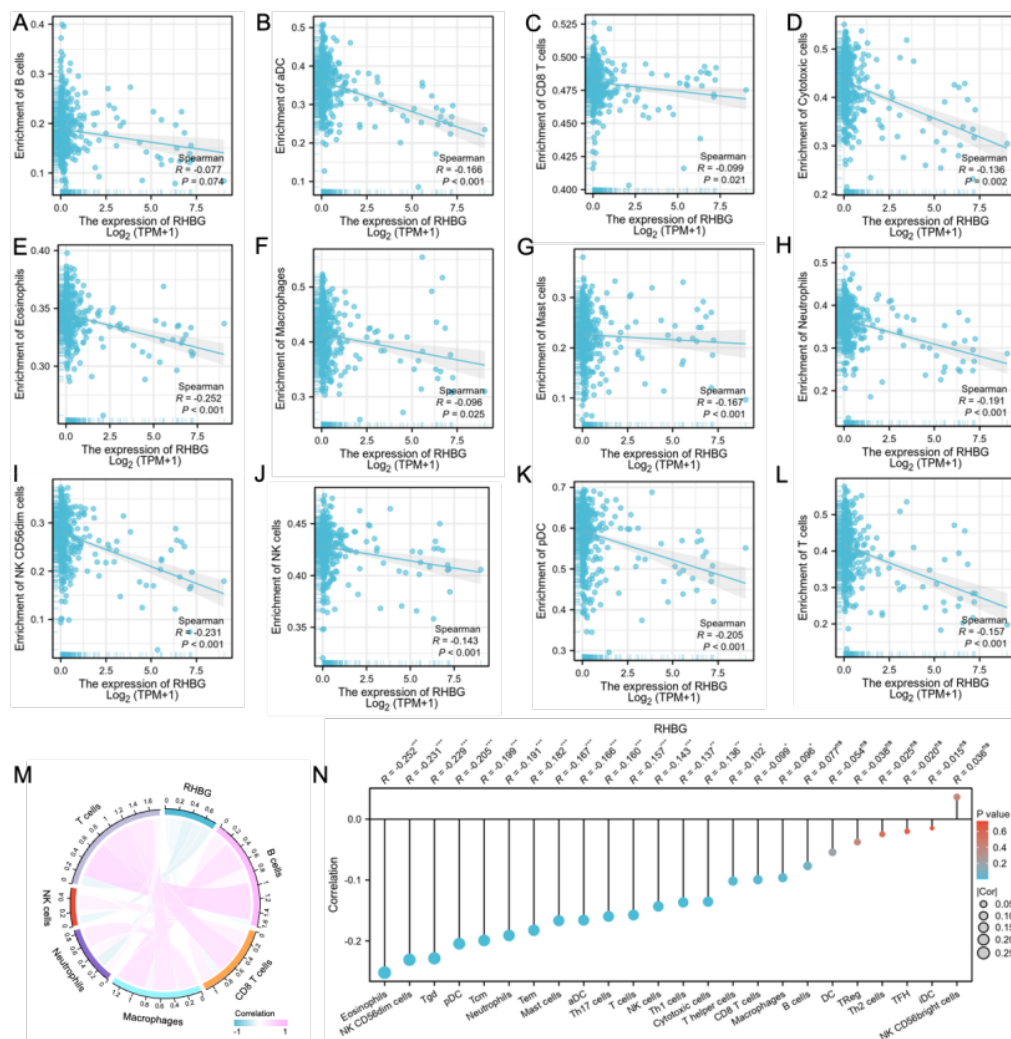


Figure 6. Correlation between renal clear cell carcinoma infiltration and RHBG. (A-L) Scatter plot of immune infiltration. (M) Chord diagram. (N) Lollipop diagram. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

4. Discussion

Renal clear cell carcinoma is the most common pathological type of renal cell carcinoma, accounting for 70% of all renal cell carcinomas, and it is also one of the pathological types of renal cell carcinoma with the worst prognosis. [25, 26] Targeted therapy is often used for advanced ccRCC patients with distant metastasis or recurrence after surgery. Although targeted therapy drugs can effectively improve the overall survival of patients, it is difficult to avoid the emergence of tumor drug resistance. Therefore, clarifying the oncogenes and mechanism of action of cancer is conducive to excavating new therapeutic targets, thereby reversing tumor resistance and improving patient survival time.

RHBG, an Rh family B glycoprotein with homology to specific ammonia transporters in primitive organisms and plants, represents a member of a new family of proteins involved in ammonia transport in the mammalian kidney. I David Weiner *et al.* found that RhBG and RhCG play important cell-specific roles in ammonium transport and signaling in these regions of the kidney. [27] Lee *et al.* used two-electrode voltage clamp and ion-selective microelectrodes to measure NH_4^+ -induced and methylammonium MA^+ current and intracellular pH (pH

(i)) changes, respectively, and found that RHBG is pH-sensitive to NH_4^+ and MA^+ transport. [28] By expressing human RHAG, RHBG and RHCG in *Xenopus* oocytes, Raif Musa-Aziz and his team discovered the mechanism by which RHBG and RHCG promote CD acid secretion by enhancing NH_3 and CO_2 transmembrane transport. [29] Connie M Westhoff and his team found that the polarized localization of RhBG and RhCG in the renal tubules and the different substrate affinities may allow these proteins to participate in transepithelial ammonia secretion and thus play an important role in acid-base regulation throughout the animal. [30] Yves Colin and his team found findings suggesting a close relationship between the sorting and anchoring of RHBG and the basolateral domain of epithelial cells. [31] I David Weiner *et al.* found that both RHBG and RHCG are highly expressed in specific cells of the male reproductive tract and that they can promote multiple components of male fertility. [32] Ashley M Toye and his team found that RHCG is the major putative ammonia transporter expressed in the human kidney under normal conditions, whereas RHBG is not expressed at high levels. [33] Existing studies have shown that RHBG is associated with ammonia transport in the kidney. However, studies on RHBG in cancer are very scarce. We first investigated the diagnostic and prognostic functions of RHBG in ccRCC

using bioinformatics methods.

At the bioinformatics level, we performed paired and unpaired analyses of ccRCC in RHBG and found that the expression level of RHBG in ccRCC was significantly lower than that in normal tissues. We then analyzed the expression level of RHBG in all cancers and found that it was low expressed in the vast majority of cancers. We found that RHBG is significant in terms of M stage, pathological stage, and hemoglobin in ccRCC patients, and it plays a very important role in the prognosis of ccRCC. In addition, the results of ROC curve and column line table indicated that RHBG had a high diagnostic value in ccRCC. Scatter plots, lollipop plots and chord plots of immune infiltration indicated that RHBG was closely related to immune cell infiltration.

5. Results

In conclusion, bioinformatics studies have shown that RHBG is lowly expressed in ccRCC and is associated with tumor M stage, pathological stage and hemoglobin aspects. In addition, the ROC curve suggested that RHBG had a high diagnostic value in ccRCC and may be used as a diagnostic marker for ccRCC. The immune infiltration scatter plot, lollipop plot and chord plot also suggested the potential correlation between RHBG and immune cell infiltration, which provided the basis and suggestion for further study of the mechanism of action.

Author Contributions

Methodology: Ying Song

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Writing – review & editing: Haiming Yu

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