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Comprehensive Analysis of ADCY Family Members in The Development, Immune Infiltration and Prognostic Value of Kidney Renal Clear Cell Carcinoma

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1. Abstract

Objective: Kidney renal clear cell carcinoma (KIRC) has a high incidence worldwide. Adenylate cyclases (ADCYs) family is a membrane-binding enzyme that passes through the GnRH signaling pathway associated with the G protein system and participates in epigenetic regulation. However, the role of different ADCYs proteins in colorectal cancer is poorly understood.

1.1. Methods: Oncomine, gene expression profile interactive analysis (GEPIA), Kaplan-Meier plotter, cBioPortal, GeneMANIA and TIMER were used to analyze the differential expression, pathological grade, prognostic value, gene changes and immune cell infiltration of ADCYs in patients with renal cell carcinoma.

1.2. Results: Compared with normal control tissues, the expression levels of ADCY1/6 were decreased and the expression levels of ADCY2/3/4/5/7/8/9 were increased in KIRC tissues. In addition, except for ADCY7, the expression of other members of the ADCY family tended to decrease with the increase of pathological grade in KIRC patients. In the prognostic analysis, ADCY1/2/5/8/9 was significantly associated with overall survival (OS) in KIRC patients, and ADCY1/4/5/6/9 was significantly associated with disease-free survival (DFS). ADCY1/5/9 may be a potential survival prognostic biomarker in patients with KIRC. Functional analysis indicated that the differentially expressed ADCYs might be related to the activation of protein kinase activity, the regulation of phospholipase activity and the activation of phosphodeoxylase activity. Moreover, the expression of ADCYs were significantly correlated with the infiltration of six immune cells in KIRC patients.

1.3. Conclusion: This study provides a new direction for potential prognostic biomarkers of KIRC patients.

2. Introduction

The latest world cancer report shows that the incidence of kidney cancer ranks ninth among male cancers and fourteenth among female cancers [1]. Although the mortality trend of kidney cancer in many highly developed countries has stabilized or even declined, in most countries with lower levels of development, the incidence of renal cancer is still rising, and these gaps seem to be widening [2]. Renal cell carcinoma (RCC) accounts for more than 90% of kidney cancers, of which clear cell carcinoma accounts for 70% and has a poor prognosis [3-5]. The treatment of renal cell carcinoma has advanced from nonspecific immunotherapy to specific immunotherapy. At present, research has found that many factors affect the occurrence and development of kidney cancer, such as VHL, NR1B2, METTL14, PTEN, PDL1, etc [6-10]. However, poor treatment effect and drug resistance are still a major problem in clinical treatment. Adenylate cyclases (ADCYs) are membrane binding enzymes that include ADCY1-9 responsible for the con-

version of adenosine triphosphate (ATP) to the second messenger cAMP [11]. They are involved in the GnRH signaling pathway associated with the G protein system and are involved in many cellular processes, including the promotion of tumor cell growth [12]. According to the similarity of gene sequence and common regulation pattern, these 9 genes were divided into 4 categories. ADCY1, 3 and 8 are characterized by being activated by Ca2+/calmodulin. ADCY2, 4 and 7 are ineffective against Ca2+, but can be activated by G protein βγ subunits. Low concentration of Ca2+ can inhibit ADCY5 and ADCY6, but this group is not sensitive to the regulation of βγ subunits. And ADCY9 can be specifically inhibited by Ca2+/calmodulin, so it is listed as a separate group [13,14]. Several studies have reported the correlation between ACDYs and tumors, such as melanoma, NSCLC, glioblastoma, esophageal carcinoma, acute myeloid leukemia, prostate cancer, colorectal cancer, hepatocellular carcinoma (HCC), cervical cancer and pancreatic cancer [12,15,22]. Nevertheless, the role of ADCYs in the occurrence and progression of cancer has not been specifically clarified. And the mechanism of abnormal expression of ADCYs in renal carcinoma and its effect on prognosis have not been fully elucidated. Further study of its bioinformatics to guide clinical treatment and improve the prognosis is imminent. In this study, a comprehensive bioinformatics analysis of the expression of ADCYs in KIRC was performed using public data to evaluate its prognostic value in KIRC patients and explore possible mechanisms of action to provide a potential target for clinical treatment.

3. Results

3.1. Transcription of ADCYs in KIRC

We analyzed the expression of ADCY family members in various tumors and normal tissue controls using the Oncomine database. By comparison, several studies have shown that ADCYs is highly expressed in renal tumors (Figure 1). As shown in multiple datasets showed that mRNA expression of ADCY2/3/4/5/7 was significantly elevated in KIRC tissues. In Gumz Renal statistics dataset, the fold change of ADCY2 in KIRC tissues compared with normal tissues was 4.9, and 1.883-fold in Beroukhim Renal statistics dataset of Non-Hereditary Clear Cell Renal Cell Carcinoma, 1.734-fold in Beroukhim Renal statistics dataset of Hereditary Clear Cell Renal Cell Carcinoma, 2.141-fold in Jones Renal statistics dataset, 2.78-fold in Yusenko Renal statistics dataset. In Lenburg Renal statistics, a 1.541-fold increase in ADCY3 expression was detected in KIRC as compared to normal control tissues. Similar increases of ADCY3 expression in KIRC were also seen in Gumz Renal statistics dataset (2.132-fold), Beroukhim Renal statistics dataset of Hereditary Clear Cell Renal Cell Carcinoma (1.942-fold), Ber-

oukhim Renal statistics dataset of Hereditary Clear Cell Renal Cell Carcinoma (1.623-fold), Yusenko Renal statistics dataset (1.407-fold) and Jones Renal statistics dataset (1.165-fold). In addition, in Lenburg Renal statistics, a 1.215-fold increase in ADCY4 expression was also detected in KIRC patients compared to the control group. Similarly, in Lenburg Renal statistics and Yusenko Renal statistics, ADCY5 expression was also 1.377-fold and 1.661-fold increased in the KIRC patients compared with the control group, respectively. ADCY7 over-expression was also detected in KIRC tissues compared with normal tissues with a 2.897-fold in Higgins Renal statistics, 2.418-fold in Beroukhim Renal statistics dataset of Non-Hereditary Clear Cell Renal Cell Carcinoma, 2.608-fold in Beroukhim Renal statistics dataset of Hereditary Clear Cell Renal Cell Carcinoma, 2.879-fold in Gumz Renal statistics, 2.660-fold in Jones Renal statistics and 4.259-fold in Yusenko Renal statistics. GEPIA dataset was further used to compare the expression level of ADCYs in KIRC and normal control tissues. As shown in Figure 2A and 2B, the expression levels of ADCY2/3/4/5/7/9 in KIRC tissues were higher than those in normal tissues. These results tend to be consistent with the results of Oncomine. To explore the possible role of ADCYs in the development of KIRC, we evaluated the correlation between ADCYs expression and pathological staging in patients with KIRC. As can be seen in Figure 3, ADCY1/2/3/4/5/6/8/9 are significantly correlated with the pathological staging of KIRC and participate in its occurrence and development, while ADCY7 has no significant correlation with the pathological staging of KIRC.

3.2. Prognostic value of ADCYs mRNA expression in KIRC patients

Gepia analysis was used to develop overall survival (OS) and disease-free survival (DFS) curves to explore and analyze the correlation between different ADCYs and clinical outcomes, so as to further evaluate the prognostic value of differential expression of ADCYs in KIRC. Figure 4A shows a significant correlation between ADCY1/2/5/8/9 and OS in patients with KIRC, and it can be seen that the higher expression level of ADCY1/2/5/8/9 has a better prognosis. The expression levels of ADCY3/4/6/7 were not statistically significant with OS (Figure 4A). In the DFS curve, ADCY1/4/5/6/9 were significantly correlated with DFS in KIRC patients, indicating that the high expression group had a better prognosis (Figure 4B). The expression levels of ADCY2/3/7/8 in DFS were not statistically significant (Figure 4B). We further analyzed the prognostic value of ADCYs in patients with KIRC using Kaplan-Meier plotter. High mRNA expression of ADCY1/2/4/5/6/8/9 was significantly associated with OS in KIRC patients (Figure 5).
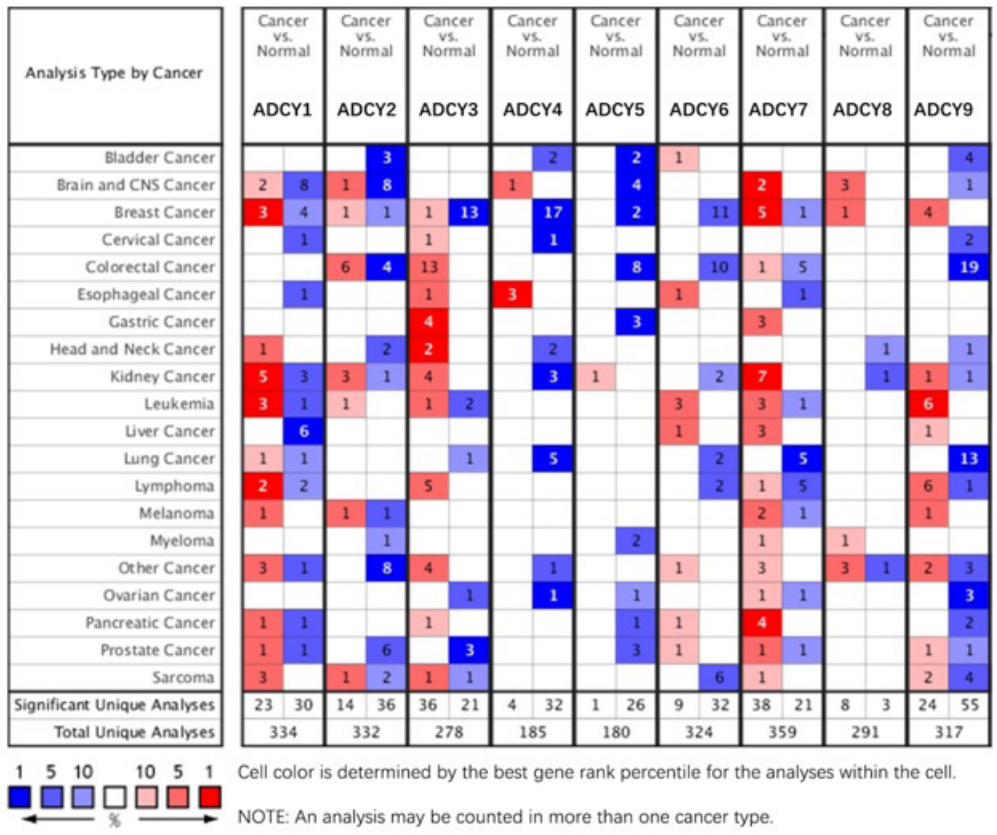
Analysis Type by Cancer	ADCY1		ADCY2		ADCY3		ADCY4		ADCY5		ADCY6		ADCY7		ADCY8		ADCY9	
	Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal		Cancer vs. Normal	
Bladder Cancer				3				2	2		1							4
Brain and CNS Cancer	2	8	1	8			1			4			2		3			1
Breast Cancer	3	4	1	1	1	13		17		2		11	5	1	1		4	
Cervical Cancer				1	1			1										2
Colorectal Cancer			6	4	13					8		10	1	5				19
Esophageal Cancer				1	1		3				1			1				
Gastric Cancer					4					3			3					
Head and Neck Cancer	1			2	2			2								1		1
Kidney Cancer	5	3	3	1	4			3	1			2	7			1	1	1
Leukemia	3	1	1		1	2					3		3	1			6	
Liver Cancer		6									1		3		1			
Lung Cancer	1	1				1		5				2		5				13
Lymphoma	2	2			5							2	1	5			6	1
Melanoma	1		1	1									2	1			1	
Myeloma				1						2			1		1			
Other Cancer	3	1		8	4			1			1		3		3	1	2	3
Ovarian Cancer						1		1	1				1	1				3
Pancreatic Cancer	1	1			1					1	1		4					2
Prostate Cancer	1	1		6		3				3	1		1	1			1	1
Sarcoma	3		1	2	1	1						6	1				2	4
Significant Unique Analyses	23	30	14	36	36	21	4	32	1	26	9	32	38	21	8	3	24	55
Total Unique Analyses	334		332		278		185		180		324		359		291		317	

1 5 10   10 5 1   Cell color is determined by the best gene rank percentile for the analyses within the cell.

%

NOTE: An analysis may be counted in more than one cancer type.

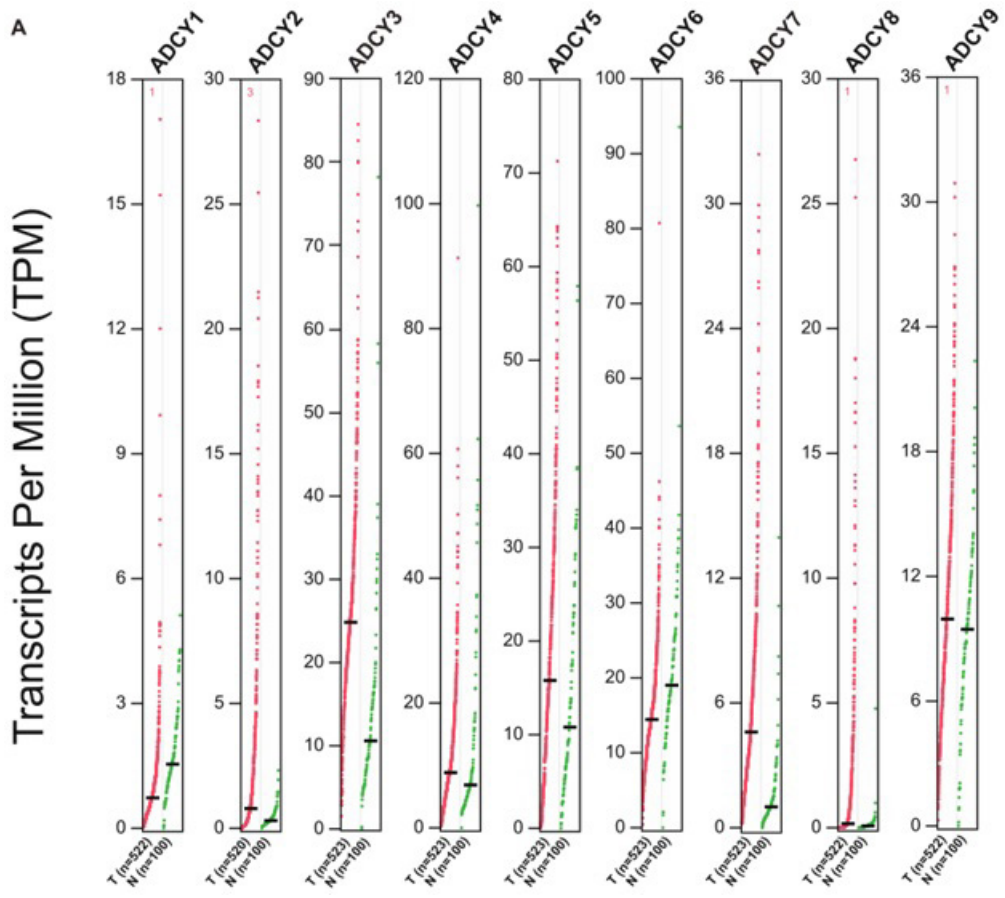**Figure 1:** Transcription of ADCYs in various tumor types (Oncomine).

Figure 2. The expression level of ADCYs in KIRC and normal control tissues (GEPIA).
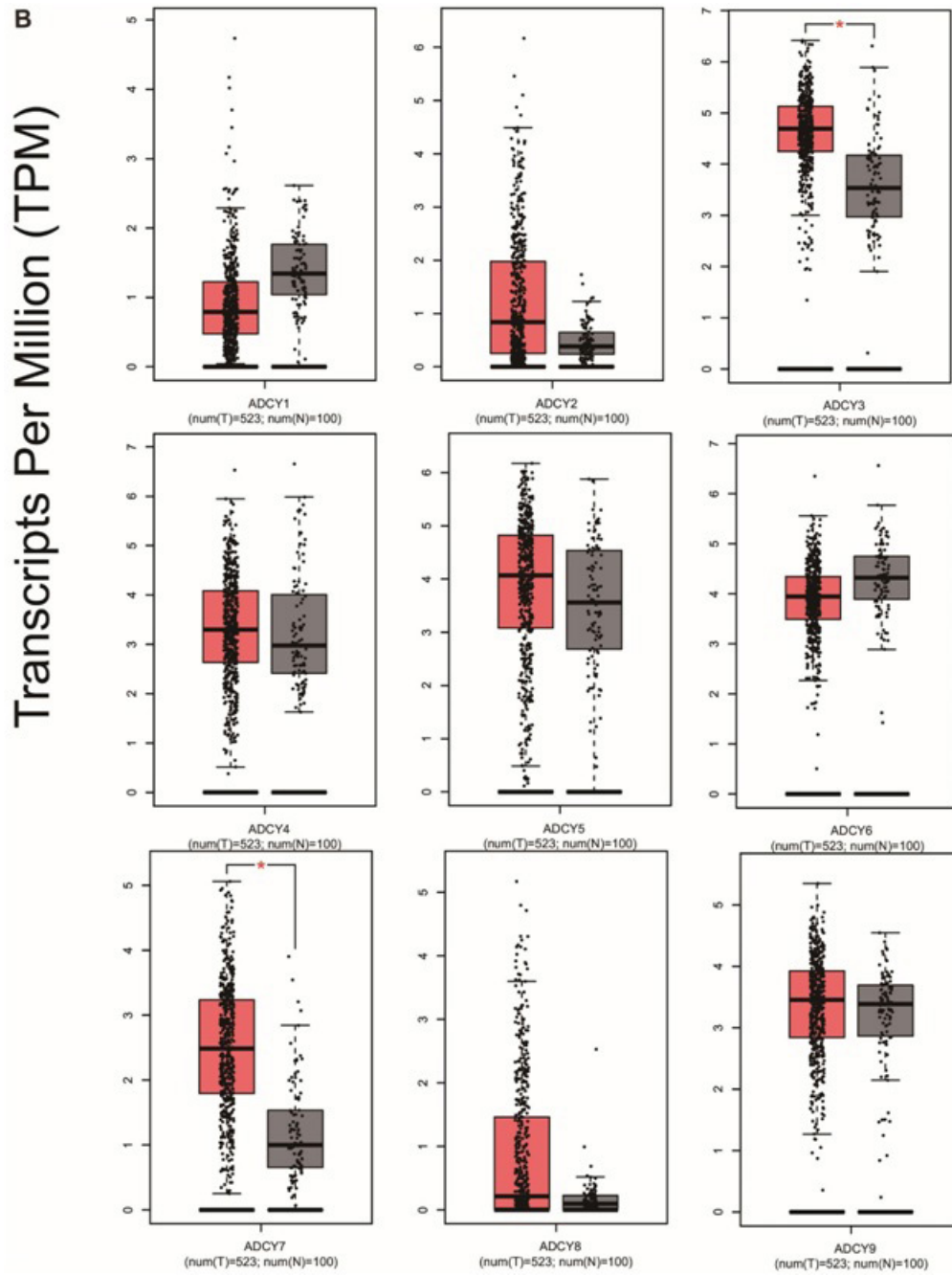
Figure 2 (**A**): Comparison of expression levels of ADCYs in KIRC versus normal tissue samples. (**B**) TPM values of ADCYs in KIRC and normal tissue samples. T represents the KIRC sample and N represents the normal tissue sample.
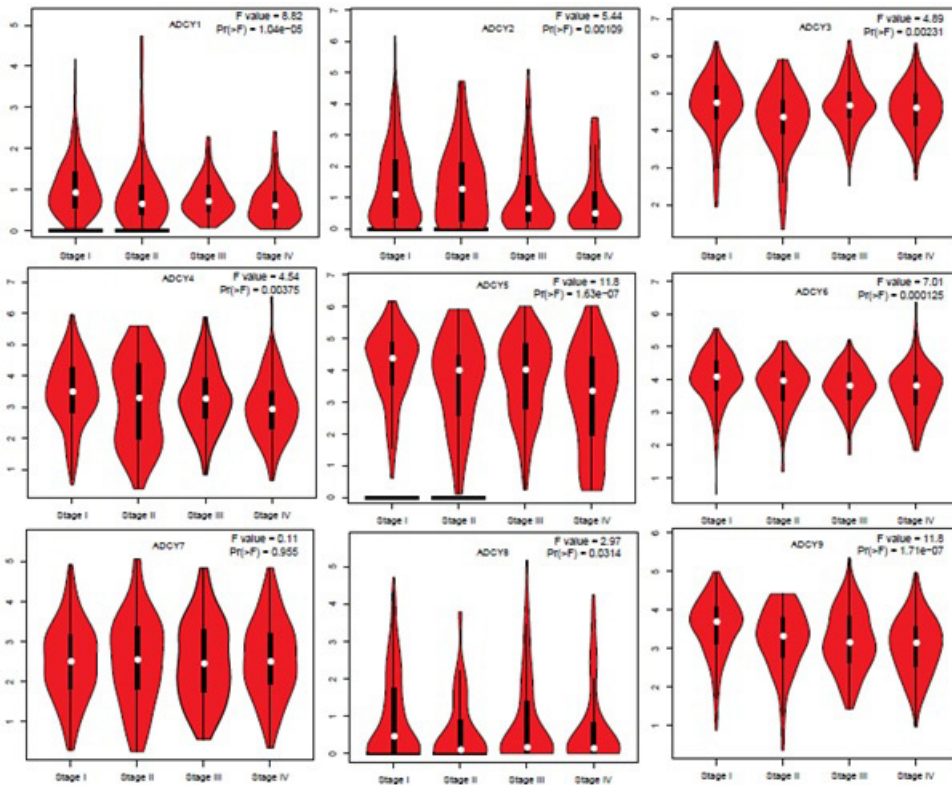
Figure 3: Association of ADCY expression with tumor stage in patients with KIRC (GEPIA).
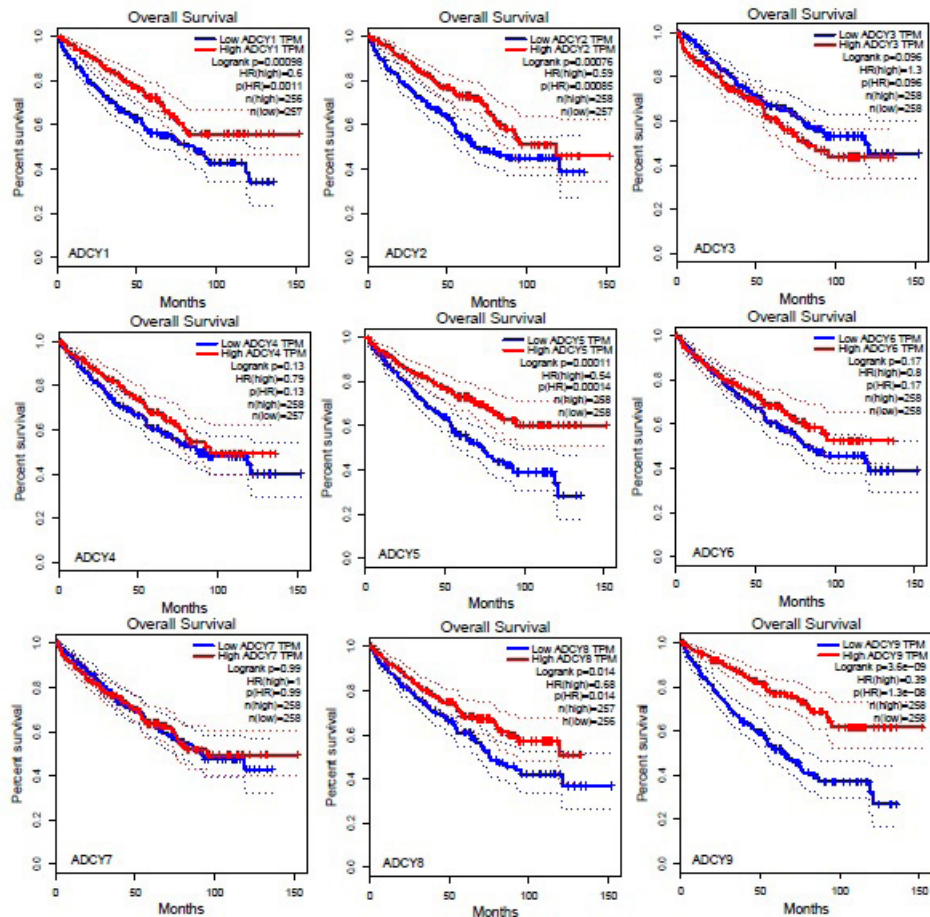

Figure 4: Prognostic value of ADCYs mRNA expression in KIRC patients (GEPIA).
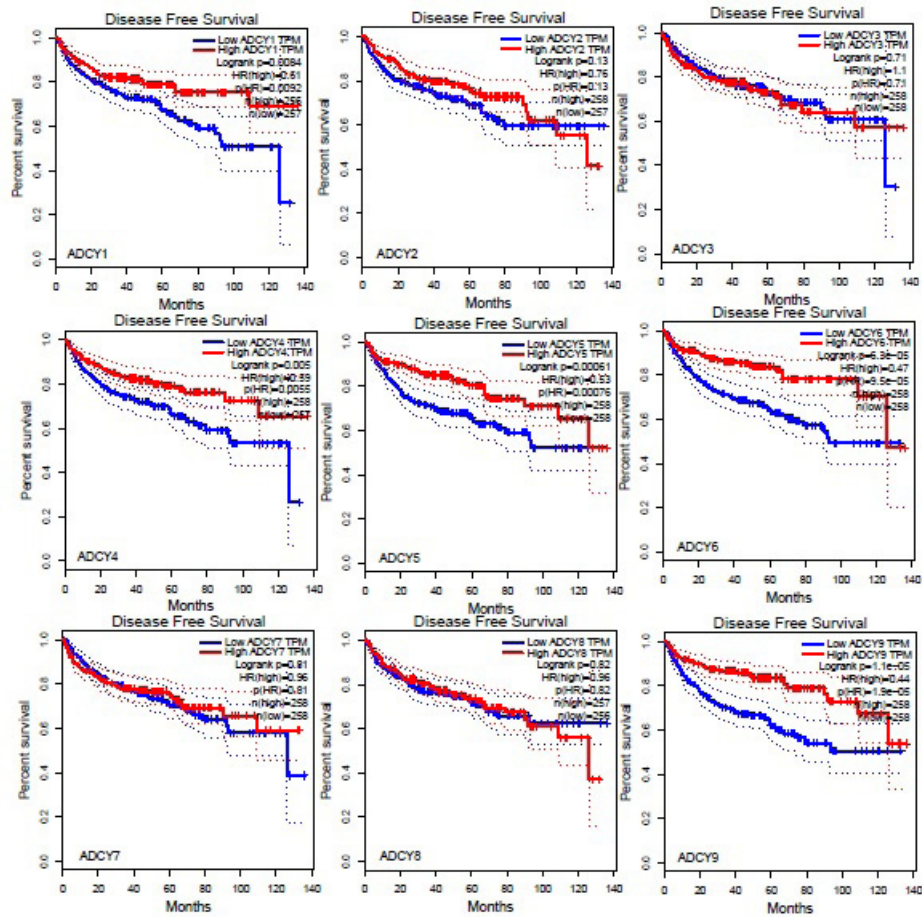
(**A**) Correlation between ADCYs expression and OS in KIRC patients.

Figure 4(B): Correlation between ADCYs expression and DFS in KIRC patients.
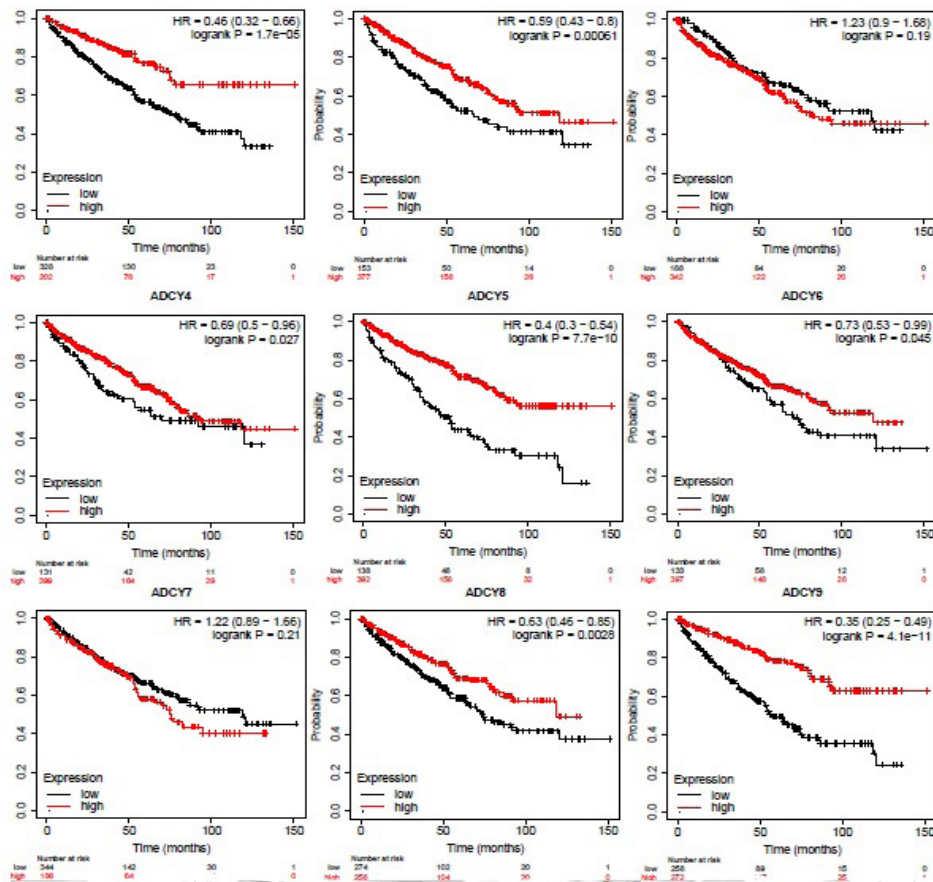
Figure 5: Prognostic value of ADCYs mRNA expression in KIRC patients (Kaplan-Meier plotter) (OS).

3.3. Analysis of gene Alteration, Expression, and Interaction of ADCYs in KIRC patients

We used the Cbioportal to analyze the molecular characteristics in ADCYs in patients with KIRC.

The data showed that ADCYS changed in 174(34%) of 510 KIRC patients. Among them, 7%, 6%, 8%, 8%, 7%, 5%, 8%, 2.9% and 8% mutations occurred in ADCY1-9, respectively (Figure 6A.6B). Among the most frequent mutations are low mRNA, high mRNA, mutation multiple changes, amplification and deep deletion (Figure 6B). Next, we used String to perform protein-protein interaction PPI network analysis on the differentially expressed ADCYs to find their possible interactions. We selected the 5 most closely related molecules, Prkacg, mtnr1b, grm3, gpr83 and gnas, with a total of 14 nodes and 86 edges (Figure 6C). Further functional exploration was conducted using Genemania, and the results showed that the differentially expressed functional ADCYs and its related molecules (such as GUCY1A2, GUCY1A3, GUCY1B3, GUCY2C, GUCY2F, NPR1, NPR2, GUCY2D, PLCB2, GNA11, RGS2, NPR3, NPPB, TRAPPC5, TRAPPC6B, GUCA2B, TRAPPC3L, NPPA, TRAPPC6A) are associated with phosphorus-oxygen lyase activity, cyclase activity, activation of protein kinase A activity, regulation of phospholipase activity, response to glucagon, cellular response to glucagon stimulus, activation of phospholipase C activity and so on(Figure 6D).
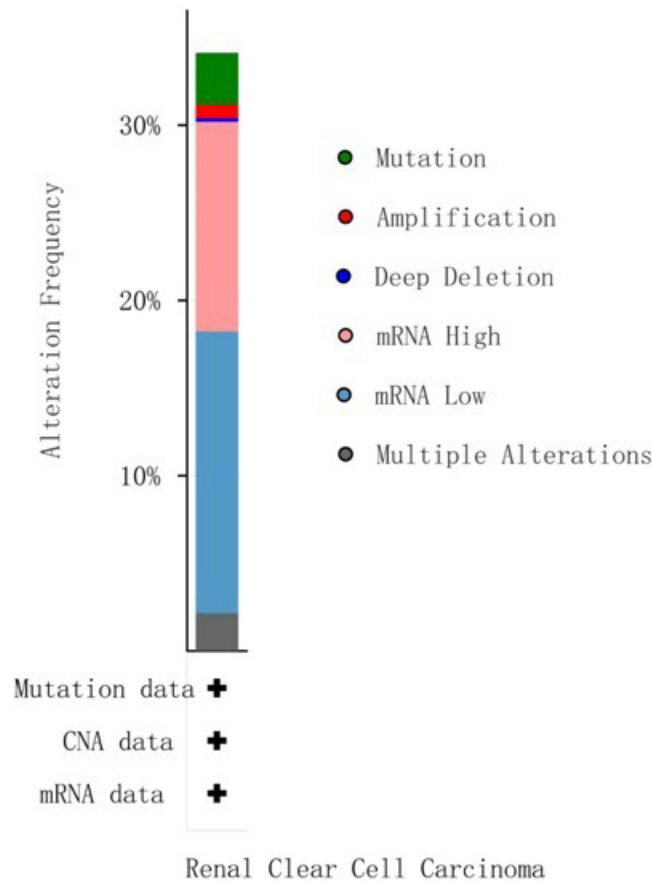

Figure 6: Mutation and expression analysis of ADCYs gene in KIRC (cBioPortal and STRING) (A, B). Protein-protein interaction networks with different expressions of ADCYs (C, D).
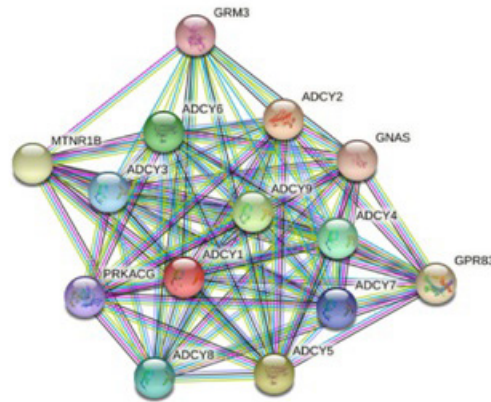

Figure 6B: Mutation and expression analysis of ADCYs gene in KIRC (cBioPortal and STRING) (A, B). Protein-protein interaction networks with different expressions of ADCYs (C, D).

Figure 6C: Mutation and expression analysis of ADCYs gene in KIRC (cBioPortal and STRING) (A, B). Protein-protein interaction networks with different expressions of ADCYs (C, D).
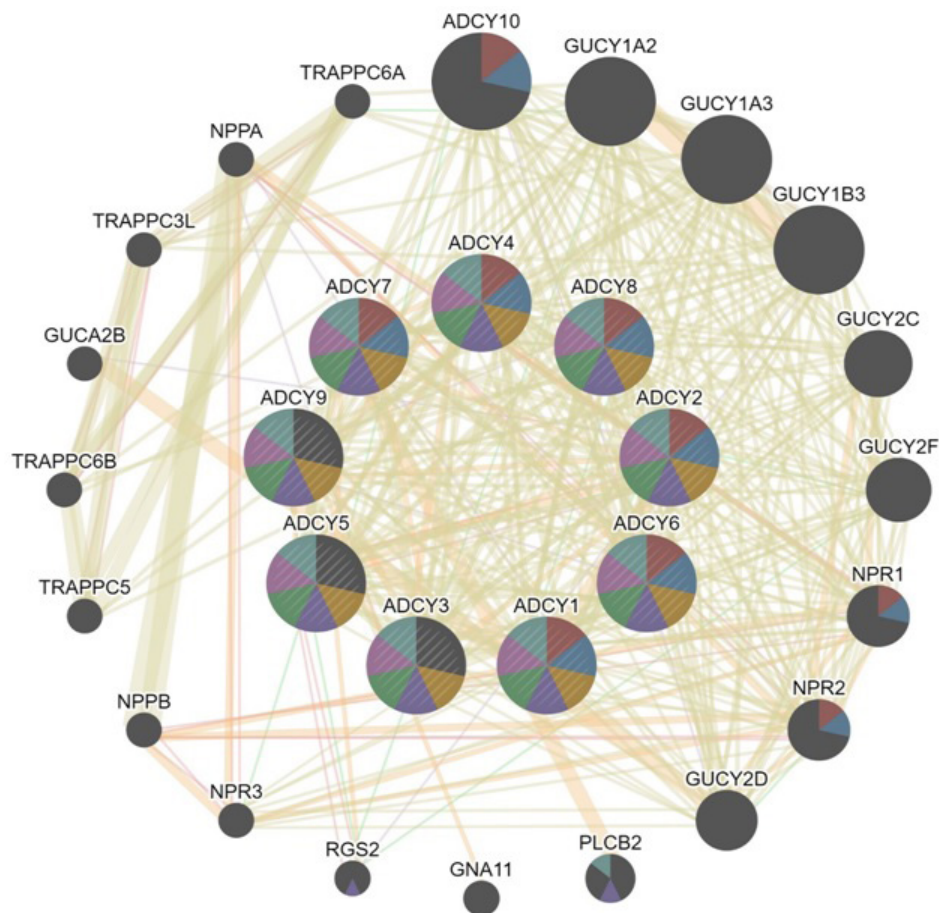

Figure 6D: Mutation and expression analysis of ADCYs gene in KIRC (cBioPortal and STRING) (A, B). Protein-protein interaction networks with different expressions of ADCYs (C, D).

3.4. Correlation Between Differential Expression of Adcys and Immune Cell Infiltration in Tissues of Patients with KIRC

A growing number of studies have found that immune cell infiltration is an important factor affecting the prognosis of cancer patients, including patients with KIRC[23]. Therefore, we used the TIME database of 6 kinds of immune cell infiltration, including B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells, to analyze the correlation of immune infiltration of ADCYs in KIRC (Figure 7). The analysis showed that the expression of ADCY1 was significantly correlated with the infiltration of 6 kinds of immune cells. ADCY5/6/9 were significantly correlat-

ed with B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils and dendritic cells. ADCY2 was significantly correlated with macrophages, neutrophils, and dendritic cells. ADCY3 was significantly correlated with CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells. ADCY4 was significantly correlated with B cells, CD8+ T cells, CD4+ T cells, macrophages and neutrophils. ADCY6 was significantly correlated with CD4+ T cells, macrophages and neutrophils. ADCY8 was significantly correlated with CD4+ T cells. Among them, except the expression of ADCY1 and 4 were negatively correlated with the infiltration of B cells, the others were positively correlated.
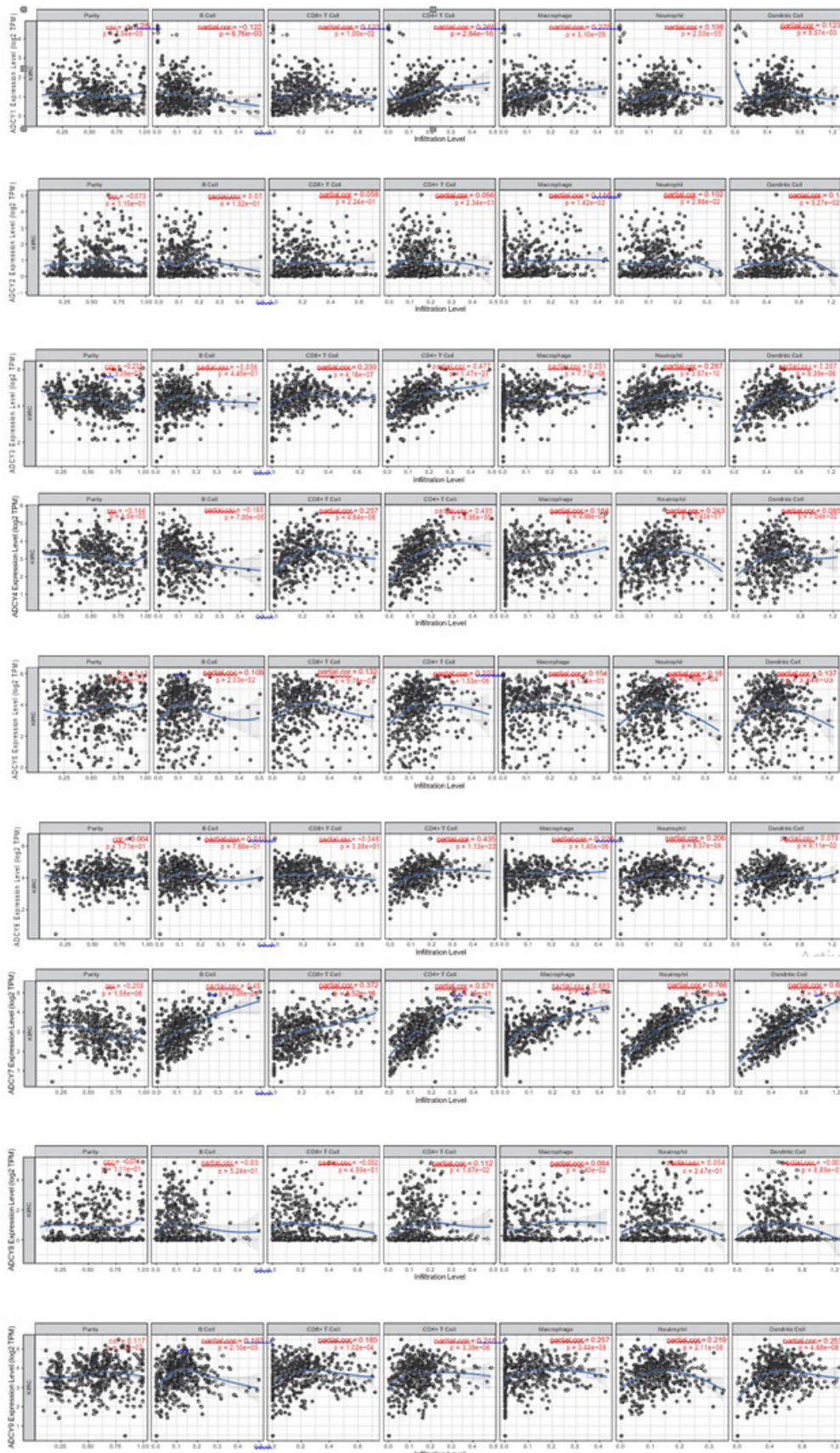
Figure 7: Correlations between ADCYs expression and immune cell infiltration (TIMER).

4. Materials and Methods

4.1. Oncomine

ADCY gene expression data were obtained from Oncomine website (www.oncomine.org). Student's t test was used to compare the expression of ADCY in clinical tumor samples and matched normal control samples. Check the following options "Analysis Type: Cancer vs. Normal Analysis" "Cancer Type: Clear Cell Renal Cell Carcinoma" and "Data Type: mRNA". The threshold defaults to P-VALUE 0.05, FOLD CHANGE 1.5, GENE RANK top 10%.

4.2. Gene Expression Profiling Interactive Analysis (GEPIA)

GEPIA used standard processing pipelines to analyze RNA sequencing expression data from the TCGA and GTEX projects [24]. In this study, GEPIA was used to analyze the correlation between the differential expression of ADCYs in KIRC tumor and normal tissues, pathological staging analysis and related prognosis analysis. Student's T-test was used for expression and pathological staging analysis. Survival analysis was performed using Kaplan-Meier curves.

4.3. Kaplan-Meier Plotter

Application of Kaplan Meier - plotter (http://kmplot.com/analysis/) analysis different ADCYs mRNA expression to the value of the prognosis of patients with KIRC [25]. The difference was statistically significant when P < 0.05.

4.4. CBioPortal

cBioPortal (http://cbioportal.org) is a web site that can visualize multidimensional cancer genomic data for clinical data analysis [26]. ADCYs expression alterations in KIRC patients based on TCGA database was analyzed by cBioPortal.

4.5. String

String (https://string-db.org/) is a website that analyzes the interactions between proteins and proteins in order to fully understand cellular mechanisms [27]. Through PPI network analysis, the potential interactions of ADCYs and other genes can be collected and integrated in String.

4.6. GeneMANIA

Genemania (http://www.genemania.org) is a Web interface for generating assumptions about gene function, analyzing gene lists, and sequencing genes for functional analysis [28]. We use it to predict the potential predictive value of ADCYs.

4.7. Timer

TIMER (https://cistrome.shinyapps.io/timer/) is a web server that provides a comprehensive analysis of 6 types of immune cell infiltrates including B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells [29]. We used TIMER to visualize the relationship between the expression of ADCYs and the level of KIRC immune infiltration.

5. Discussion

The role of ADCY family members in several cancers has been demonstrated [12,15-22], but its unique role in KIRC remains to be further elucidated. This study comprehensively analyzed the ADCYs in KIRC from the aspects of expression level, pathological grade, survival prognostic value, gene mutation and immune cell infiltration.

Analysis of the above results found that both the TCGA database and the Genotype-Tissue Expression (GTEx) databases showed that ADCY2/3/4/5/7/8/9 are highly expressed in tumor tissues, while ADCY1/6 are low in tumor tissues. In addition, the analysis suggested that, except for ADCY7, other ADCY factors were significantly correlated with the pathological stage of KIRC, and the results were statistically significant. They may play an important role in the occurrence and development of KIRC patients. OS survival analysis showed that the high expression of ADCY1/2/5/8/9 had a better prognosis. The prognostic analysis of DFS showed that the high expression of ADCY1/4/5/6/9 in tumor tissues had a better prognosis. K-M survival analysis also showed that ADCY1/2/4/5/6/8/9 high expression had better OS. Comparing the results, it is found that the highly expressed of ADCY1, 5 and 9 have better OS and DFS at the same time. Combined with the above expression level analysis, ADCY1 is low in KIRC, and the prognosis of OS and DFS is poor. Analysis from a genetic perspective shows that the differentially expressed ADCYs in KIRC often undergo genetic changes, especially low mRNA expression. These genes are considered to have the potential to activate protein kinase activity, regulate phospholipase activity and activate phosphor-oxygen lyase activity, which play important roles in regulating the proliferation, invasion, survival prognosis and even immune disorders of various cancer cells [30-33]. The role of tumor microenvironment(TME) in promoting tumor occurrence and development is increasingly emphasized [34,35]. CD4+ T cells and CD8 + T cells can enhance the anti-tumor activity of CTLs, enhance the penetration of CTLs into the core of tumors, and participate in promoting the killing of cancer cells [36-38]. Immunotherapy also plays an important role in the current and future treatment of kidney cancer [39]. Consistently, our analysis results show that the expression of ADCYs may be significantly related to the infiltration of the above 6 immune cells, reflecting the immune status of KIRC patients, and providing new potential directions for clinical treatment. As ACYD1,5,9, which are significantly related to prognosis in both OS and DFS survival analyses, deserve attention. Adcy1 is an important regulator that catalyses the production of cyclic adenosine 3', 5' -monophosphate (cAMP) from ATP, and then participates in cell growth, differentiation, proliferation, apoptosis , metabolism and other cellular reactions [40,41]. Studies have reported that ADCY1 is associated with the survival prognosis of melanoma, non-small cell lung cancer, and glioma [15-17]. Yang Lx et al. pointed out that the high expression of ADCY1

may be related to chemotherapy resistance of esophageal cancer cells [18,42]. ADCY5 is a hub gene in Colorectal cancer and may be involved in the development of triple-negative breast cancer through hormone-related pathways [43,44]. ADCY9 was differentially expressed in endometrial cancer [45]. Meanwhile, Hua Yi et al. found that ADCY9 is highly expressed in colon cancer tumor tissues and is related to the TNM staging [46,47]. The present study also showed that ADCY1/5/9 was significantly associated with prognosis, pathological grade, and immune infiltration in patients with KIRC. Low expression of ADCY1/5/9 may be a poor prognostic factor for KIRC survival. In the meantime, ADCY2 and 8 showed significant correlations with OS in both GEPIA and K-M Plotter databases, ADCY4 and 6 were significantly correlated with DFS in GEPIA, which are also noteworthy. ADCY2 was differential expression in oral cancer, prostate cancer and metastatic colon cancer [48-50]. ADCY8 mutations was associated with a significant decrease in tumor PD-L1 expression and may affect early lung squamous cell carcinoma clinical outcomes and personalized targeted immunotherapy strategies [51]. Nicole M Warrington et al. believe that ADCY8 has genetic polymorphism, which is related to the risk of NF1 glioma in a gender-specific manner, which increases the risk for women and reduces the risk for men [52]. It has also been reported that ADCY8 is differentially expressed in endometrial cancer and is involved in the occurrence and development of HCC [45,53]. ADCY4 is differentially expressed in breast cancer, lung squamous cell carcinoma, lung adenocarcinoma and hepatocellular carcinoma [54-57]. Weijing Li et al. found that ADCY6 can be used as a prognostic factor related to the immune process regulated by DNA methylation in luminal-like breast cancer, and patients with ADCY6 down-regulation and hypermethylation have a better prognosis [58]. At the same time, ADCY6 is also differentially expressed in lung cancer and laryngeal cancer [16,59]. ADCY2/8/4/6 also have the potential to be a reliable prognostic factor in KIRC.

In this study, ADCY3 and 7 were not significantly associated with the prognosis of KIRC, but were differentially expressed in KIRC and correlated with pathological grade and multiple immune cell infiltrates. Which may be involved in the progression of KIRC. Several studies showed that ADCY3 overexpression may play a tumor-promoting effect through the cAMP/PKA/CREB pathway. By increasing the levels of cAMP and phosphorylated cAMP response element binding protein (CREB), it increases the mRNA levels and activities of matrix metalloproteinase 2 (MMP2) and MMP9 [60-62]. Chunling Li et al. demonstrated that the expression of ADCY7 promotes the development of leukemia by reducing the apoptosis of AML cells, which is inversely correlated with the overall survival of AML patients [19]. ADCY7 can be mediated by miR-192 to affect intracellular cAMP levels, and affect the differentiation of APL cells induced by ATRA, further affecting its recurrence [63]. The differential expression of ADCY3 and 7

in pathological grade and immune infiltration may also be related to the occurrence and development of KIRC, which needs further exploration and discussion. Our research still has some limitations. The data analyzed in this research are obtained from online databases. Extensive cell biology experiments and clinical studies are still needed to validate our findings and explore the potential mechanisms and roles of different ADCYs in KIRC to guide clinical practice.

6. Funding

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