Heliyon 8 (2022) e12191

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

APOBEC3G expression correlates with unfavorable prognosis and immune infiltration in kidney renal clear cell carcinoma

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ARTICLE INFO

Keywords:
APOBEC3G
KIRC
Prognosis
Immune infiltration
PD-L1

ABSTRACT

Background: Kidney renal clear cell carcinoma (KIRC) is the most common pathological subtype of renal cell cancer. APOBEC3 activity has been identified in a variety of human cancers. Although its involvement in cancer has been studied widely, its influence on the tumor immune microenvironment remains poorly understood. Therefore, this study aimed to focus on the effect of APOBEC3 on tumor immune microenvironment of KIRC.

Methods: In this study, we comprehensively analyzed the expression and prognostic significance of the APOBEC3 family in pan-cancer using multiple databases. The functions of key APOBEC3 family members were further investigated in KIRC, with APOBEC3G determined to be a candidate biomarker for unfavorable prognosis. We subsequently explored the correlation of APOBEC3G with the tumor immune environment in KIRC by analyzing the Cancer Genome Atlas (TCGA) dataset, then validated the prognostic significance and PD-L1 correlation of APOBEC3G by using tissue microarrays which included 233 primary tumor samples from patients with renal clear cell carcinoma.

Results: The APOBEC3 family was overexpressed in KIRC and high APOBEC3 expression predicted poor prognosis. In addition, APOBEC3G was positively correlated with the expression of immunoinhibitors such as TIGIT, LAG3, CD96, PD-1, and CTLA4. In addition, APOBEC3G had a positive correlation with immunosuppressive cells, including regulatory T cell and myeloid-derived suppressor cell. Finally, based on 233 clinical samples, we validated that high expression of APOBEC3G contributed to a poor prognosis for KIRC patients and the positive relationship between APOBEC3G and PD-L1 expression. High APOBEC3G expression was also found to be more common in patients with sarcomatoid histology ($P = 0.0026$).

Conclusions: Our study showed that APOBEC3G was a prognostic biomarker correlated with the immune response in KIRC. In addition, APOBE3G had a positive correlation with PD-L1 expression and sarcomatoid histology, perhaps suggesting the potential impact of APOBEC3G on immunotherapy.

1. Introduction

Kidney renal clear cell carcinoma (KIRC) is the most common pathological subtype of renal cell cancer [1]. Since 2015, renal cell cancer treatment has shifted from targeted therapy to immunotherapy [2]. As newly emerging cancer therapeutics, immune checkpoint inhibitors (ICIs), such as cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) and programmed cell death protein 1 and its ligands (PD-1/PD-L1), show greater potential in advanced and metastatic KIRC treatment than targeted therapy such as the vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibitor axitinib. Compared with sunitinib, combined therapies (e.g., PD-1/PD-L1 inhibitors with anti-VEGF agents

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https://doi.org/10.1016/j.heliyon.2022.e12191
Received 6 August 2022; Received in revised form 17 October 2022; Accepted 30 November 2022

or anti-CTLA-4 antibodies) significantly increase the survival of patients with renal cell carcinoma [3, 4, 5, 6]. Nevertheless, only a small proportion of patients benefit from ICIs therapy and progress often leads to treatment failure, emphasizing the urgent need to identify useful markers or methods that can predict patients respond to this novel treatment strategy for optimal therapeutic outcome [7]. At the same time, identifying the complex mechanisms underpinning immunotherapy resistance is essential for improving patient treatment outcome.

APOBEC3 belongs to the large apolipoprotein B mRNA editing enzyme catalytic polypeptide-like (APOBEC) family, and is known for its unique function in antiviral immunity [8]. While a single APOBEC3 gene is present in rodents, seven APOBEC3 genes are encoded on chromosome 22 in humans (i.e., APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H) [9]. APOBEC3 family members (A3s) contain single-stranded DNA cytosine-to-uracil deaminase enzymes that cause strand breaks and base substitutions, and are usually stimulated by cytokines released during inflammation to defend against various viral infections [9, 10]. In addition to the antiviral effect, the activities of A3s have been clarified in the human genome and cancer in recent years [11]. For example, APOBEC3 activity is implicated in genome mutagenesis in human cancer [12, 13, 14]. Furthermore, dysregulation of A3s is thought to play an important role in tumorigenesis, malignant progression, and drug resistance of malignant cancers [15, 16, 17, 18]. APOBEC3G promotes immune cell infiltration and its high expression is predictive of a more favorable prognosis in cancer patients [14]. APOBEC3B is also predictive of immunotherapy response in non-small cell lung cancer based on its correlation with immune gene expression [19]. However, our understanding of the expression, prognostic significance, and underlying mechanism of the APOBEC3 family in KIRC remains poor.

In this study, we explored the expression and prognostic value of the APOBEC3 family in various cancers using the Oncomine and GSCALite databases. We examined A3s in KIRC using the GEPIA2 and Kaplan-Meier plotter databases and identified genomic alterations, methylation, and co-expressed gene sets of APOBEC3 in KIRC using cBioPortal and Gene Set Cancer Analysis (GSCA). Functional enrichment analysis of APOBEC3 was performed using Metascape. The TIMER and TISIDB databases were used to investigate the correlations between APOBEC3 expression and immune cell infiltration, as well as the correlations between immunoinhibitors and APOBEC3 expression in KIRC. Among all APOBEC proteins, APOBEC3G has arguably the strongest antiviral effect and most of the published work concerning the antiviral activities of APOBEC proteins involves APOBEC3G [20]. A positive correlation of APOBEC3G expression with several T cell genes in ovarian cancer was confirmed later [14]. More importantly, APOBEC3G showed the highest correlation with immune infiltration and immunoinhibitors in our study, suggesting the importance of this family member. To further identify the pivotal role of APOBEC3G in the tumor immune microenvironment (TIME), the association between APOBEC3G expression and immunosuppression in KIRC was investigated using the ESTIMATE and ssGSEA algorithms. Finally, tissue microarrays (TMAs) of 233 primary tumor samples from KIRC patients were used to validate the prognostic value of APOBEC3G, and the correlation between PD-L1 and APOBEC3G expression was confirmed in this cohort.

2. Materials and methods

2.1. Analysis of differential expression and prognostic significance of APOBEC3 family members in pan-cancer

Differential expression of APOBEC3 in different types of cancers were obtained from the Oncomine database (https://www.oncomine.org), which is a comprehensive database of gene expression studies [21]. GSCALite (http://bioinfo.life.hust.edu.cn/web/GSCALite/), a web-based platform integrated TCGA cancer genomics data for gene set cancer analysis, was used to further analysis of differential expression and prognostic value of APOBEC3 family members across 33 cancer types [22].

2.2. Analysis of the correlation between APOBEC3 mRNA levels and the clinicopathological parameters of patients with KIRC

GEPIA2 (http://gepia2.cancer-pku.cn/#index), a updated web server for researchers to perform not only tumor/normal differential expression but also more interactive analysis based on data from the TCGA and GTEx projects using a standard processing pipeline [23]. In this study, we used GEPIA2 to validate the transcriptional level of APOBEC3 genes in kidney renal clear cell carcinoma compared to normal renal tissue. In addition, APOBEC3 family expression characteristics between different clinical staging of KIRC were also explored by GEPIA2.

2.3. Survival analysis

Kaplan-Meier plotter database (www.kmplot.com) is a database with gene expression data and clinical survival data of various kinds of cancers, and TISIDB (http://cis.hku.hk/TISIDB/index.php) is an integrated portal with genomics, transcriptomics and clinical data of cancers [24, 25], both of which were used to explore the correlations between APOBEC3 family members and overall survival (OS) in patients with KIRC.

2.4. Analysis of gene alterations of APOBEC3 family in KIRC

c-BioPortal (https://www.cbioportal.org/), an open-access platform providing multi-dimensional exploration of cancer genomic data from more than 5, 000 tumor samples [26], was used to acquire information about genetic mutation of APOBEC3 family in KIRC. GSCA (http://bioinfo.life.hust.edu.cn/GSCA/#/) is an update version of GSCALite, which integrates genomic data across 33 cancer types from TCGA for gene set cancer analysis [22]. It was used to investigate the methylation state of APOBEC3 family and the correlation between APOBEC3 methylation and mRNA expression.

2.5. Function analysis

We used cBioPortal to identify the correlation of APOBEC3 with each other through calculating their mRNA expressions, and the top 100 co-expressed genes of each APOBEC3 family member in kidney renal clear cell carcinoma (TCGA, Firehose Legacy) subdataset which included 446 samples [27]. A venn diagram was then drawn for showing the overlapped co-expressive genes among APOBEC3 family members. To understand the functions of APOBEC3 and the genes significantly related to APOBEC3 alterations, an online web tool named Metascape (http://metascape.org) was used for functional enrichment analysis of APOBEC3 by importing their co-expressed gene lists [28].

2.6. The correlation between APOBEC3 family and immune cell infiltration analysis

TIMER (http://timer.cistrome.org), an available online portal allowing comprehensive analysis tumor-infiltrating immune cells across various cancer types, was used to reveal the correlations between immune infiltration level and APOBEC3 family expression [29].

2.7. The correlation between APOBEC3 family and immunoinhibitor analysis

TISIDB (http://cis.hku.hk/TISIDB/), an integrated repository portal for tumor-immune system interactions [25], was used to clarification of correlations between APOBEC3 family and immunoinhibitors expression.

2.8. Analysis of the correlation between APOBEC3G and TIME

LinkedOmics (http://www.linkedomics.org/login.php) is a public portal analysis tool comprising multi-omics data from 32 TCGA cancer types [30], which was used to examine both positively and negatively correlated gene sets of APOBEC3G in KIRC. To further characterize the potential role of APOBEC3G in tumor immunity, we downloaded 539 RNA-seq FPKM (Fragments per kilobase per million) data from TCGA-KIRC project to analyze the relationship between APOBEC3G expression and TIME by using ESTIMATE algorithm [31], and the correlation between 24 immune cell infiltrates and APOBEC3G expression was examined by ssGSEA algorithm [32, 33]. Subsequently, these TCGA samples were grouped by the median values of APOBEC3G to compare the level of different immune cells infiltration. Finally, the correlation between APOBEC3G expression and MDSC (myeloid-derived suppressor cell) abundance was explored by TISIDB.

2.9. Institutional dataset

This study used two kinds of renal cancer tumour microarray slides (HKidE180Su02; HKidE180Su03) which included 233 primary tumor samples from patients with renal clear cell carcinoma. Immunohistochemistry for APOBEC3G and PD-L1 were performed on these tissue microarrays (TMAs). The slide was placed in a dry oven (PH-070A, Shanghai, China) at 63 °C for 1 h to deparaffinize. Then rehydrated (LEICAST5020, LEICA) tissue sections were incubated in heat-mediated antigen retrieval by immersing them into staining dish with boiled citric acid solution (pH = 6.0) the formulation of which included 0.1 mol/L sodium citrate solution (82 mL), 0.1 mol/L citric acid solution (18 mL), deionized water (900 mL). After cooling to room temperature by placing them in distilled water for 30 min, we bathed these slides in Peroxidase-Blocking Reagent for 15 min at room temperature to finish blocking. Afterwards, to cover the slides with primary antibody (APOBEC3G, HPA001812, 1:500, Atlas Antibodies; PD-L1, GT2280, ready-to-use, Gene Tech) diluting with antibody diluents (Antibody Diluent with Background Reducing Components, S3022, DAKO) and incubate overnight at 4 °C in a humidified chamber followed by equilibration to room temperature for 45 min. Next, the slides were put into the DAKO automatic immunohistochemical staining system instrument to run the procedures of blocking, secondary antibody combination (Envision+/HRP, Rabbit, DAKO) and DAB staining (Automatic immunohistochemical staining system instrument, Autostainer Link 48, DAKO). Tissue sections were then bathed for 40 s in hematoxylin solution (Mayer's hematoxylin, GT100540, Gene), 2 s in 0.25% acid alcohol (1 mL hydrochloric acid in 400 mL of 70% ethanol) to counterstain nuclei. Between steps, we washed the slides three times, 1 min each, with PBS (pH = 7.2). After the experiment, the staining intensity and staining range of each site were evaluated by two observers. The standard for the staining intensity was as follows: no staining (negative), weak staining (1/+), moderate staining (2/++), strong staining (3/+++). The staining area score is defined as 0 (no staining area), 1 (lower 30%), 2 (30%–70%), 3 (higher 70%). APOBEC3G protein expression of each patient was quantified a result of staining intensity score multiplied by staining area score.

3. Results

3.1. Expression and prognosis of APOBEC3 family in pan-cancer

Seven APOBEC3 family members have been identified in human. Their transcriptional levels in both cancers and normal samples were investigated in 20 types of cancers by using Oncomine database. In total, 346 studies involving APOBEC3A, 384 studies involving APOBEC3B, 397 studies involving APOBEC3C, 252 studies involving APOBEC3D, 387 studies involving APOBEC3F, 427 studies involving APOBEC3G, and 141 studies involving APOBEC3H were identified. The number for the significant unique analyses indicates that the corresponding gene is a significantly different gene in studies, whose expression levels meeting our selection threshold ($P < 0.05$, expression fold-change >1.5, expressed gene rank in top 10%) (Figure 1A). Red background with numbers indicates gene up-regulated and blue background shows the down-regulated case. The counting result was APOBEC3A (5:11), APOBEC3B (38:10), APOBEC3C (4:6), APOBEC3D (3:3), APOBEC3F (7:2),

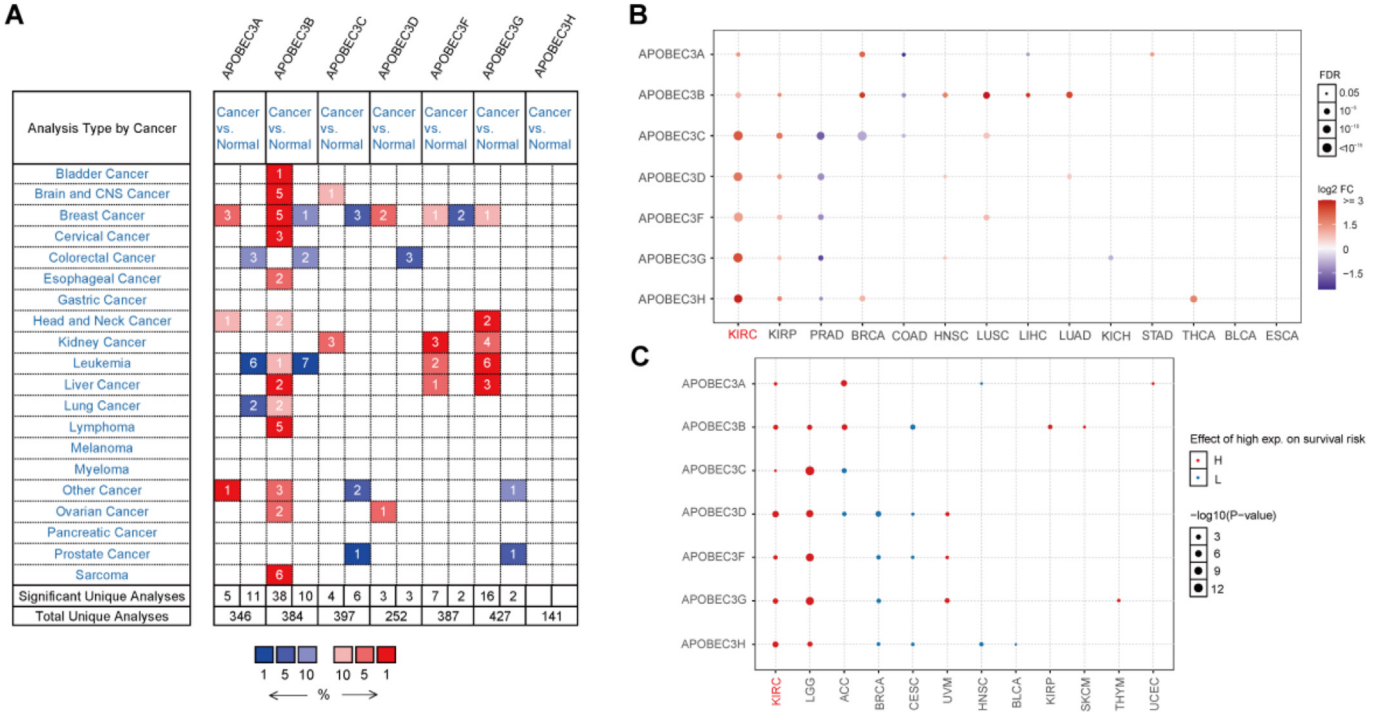

Figure 1. Expression and prognostic value of APOBEC3 in pan-cancer. A Differential expression of APOBEC3 family in 22 different cancer types (Oncomine). B APOBEC3 expression levels in TCGA cancers by GSCALite. C Prognostic significance of APOBEC3 family in TCGA cancers by GSCALite.

APOBEC3G (16:2) (Figure 1A). To further investigate the potential value of APOBEC3 family members in cancer research, we compared their expression levels in the Cancer Genome Atlas (TCGA) dataset and explored the effect of high APOBEC3 expression on survival risk in multiple tumors using GSCALite. Results showed that the prognostic value of the seven APOBEC3 family members varied by cancer type. However, the differential expression and prognostic value of the APOBEC3 family was most significant in KIRC, which warrants further study (Figure 1B&1C).

3.2. Differential expression and prognostic significance of APOBEC3 in KIRC

To clarify the differential expression of the APOBEC3 family in KIRC, we compared the expression levels of the seven APOBEC3 family members in tumor and normal renal tissue using GEPIA2. Results showed that the mRNA levels of A3s were higher in KIRC tumor tissue than in normal tissue, and the differences in APOBEC3C, APOBEC3D, APOBEC3G, and APOBEC3H reached statistical significance (Figure 2A). Regarding the

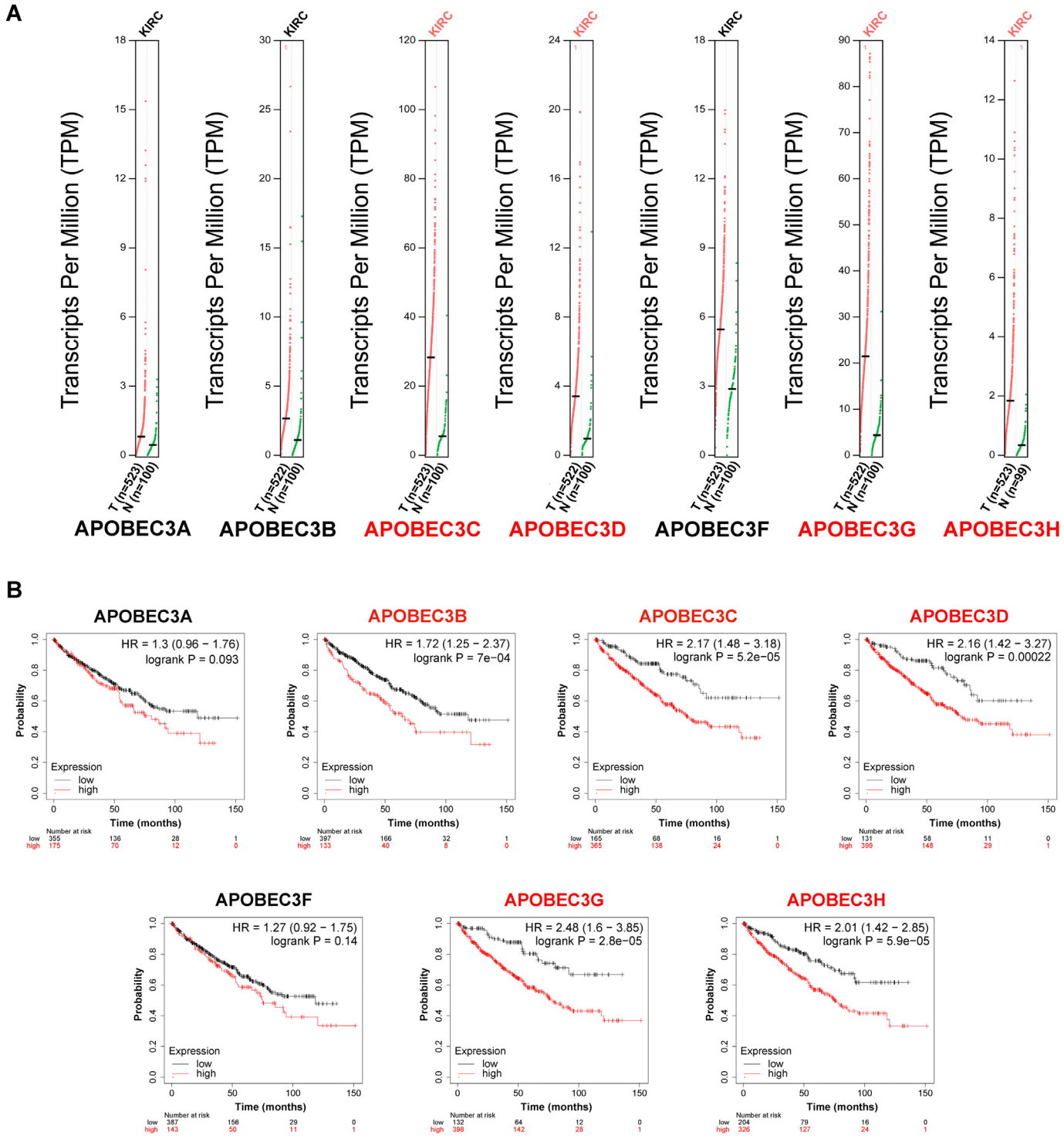

Figure 2. Overexpressed APOBEC3 family members exhibited unfavorable prognosis in KIRC. A APOBEC3 expression in KIRC and normal tissue (GEPIA2). B Kaplan-Meier plots of OS according to expression of APOBEC3 family in patients with KIRC (Kaplan-Meier plotter).

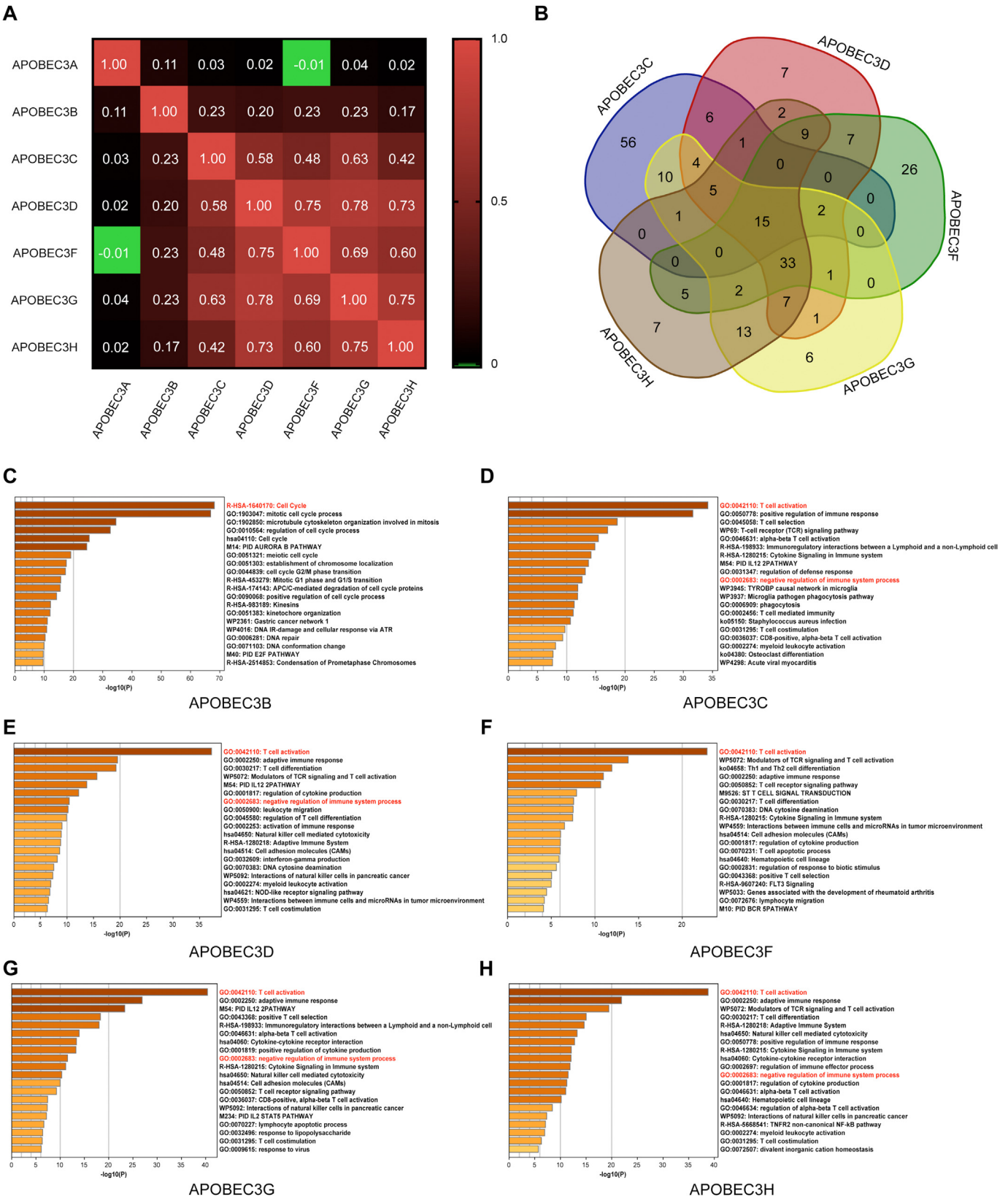

Figure 3. Co-expressed gene set and enrichment analysis of APOBEC3 in KIRC. A Person's correction between different APOBEC3 in KIRC. B Venn diagram showing the distribution of co-expression gene data sets. C Bar graph showing results of functional enrichment analysis of APOBEC3B (Metascape). D Bar graph showing results of functional enrichment analysis of APOBEC3C (Metascape). E Bar graph showing results of functional enrichment analysis of APOBEC3D (Metascape). F Bar graph showing results of functional enrichment analysis of APOBEC3F (Metascape). G Bar graph showing results of functional enrichment analysis of APOBEC3G (Metascape). H Bar graph showing results of functional enrichment analysis of APOBEC3H (Metascape).

association between A3s expression and clinical stage of KIRC, the APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3G, and APOBEC3H groups differed significantly, whereas the APOBEC3A and APOBEC3F groups did not (Supplementary material: Figure S1). The critical efficiency of A3s in KIRC patient survival was confirmed using Kaplan-Meier plotter (Figure 2B) and TISIDB (Supplementary material: Figure S2). Overall survival (OS) analysis indicated that high expression levels of APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3G, and APOBEC3H were predictive of poor prognosis in KIRC.

3.3. Genetic variation of APOBEC3 and correlation between APOBEC3 methylation and mRNA expression in KIRC

Genetic alteration is an important factor in the regulation of gene expression and is closely related to gene function and cancer progression [34]. In this study, we explored the frequency and type of APOBEC3 alterations in 446 renal clear cell carcinoma samples by cBioPortal. The APOBEC3 family members were highly conserved and rarely mutated in the samples (Supplementary material: Figure S3A). Based on GSCA, we further determined APOBEC3 family methylation and its correlation with gene expression in KIRC. Results showed that the methylation levels of APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3F, and APOBEC3H were significantly lower in tumor tissue than in normal tissue (Supplementary material: Figure S3B), and their methylation levels were negatively associated with mRNA expression (Supplementary material: Figure S3C). These findings suggest that increased APOBEC3 expression in KIRC may be attributable to reduced methylation levels. Although the specific molecular mechanisms underlying the upregulation of APOBEC3G remain unclear, it has already been shown that APOBEC3G can be upregulated by T cell activation [14].

3.4. Functional analysis of APOBEC3 in KIRC

We analyzed mRNA expression correlation within the APOBEC3 gene family via the cBioPortal online tool for KIRC (TCGA, Firehose Legacy). Results showed strong positive correlations between the APOBEC3C,

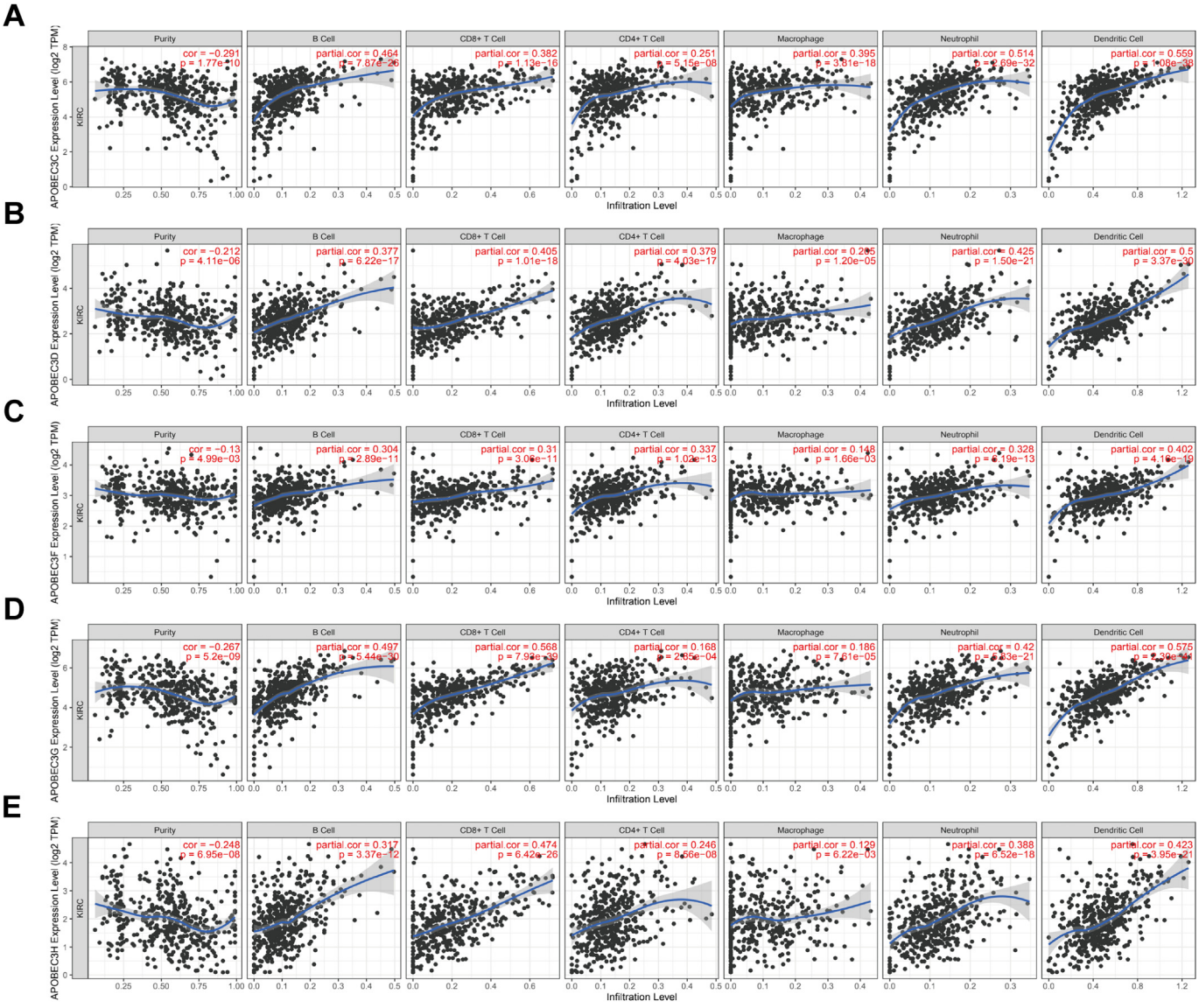

Figure 4. The relationship between APOBEC3 expression and immune cell infiltration (TIMER). A Correlation of APOBEC3C expression with B cell, CD8+ T cell, CD4+ T cell, macrophage, neutrophil, dendritic cell infiltration. B Correlation of APOBEC3D expression with B cell, CD8+ T cell, CD4+ T cell, macrophage, neutrophil, dendritic cell infiltration. C Correlation of APOBEC3F expression with B cell, CD8+ T cell, CD4+ T cell, macrophage, neutrophil, dendritic cell infiltration. D Correlation of APOBEC3G expression with B cell, CD8+ T cell, CD4+ T cell, macrophage, neutrophil, dendritic cell infiltration. E Correlation of APOBEC3H expression with B cell, CD8+ T cell, CD4+ T cell, macrophage, neutrophil, dendritic cell infiltration.

APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H genes (Figure 3A). The Venn diagram in Figure 3B shows the intersection of these five co-expressed gene sets. To determine whether they were functionally related, we identified the top 100 co-expressed genes. As shown in Figure 3C, functional enrichment analysis indicated that APOBEC3C, APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H were involved in T cell activation and regulation of immune response (Figure 3D-H). Unexpectedly, APOBEC3C, APOBEC3D, APOBEC3G, and APOBEC3H were also related to the negative regulation of immune system process (GO:0002683) (Figure 3D, E, G and H). We performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, which indicated that APOBEC3B may affect prognosis in KIRC patients by participating in cell cycle regulation (Figure 3C).

3.5. Relationship between APOBEC3 expression and immune infiltration in KIRC

We explored the correlation between immune-related APOBEC3 family members (APOBEC3CD/F/G/H) expression and tumor-infiltrating lymphocyte abundance in KIRC using the TIMER algorithm. Results showed that all five members were positively correlated with the six immune cell types (i.e., B cell, CD8+ T cell, CD4+ T cell, macrophage, neutrophil, and dendritic cell), with APOBEC3F showing the lowest correlation with immune cell infiltration and APOBEC3G showing the highest correlation with B cell, CD8+ T cell, and dendritic cell infiltration (Figure 4A-E).

3.6. The relationship between immunoinhibitors and APOBEC3G expression in KIRC

Immune checkpoints are considered the "off switches" of immune cell function [35]. In-depth research on the regulation of human immune function and its relationship with APOBEC3 expression will help us gain a deeper understanding of the key mechanisms underlying APOBEC3C/D/F/G/H immune regulation. Therefore, we used the TISIDB database to assess the association between APOBEC3 expression and 22 immunoinhibitors collected from Charoentong's study [36]. Results showed that most immunoinhibitors were positively correlated with APOBEC3 expression, including TIGIT, LAG3, CD96, PD-1, and CTLA4. Remarkably, APOBEC3G was still the most significant member of APOBEC3 family (Table 1 & Figure 5A). Thus, we next explored the co-expression gene set of APOBEC3G in renal clear cell carcinoma using LinkedOmics (Figure 5B). In total, 20 159 genes were associated with APOBEC3G in KIRC, including 2 535 negatively correlated genes and 17 624 positively correlated genes (Spearman correlation). The top 50 positively correlated genes are shown in Figure 5C, which include TIGIT, LAG3, CD96, and PD-1 (Figure 5E-H). The top 50 negatively correlated genes in KIRC are shown in Figure 5D. Functional analysis of the above 100 genes showed that APOBEC3G was associated with regulation of T cell activation and cancer immunotherapy by PD-1 blockade (Figure 5I).

3.7. Correlation of APOBEC3G and TIME

APOBEC3G is not only an antiviral molecule but also displays strong activity in mediating tumor immunity [14, 37, 38]. Thus, to further clarify its role, we downloaded RNA-seq data from the TCGA-KIRC project to analyze the relationship between APOBEC3G and TIME. As shown in Figure 6A, APOBEC3G expression was strongly positively correlated with immune and estimate scores. Furthermore, ssGSEA was performed to assess the association of 24 immune cell subtypes with APOBEC3G expression (Figure 6B). Grouping analysis based on APOBEC3G expression showed that the infiltration level of regulatory T cell (Treg cell) was higher in the high expression group than in the low expression group (Figure 6C). Molecular markers (FOXP3 and CCR8) of Treg cell were also positively correlated with APOBEC3G expression and represented unfavorable survival in KIRC (Figure 6D and E). By analyzing the effect of APOBEC3G on prognosis under different immune cell infiltration conditions, we found that overexpression of APOBEC3G was associated with poor outcome in KIRC patients with enriched regulatory T-cells while such a correlation was not significant in patients with decreased regulatory T-cells (Figure 7). Thus, APOBEC3G may promote the infiltration of Treg cell in tumor environment, which generates tumor immune escape and poor survival outcome. APOBEC3G was also positively associated with infiltration of myeloid-derived suppressor cell

Table 1. Correlations between 22 immunoinhibitors and APOBEC3 expression (TISIDB).

immunoinhibitors	APOBEC3C		APOBEC3D		APOBEC3F		APOBEC3G		APOBEC3H	
	rho	p value	rho	p value	rho	p value	rho	p value	rho	p value
TIGIT	0.592	<2.2e-16	0.746	<2.2e-16	0.534	<2.2e-16	0.789	<2.2e-16	0.728	<2.2e-16
LAG3	0.503	<2.2e-16	0.753	<2.2e-16	0.594	<2.2e-16	0.769	<2.2e-16	0.755	<2.2e-16
CD96	0.616	<2.2e-16	0.72	<2.2e-16	0.492	<2.2e-16	0.76	<2.2e-16	0.719	<2.2e-16
PD-1	0.509	<2.2e-16	0.747	<2.2e-16	0.564	<2.2e-16	0.75	<2.2e-16	0.752	<2.2e-16
CTLA4	0.456	<2.2e-16	0.664	<2.2e-16	0.508	<2.2e-16	0.658	<2.2e-16	0.666	<2.2e-16
CD244	0.437	<2.2e-16	0.606	<2.2e-16	0.397	<2.2e-16	0.643	<2.2e-16	0.63	<2.2e-16
BTLA	0.56	<2.2e-16	0.618	<2.2e-16	0.401	<2.2e-16	0.639	<2.2e-16	0.583	<2.2e-16
LGALS9	0.472	<2.2e-16	0.493	<2.2e-16	0.334	2.72E-15	0.523	<2.2e-16	0.557	<2.2e-16
PDCD1LG2	0.529	<2.2e-16	0.408	<2.2e-16	0.232	6.64E-08	0.476	<2.2e-16	0.341	6.08E-16
IL10	0.465	<2.2e-16	0.347	1.77E-16	0.18	2.87E-05	0.427	<2.2e-16	0.35	8.49E-17
IL10RB	0.29	1.06E-11	0.23	8.41E-08	0.251	4.92E-09	0.415	<2.2e-16	0.349	1.07E-16
CSF1R	0.534	<2.2e-16	0.377	<2.2e-16	0.207	1.43E-06	0.379	<2.2e-16	0.332	4.26E-15
IDO1	0.218	3.92E-07	0.219	3.33E-07	0.26	1.15E-09	0.301	1.53E-12	0.285	2.38E-11
HAVCR2	0.27	2.87E-10	0.233	5.97E-08	0.181	2.78E-05	0.288	1.55E-11	0.17	7.79E-05
CD160	0.052	0.23	0.304	9.67E-13	0.265	5.73E-10	0.273	1.62E-10	0.399	<2.2e-16
TGFB1	0.374	<2.2e-16	0.218	3.81E-07	0.252	3.87E-09	0.248	7.22E-09	0.17	7.93E-05
ADORA2A	0.041	0.34	0.216	5.10E-07	0.353	3.34E-17	0.241	1.89E-08	0.245	1.01E-08
CD274	0.178	3.70E-05	0.178	3.64E-05	0.099	0.022	0.134	0.00199	0.036	0.411
TGFBR1	0.144	0.000832	-0.049	0.257	-0.218	4.14E-07	0.005	0.91	-0.082	0.0586
PVRL2	-0.076	0.0813	-0.11	0.0111	-0.073	9.42E-02	-0.092	0.0338	-0.03	0.485
VTCN1	-0.105	0.0151	-0.134	0.00197	-0.266	4.68E-10	-0.168	9.80E-05	-0.112	9.71E-03
KDR	-0.092	3.31E-02	-0.316	9.88E-14	-0.198	4.03E-06	-0.219	3.45E-07	-0.283	3.34E-11

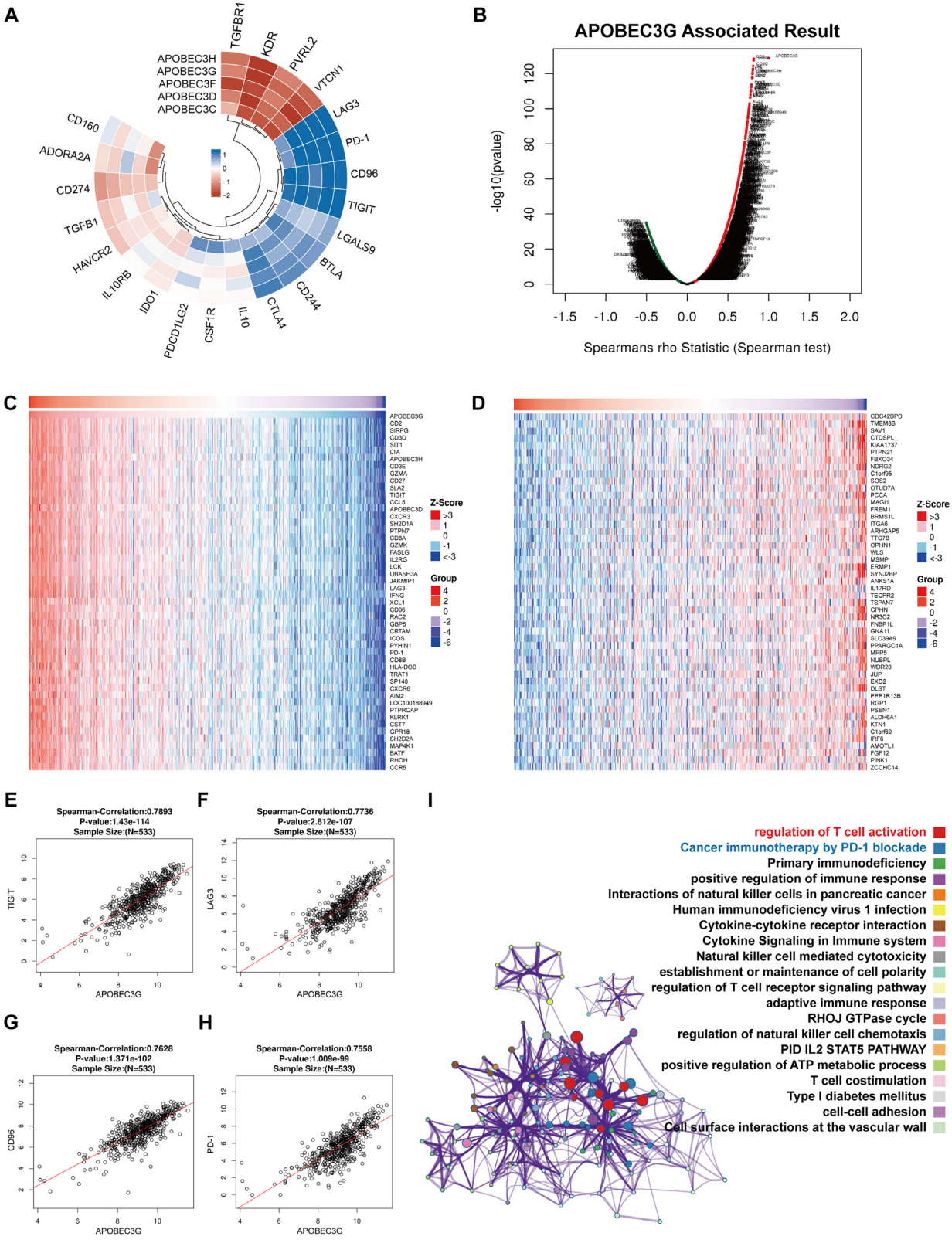

Figure 5. The correlation between APOBEC3 expression and immunoinhibitors and the function of APOBEC3G in KIRC. A Circular heatmap showing the degree of correlation between five APOBEC3 family members and immunoinhibitors (TISIDB). B Correlated genes of APOBEC3G in KIRC (LindedOmics). C The top 50 positively correlated genes of APOBEC3G in KIRC. D The top 50 negatively correlated genes of APOBEC3G in KIRC. E The scatter diagram showing the correlation between APOBEC3G expression with TIGIT. F The scatter diagram showing the correlation between APOBEC3G expression with LAG3. G The scatter diagram showing the correlation between APOBEC3G expression with CD96. H The scatter diagram showing the correlation between APOBEC3G expression with PD-1. I Pathway and process enrichment analysis of the top 100 correlated genes of APOBEC3G by metascape (Metascape).

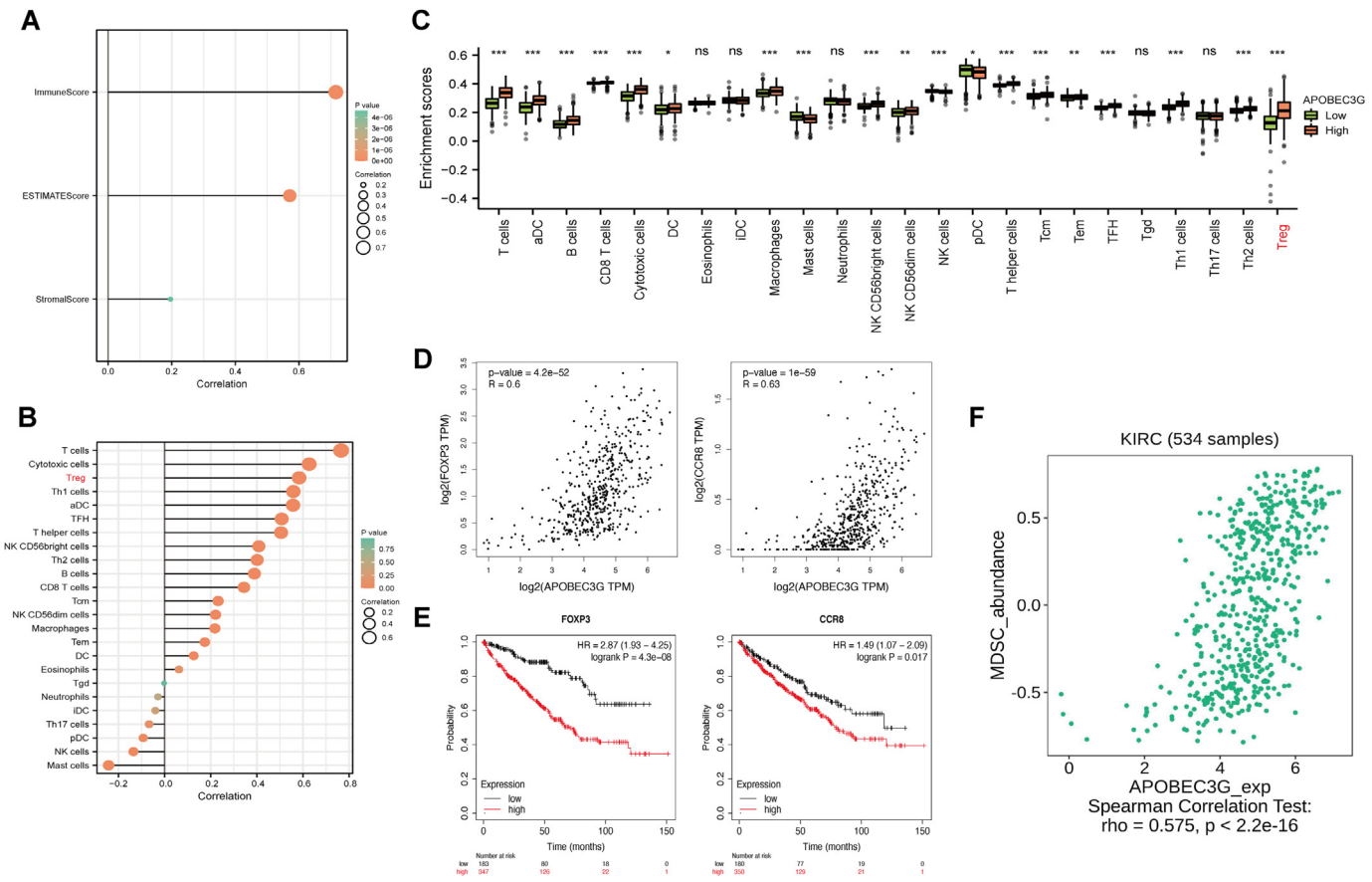

Figure 6. Relationship between APOBEC3G expression and tumor immune microenvironment. A The expression of APOBEC3G has a positive correlation with immune score. B The expression of APOBEC3G has a positive correlation with 24 immune cell subtypes including Treg cell. C Differences in 24 immune cell subtypes infiltration between APOBEC3G high-expression group and APOBEC3G low-expression group. D The correlation of APOBEC3G expression with FOXP3 and CCR8. E Overexpression of FOXP3 and CCR8 predicts poor overall survival in KIRC. F The correlation between APOBEC3G and MDSC infiltration (TISIDB).

(MDSC), which is a pivotal factor responsible for immunosuppression in cancer (Figure 6F) [39].

3.8. Expression and outcomes of APOBEC3G and association of APOBEC3G with PD-L1 in institutional dataset

As APOBEC3G may promote immunosuppression, we hypothesized that higher APOBEC3G expression may be correlated with more severe disease in renal clear cell carcinoma. Therefore, we employed an institutional dataset of 233 samples with renal clear cell carcinoma to investigate the prognostic significance of APOBEC3G expression in localized KIRC. In the cohort, total staining scores <4 and ≥ 4 were defined as the APOBEC3G$_{low}$ and APOBEC3G$_{high}$ groups, respectively. Representative staining images are shown in Figure 8A. Results demonstrated that APOBEC3G protein expression was positively correlated with age and grade of patients with KIRC (Supplementary material: Table S1). In addition, in tumors with sarcomatoid features (n = 9), 88.9% had high APOBEC3G expression, while the proportion was 33.9% in tumors without sarcomatoid features (n = 224) (P = 0.0026) (Figure 9A-I). We assessed survival risk with a median follow-up time of 7.1 years for patients with KIRC. The OS rate in the APOBEC3G$_{high}$ group was lower than that in the APOBEC3G$_{low}$ group (Figure 8B). The expression of PD-L1 was assessed in the same cohort to validate its correlation with APOBEC3G. Results showed that APOBEC3G expression was positively correlated with PD-L1 expression (Figure 8C).

4. Discussion

In this study, we explored the expression and prognosis of the APOBEC3 family in pan-cancer. Detailed analysis showed that the mRNA levels of the APOBEC3 family members were significantly higher in KIRC tumor samples than in normal renal tissue samples, and their expression levels were correlated with clinical stage, suggesting that APOBEC3 expression is closely related to patient condition. Survival analysis also demonstrated that patients with high APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3G, and APOBEC3H expression had poor prognosis, while high APOBEC3A and APOBEC3F expression had little effect on prognosis, consistent with the TISIDB results. Thus, APOBEC3B/C/D/G/H may serve as potential biomarkers for poor prognosis in KIRC. However, additional studies and clinical samples are needed to validate this conclusion.

Based on functional enrichment analysis, all APOBEC3 members were strongly correlated with immune regulation, except APOBEC3B, which was related to cell cycle regulation. APOBEC3B is reported to increase mutational burden in cancer patients [40]. Tumor mutational burden (TMB) can affect tumor immunotherapy [41, 42], thus whether APOBEC3B impacts immunotherapy response deserves further investigation. Previous evidence also suggests that a dysfunctional immune system and loss of cell proliferation control can promote cancer development [43, 44].

Our results also showed that APOBEC3 members were positively correlated with immune infiltration, with APOBEC3G showing the highest correlation with B cells, CD8+ T cells, and dendritic cells. APOBEC3C/D/F/G/H can activate immune system, embodied by promoting the infiltration of many kinds of immune cells. However, unlike many other solid cancers, CD8+T cell infiltration in KIRC represents poor prognosis, suggesting that APOBEC3G may play a crucial role in the functional status of immune cells [45]. Therefore, to clarify the antitumor immunological role of APOBEC3, we analyzed the correlations between APOBEC3 and immunoinhibitors. Results showed that four

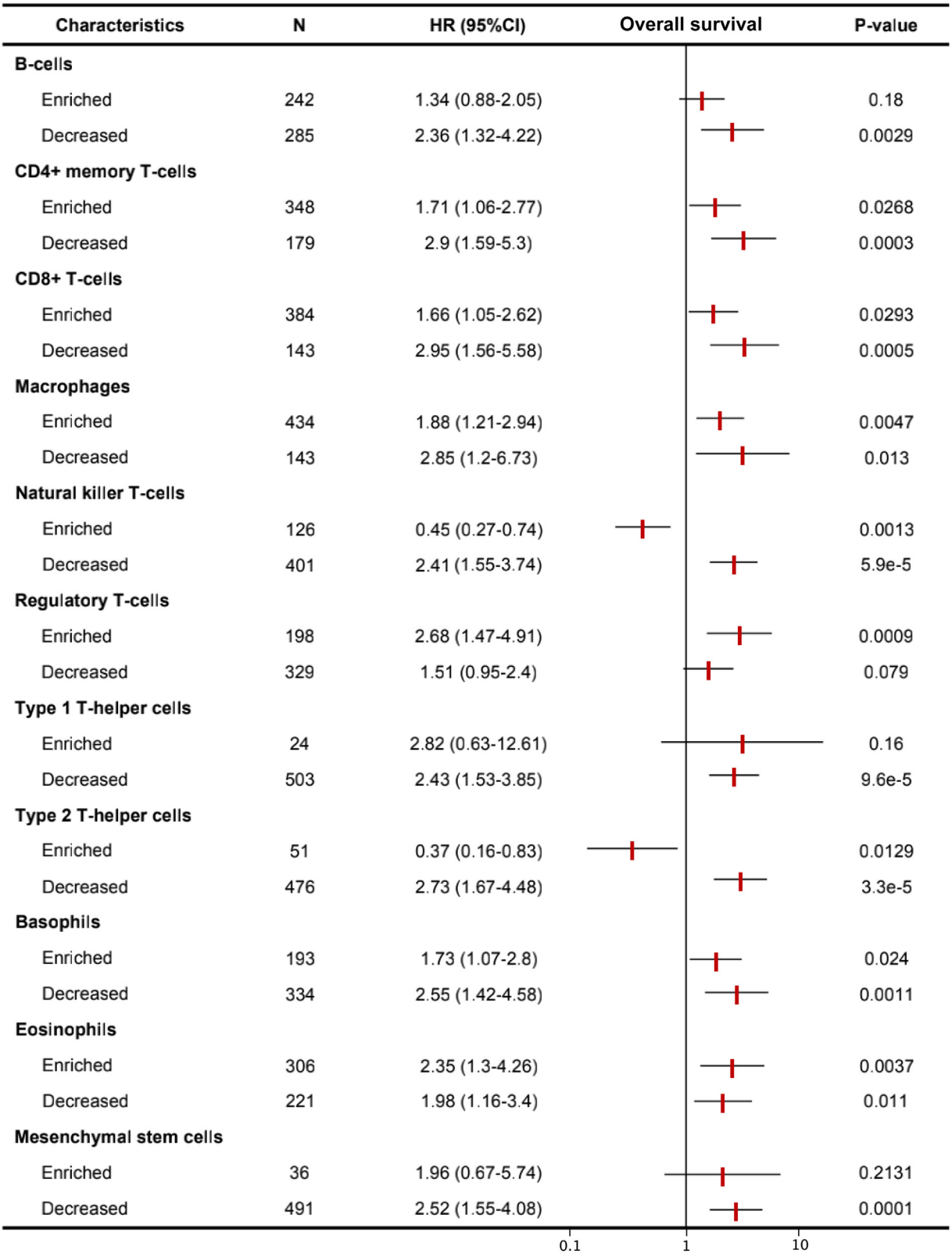

Figure 7. The forest plot of hazard ratio (HR) for OS in high expression APOBEC3G group compared to low expression APOBEC3G group under different immune cell infiltration levels.

immune-related genes (APOBEC3C/D/G/H) were positively correlated with immunoinhibitor expression, among which APOBEC3G had the strongest correlation and TIGIT, LAG3, CD96, PD-1, and CTLA4 were the top five correlated immunoinhibitors. In addition, we found that infiltration of tumor-promoting immune cells, including Treg cell and MDSC, was positively associated with APOBEC3G expression, which may induce immunosuppression and immune escape in KIRC patients. Significantly, the effect of APOBEC3G on prognosis depended on Treg

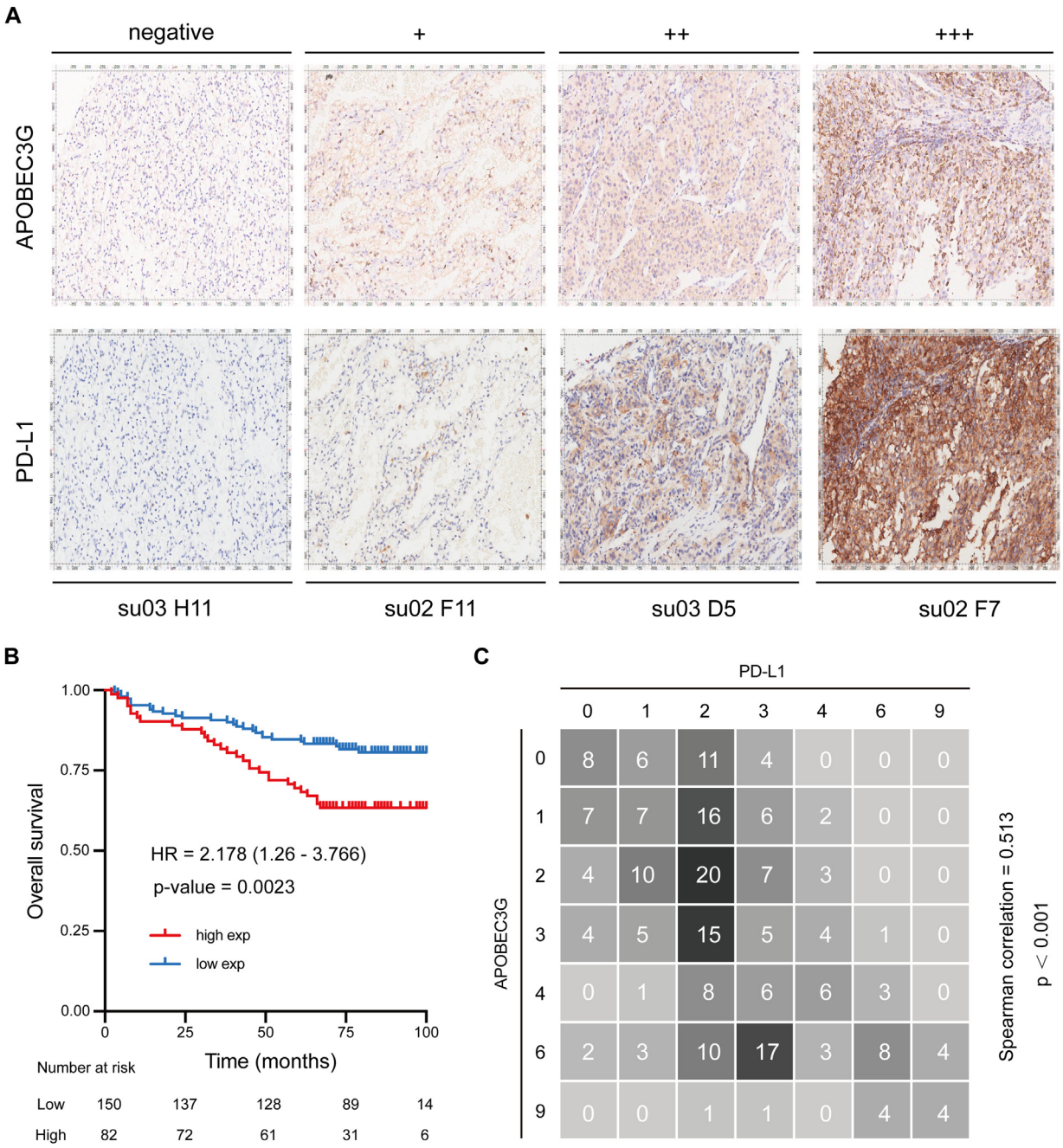

Figure 8. APOBEC3G expression correlated with a poor prognosis and the relationship between APOBEC3G and PD-L1 expression in institutional dataset. A Representative staing images of APOBEC3G and PD-L1. B Kaplan Meier plotter of OS according to APOBEC3G expression in clinical patients with renal clear cell carcinoma (Log-rank test). C The correlation between APOBEC3G and PD-L1 expression (Spearman correlation).

cell infiltration. Increasing evidence suggests that the immunosuppressive tumor microenvironment is closely related to tumor progression, metastasis, and cancer immunotherapy response [46]. The immune score is a standard test to quantify the density of T cells and cytotoxic T cells in the tumor microenvironment, which is important for tumor prognosis [47]. There is substantial literature evidence that tumor-infiltrating T lymphocytes, especially CD8 + T cell density at the invasive tumor margin, is closely associated with the survival benefit of other solid tumors such as kidney, bladder, and melanoma, and immune checkpoint inhibitors kill tumors by releasing already existing immune responses [48]. The characteristics of APOBEC3G in promoting immune infiltration and immune checkpoint expression suggest that APOBEC3G may play an important role in immunotherapy.

Given the above unexpected findings, we re-examined the function of APOBEC3G in the KIRC immune system and its potential as a biomarker for predicting immunotherapy benefit. Thus, 233 renal clear cell carcinoma samples were used to validate the prognostic value of APOBEC3G expression. As anti-PD-1/PD-L1 therapy occupies an important position in the treatment of patients with advanced renal cell carcinoma, we also analyzed the correlation between APOBEC3G and PD-L1 expression in tumor tissue. Analysis of the clinical samples showed that high APOBEC3G expression was correlated with poor patient survival, and

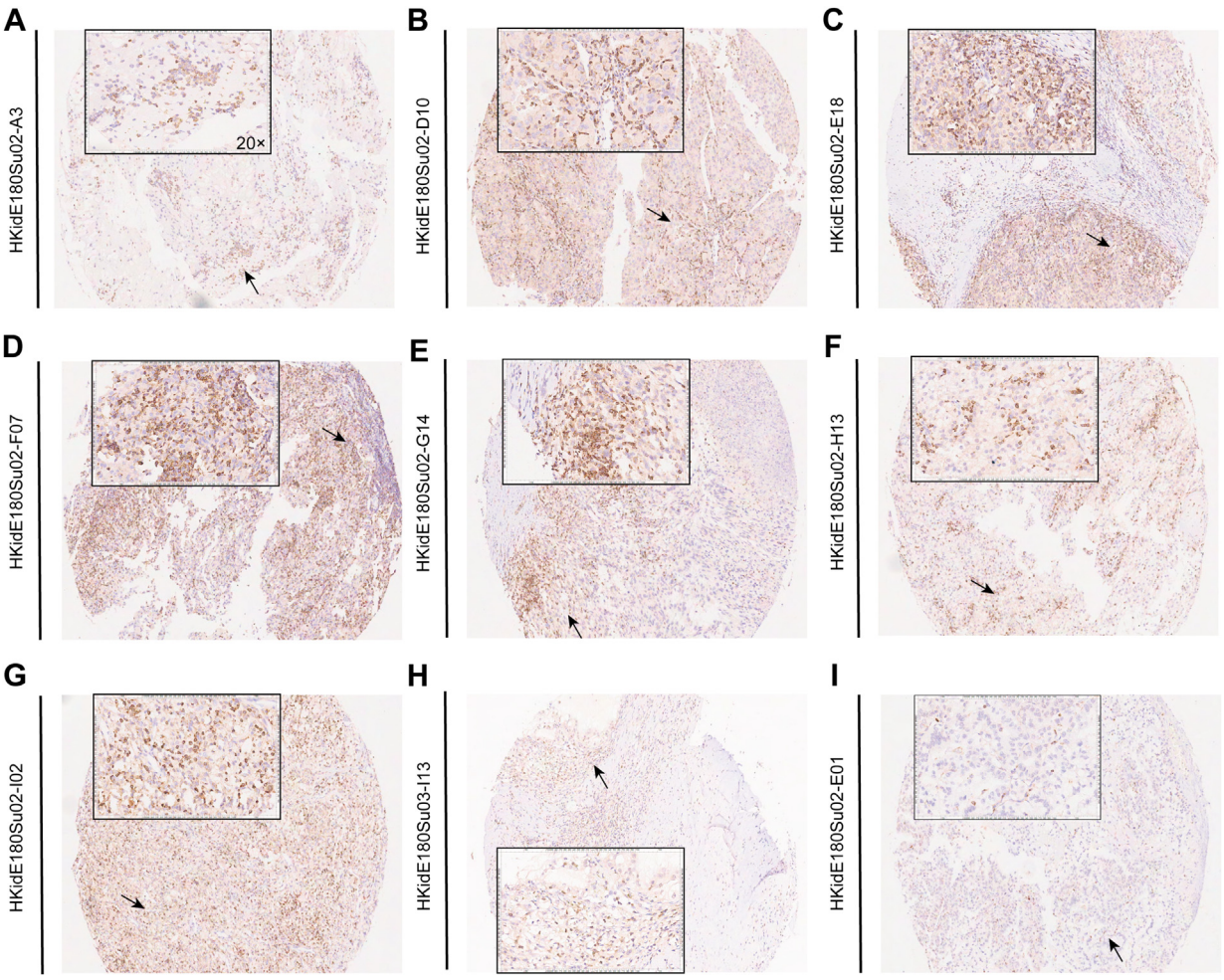

Figure 9. APOBEC3G staining level in 9 tumor samples with sarcomatoid differentiation. APOBEC3G$_{high}$ group: (A) HKidE180Su02-A03, (B) HKidE180Su02-D10, (C) HKidE180Su02-E18, (D) HKidE180Su02-F07, (E) HKidE180Su02-G14, (F) HKidE180Su02-H13, (G) HKidE180Su02-I02, (H) HKidE180Su03-I13; APOBEC3G$_{low}$ group: (I) HKidE180Su02-E01.

APOBEC3G was positively correlated with PD-L1 expression. Previous research has indicated that PD-L1 is up-regulated with APOBEC3 overexpression [49], as supported by our observations. We also found that the majority of tumors with sarcomatoid differentiation (88.9%) exhibited high APOBEC3G expression. In contrast, prior research has reported that sarcomatoid differentiation is associated with increased responsiveness to ICIs [50]. Although limited by small sample size, our study is the first to characterize APOBEC3G expression in sarcomatoid renal clear cell carcinoma. Thus, APOBEC3G may have a profound effect on immunotherapy.

The APOBEC3 family is an important class of enzymes in antiviral immunity [8]. Given the strong evidence that viral infection is closely related to the occurrence and development of cancer [8], the role of APOBEC3 in cancer immunity is worth exploring, especially in virus-associated cancers and those receiving clinical immunotherapy. KIRC is characterized by high immune infiltration and is sensitive to immunotherapy and targeted therapy [51, 52, 53]. However, the impact of APOBEC3 expression on tumor immunity in KIRC remains unknown. Whether the immune system status of KIRC patients varies with APOBEC3 expression and whether APOBEC3 expression is correlated with subsequent prognosis deserves further attention. Thus, our findings should help elucidate the important role of APOBEC3 in tumor immunity and contribute to optimal application of immunotherapy. Previous reports have proposed that APOBEC3G could increase expression level of the miRNA-targeted mRNA by releasing target mRNA from bound miRNA to facilitate tumor progression. For example, APOBEC3G downregulates miR-29 expression and hinders miR-29 activity in repressing MMP2 and thereby drives colorectal cancer hepatic metastasis [54, 55, 56]. In spite of this, underlying mechanisms of APOBEC3G in tumor regulation are needed further studies to elucidate. For the relationship between APOBEC3G and immune infiltration, it has already been shown that APOBEC3G can be upregulated by T cell activation, which is consistent with our study. Co-expression of APOBEC3G and some markers of tumor-infiltrating T lymphocytes has confirmed this fact. Moreover, our findings indicated a positive correlation between APOBEC3G expression and immunoinhibitors, which was suggestive of exhausted T cells. APOBEC3G may be closely associated with the formation of the immunosuppressive tumor microenvironment, however, there is still much to learn about the detailed mechanisms about how APOBEC3G induces a depression of immune function in KIRC [14, 45]. In recent years, immune checkpoint-targeted immunotherapy has shown great advantages in KIRC. As common genomic associated with immunotherapy showed no significant advantages, the status of the TIME is a vital influencing factor in mediating clinical efficacy of immunotherapy [57, 58]. Whether APOBEC3G could be an effective candidate biomarker targeting KIRC populations that will benefit from immunotherapy still need to be validated.

5. Conclusions

APOBEC3G was identified as a potential prognostic biomarker and closely related with the infiltration and function of immune cells in KIRC.

In addition, our results revealed the correlation of APOBE3G with PD-L1 expression and sarcomatoid differentiation. Further research of APOBEC3G may help refine our understanding its potential influence of immunotherapy response.

Declarations

Author contribution statement

Wencheng Ding & Lingli Gui: Conceived and designed the experiments.

Ting Peng & Binghan Liu: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Shitong Lin, Canhui Cao, Ping Wu, Wenhua Zhi, Ye Wei, Tian Chu: Contributed reagents, materials, analysis tools or data.

Funding statement

This work was supported by National Key Research and Development Program of China [2021YFC2701201], Natural Science Foundation of China [82072895 & 82141106].

Canhui Cao was supported by China Postdoctoral Science Foundation [2021M702223].

Data availability statement

Data associated with this study has been deposited at GSCALite & GSCA (http://bioinfo.life.hust.edu.cn/GSCA/#/), Kaplan-Meier plotter (www.kmplot.com), Oncomine (https://www.oncomine.org), GEPIA2 (http://gepia2.cancer-pku.cn/#index), TIMER (http://timer.cistrome.org), LinkedOmics (http://www.linkedomics.org/login.php), c-BioPortal (https://www.cbioportal.org/), TISIDB (http://cis.hku.hk/TISIDB/index.php).

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e12191.

Acknowledgements

Not applicable.

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