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Research article

Exploring the crosstalk of immune cells: The impact of dysregulated RUNX family genes in kidney renal clear cell carcinoma

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ABSTRACT

Background: Abnormally expressed Runt-associated transcription factor (RUNX) family has been reported in multiple tumors. Nevertheless, the immunological role of RUNX family in kidney renal clear cell carcinoma (KIRC) remains unknown.
Methods: We studied the RNA-seq data regarding tumor and healthy subjects from several public databases in detail for evaluating the prognostic and immunological functions owned by three RUNX genes in cancer patients. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) and immunohistochemical (IHC) staining served for detecting their expressions in tumor and normal samples.
Results: We observed that KIRC patients presented high expressions of RUNX1, RUNX2, and RUNX3. The expressions of three genes were validated by qRT-PCR, which was same as bioinformatical results. Prognostic analysis indicated that the overexpression of RUNX1 and RUNX2 negatively affects the outcomes in patients with KIRC. Related functional predictions indicated that the RUNXs and co-expression genes were significantly related to the immune response pathway. Moreover, three RUNX members were associated with immune infiltration cells and their related gene markers. The expression of RUNX family in several immune cells is positively or negatively correlated, and its dysregulation is obviously associated with the differential distribution of immune cells. RUNX family genes were abnormally expressed in KIRC patients, and were closely related to the crosstalk of immune cells.
Conclusions: Our findings may help to understand the pathogenesis and immunologic roles of the RUNX family in KIRC patients from new perspectives.

1. Introduction

Kidney renal clear cell carcinoma (KIRC), a representative subtype of kidney cancer, is characterized by high mortality, poor

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clinical prognosis, and an unclear pathogenesis [1]. Additionally, patients with advanced KIRC often experience survival times of less than five years, which is lower compared to patients with other tumors, partly due to its resistance to traditional radiotherapy and chemotherapy [2]. Therefore, clarifying the etiology of KIRC and discovering new therapeutic targets can essentially assist in improving the outcomes of patients with KIRC.

Runt-related transcription factors (RUNXs) are an evolutionarily conserved gene family that is widely expressed in tissues and cells [3]. The genomic structures of the RUNX genes (RUNX1, RUNX2, and RUNX3) are highly homologous and form a heterodimer complex with a representative non-DNA-binding core-binding factor (CBF-β) [4]. All three RUNX genes have been observed to exhibit aberrant expression in multiple stages of various tumors [5]. RUNX1 has been proved to be related to the differentiation of hematopoietic stem cells, although it also plays both carcinogenic and anticancer roles in many cancers [6]. RUNX2 is important for bone development and is related to cancer and bone metastases [7]. Meanwhile, RUNX3 inhibits tumor progression in multiple solid tumors [8]. Moreover, previous bioinformatics analysis has reported that RUNX1 is a potential therapeutic target in KIRC patients, and RUNX1 Promoter methylation level was positively correlated with survival. However, the crosstalk between RUNX family genes and immune cells and the specific molecular functions of these RUNX members in KIRC patients still need to be further explored [9].

Researches have attached increasing importance to the effect of the tumor microenvironment (TME) in tumor progression [10]. Our previous research demonstrated that renal cancer cells modulate peripheral cells by secreting various inflammation-related factors and reshaping their microenvironment functionally [11]. Previous reports have implicated tumor-infiltrating immune cells (TIICs) in the metastasis and drug resistance of renal cancer [12]. The three RUNX genes play critical roles in the differentiation regarding immune cells ($CD8^+$ T cells and effector B cells) [5], suggesting their potential role in immune regulation. We previously reported that RUNX1 may affect the progression of the tumor microenvironment (TME), particularly cancer-associated fibroblasts [13].

However, the specific contributions of the RUNX genes to tumor progression, TIICs function, and the TME in KIRC are yet to be elucidated. Here, we focused on evaluating the biological function of RUNX family in KIRC patients and their crosstalk to TIICs.

2. Materials and methods

2.1. Collecting clinical samples

Our study adhered to the Helsinki Declaration principles and got approval from the Research Ethics Committee of the Second Affiliated Hospital of Anhui Medical University (SL-YX2022-065). After acquiring all patients' written informed consent, we collected 60 samples of renal carcinoma and adjacent normal tissues and placed them at −80 °C until use.

2.2. RNA isolation and qRT-PCR

The TRIzol® reagent (Invitrogen; Thermo Fisher Scientific) served for extracting total RNA. The qRT-PCR procedures followed previous description [14], and the primers used in the qRT-PCR experiments were produced by Novabio. The sequences of these primers are listed in Table 1.

2.3. Immunohistochemical and immunoblotting analyses

RUNX1, CD4, CD25 and Foxp3 immunohistochemical staining was achieved using a rabbit anti-human RUNX1 Ab (Abcam, Cambridge, UK), a rabbit polyclonal antibody to CD4 (Affinity), a rabbit polyclonal antibody to CD25 (Affinity), and a Rabbit polyclonal antibody to Foxp3 (Affinity), following the manufacturers' instructions. We obtained the quantitative scoring based on immunohistochemistry results (positive light microscope; magnification, x200; Zen blue 3.1 software; Zeiss AG), then set the scoring criteria based on the percentage of positively stained cells (1 for ≤25 %, 2 for 26–50 %, 3 for 51–75 %, and 4 for >75 %) and the staining intensity (0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining). The final score was calculated by summing these two components [14].

2.4. Data collection and processing

RNA-seq data as well as clinical survival information regarding KIRC samples were obtained from TCGA database (https://portal.gdc.cancer.gov/) for subsequent analysis. The 'limma' R-package served for analyzing the differentially expressed genes (DEGs) between them. Next, the "cluster profiler" R-package was used to analyze the related biological functions of RUNX genes, including Gene

Table 1
Primer sequences used for qRT-PCR amplification.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
RUNX1	5′-AGGTTTGTCGGTCGAAGTGGAA-3′	5′-GATTTTGATGGCTCTGTGGTAGG-3′
RUNX2	5′-CCCAGTATGAGAGTAGGTGTCC-3′	5′-GGGTAAGACTGGTCATAGGACC-3′
RUNX3	5′-AATGCTCTTCATTGGTTGTCTCTG-3′	5′-AGTGTGTCTCTGGTGTATGGTTCTG-3′
GAPDH	5′-GTCTCCTCTGACTTCAACAGCG-3′	5′-ACCACCCTGTTGCTGTAGCCAA-3′

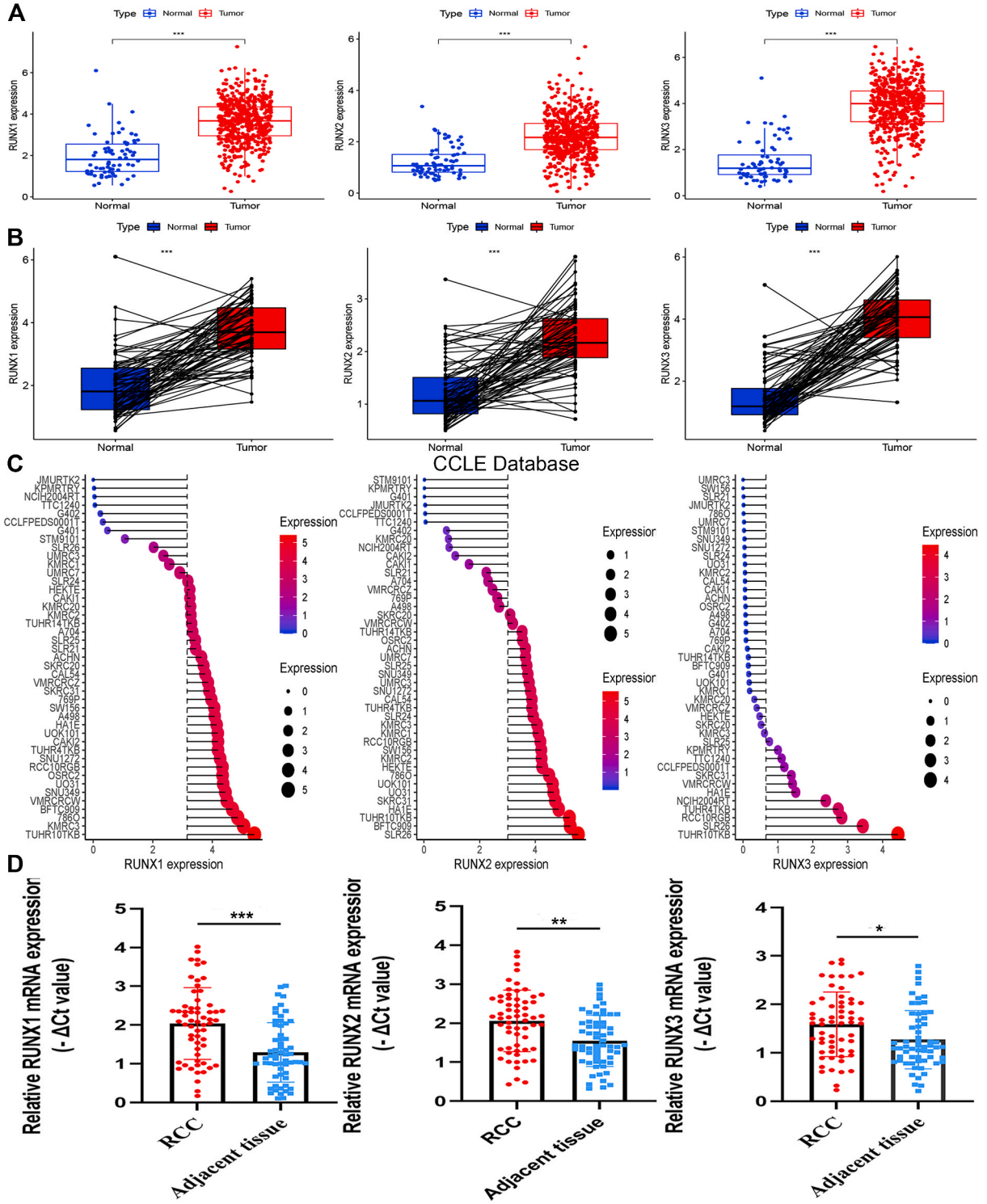

Fig. 1. RUNXs is overexpressed in KIRC. Unpaired (A) and paired (B) expression data analyses indicated that RUNXs expression was markedly higher in KIRC. (C) The mRNA levels of RUNX family factors in cancer cell lines (CCLE database). (D) The expression of RUNXs in renal cancer, detected by RT-qPCR.

Set Enrichment Analysis (GSEA), Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. Correlation analysis of RUNX genes with immune cell infiltration was evaluated by CIBERSORT and ESTIMATE algorithms. Additionally, the immunotherapy response of the RUNX family was evaluated using the Cancer Immune Atlas (TCIA).

2.5. UALCAN database and gene expression analysis

The UALCAN database is a valuable tool for online cancer data analysis [15]. For examining the functions possessed by RUNXs in KIRC patients, we used gene expression data from patients with renal cancer based on TCGA analysis.

2.6. Human protein atlas (HPA) database analysis

The Human Protein Atlas (HPA) database contains the atlas of the subcellular protein group, which can study the association between individual genes and protein expression [16].

2.7. WebGestalt database and enrichment analysis

The WebGestalt database is an online network tool that provides multiple functions [17]. In this study, we analyzed the enrichment of the three RUNX genes and their neighboring genes using this database.

2.8. TIMER database and estimating immune infiltration abundance analysis

The TIMER database conducts systematic analysis on different types of cancers, and adopts the multiple immune deconvolution methods to estimate immune infiltration abundance [18]. Moreover, we explored the correlation between RUNX gene expression in KIRC and representative immune markers, as reported in a previous study [19].

2.9. GEPIA database and prognostic analysis

The GEPIA database contains a vast array of RNAseq data from TCGA and GTEx databases, which allows for advanced data

Table 2
The mRNA expression of RUNXs and their correlation with clinicopathological parameters of patients with RCC.

Clinicopathological parameters		N	Relevant expression of RUNX1			Relevant expression of RUNX2			Relevant expression of RUNX3		
			Mean ± SD	t/F	p-Value	Mean ± SD	t/F	p-Value	Mean ± SD	t/F	p-Value
Tissue	Adjacent tissues	60	1.29 ± 0.77	4.794	<0.001*	1.43 ± 0.68	4.897	<0.001*	1.27 ± 0.60	2.742	0.007*
	RCC	60	2.04 ± 0.93			2.09 ± 0.79			1.59 ± 0.69		
Age,years	≤55	29	2.13 ± 0.91	0.783	0.437	2.11 ± 0.80	0.140	0.889	1.59 ± 0.65	0.055	0.957
	>55	31	1.95 ± 0.95			2.09 ± 0.79			1.58 ± 0.69		
Gender	Male	32	2.05 ± 0.81	−0.097	0.923	2.10 ± 0.81	0.079	0.938	1.59 ± 0.71	0.017	0.987
	Female	28	2.02 ± 1.06			2.08 ± 0.78			1.59 ± 0.63		
Location	Left	26	2.10 ± 0.81	0.454	0.652	2.06 ± 0.84	−0.209	0.835	1.64 ± 0.68	0.484	0.630
	Right	34	1.99 ± 1.01			2.11 ± 0.76			1.55 ± 0.66		
WHO/ISUP grade	G1-G2	34	2.09 ± 1.05	0.495	0.623	2.21 ± 0.79	1.372	0.175	1.51 ± 0.67	−1.016	0.314
	G3-G4	26	1.97 ± 0.74			1.93 ± 0.76			1.69 ± 0.66		
Clinical stage	I-II	35	2.03 ± 1.04	−0.094	0.926	2.18 ± 0.80	1.010	0.317	1.49 ± 0.68	−1.414	0.163
	III-IV	25	2.05 ± 0.76			1.97 ± 0.77			1.73 ± 0.73		
Tumor size	≤4 cm	31	2.14 ± 1.00	0.855	0.396	2.24 ± 0.80	1.565	0.123	1.62 ± 0.69	0.408	0.685
	>4 cm	29	1.93 ± 0.84			1.93 ± 0.75			1.55 ± 0.64		

RCC, renal cell carcinoma; ccRCC, clear cell RCC; pRCC, papillary RCC; cRCC, chromophobe RCC; RUNX1,Runt-related transcription factor 1; RUNX2, Runt-related transcription factor, RUNX3, Runt-related transcription factor; WHO/ISUP, World Health Organization/International Society of Urologic Pathology; SD, standard deviation; *$p < 0.05$ was considered statistically significant.

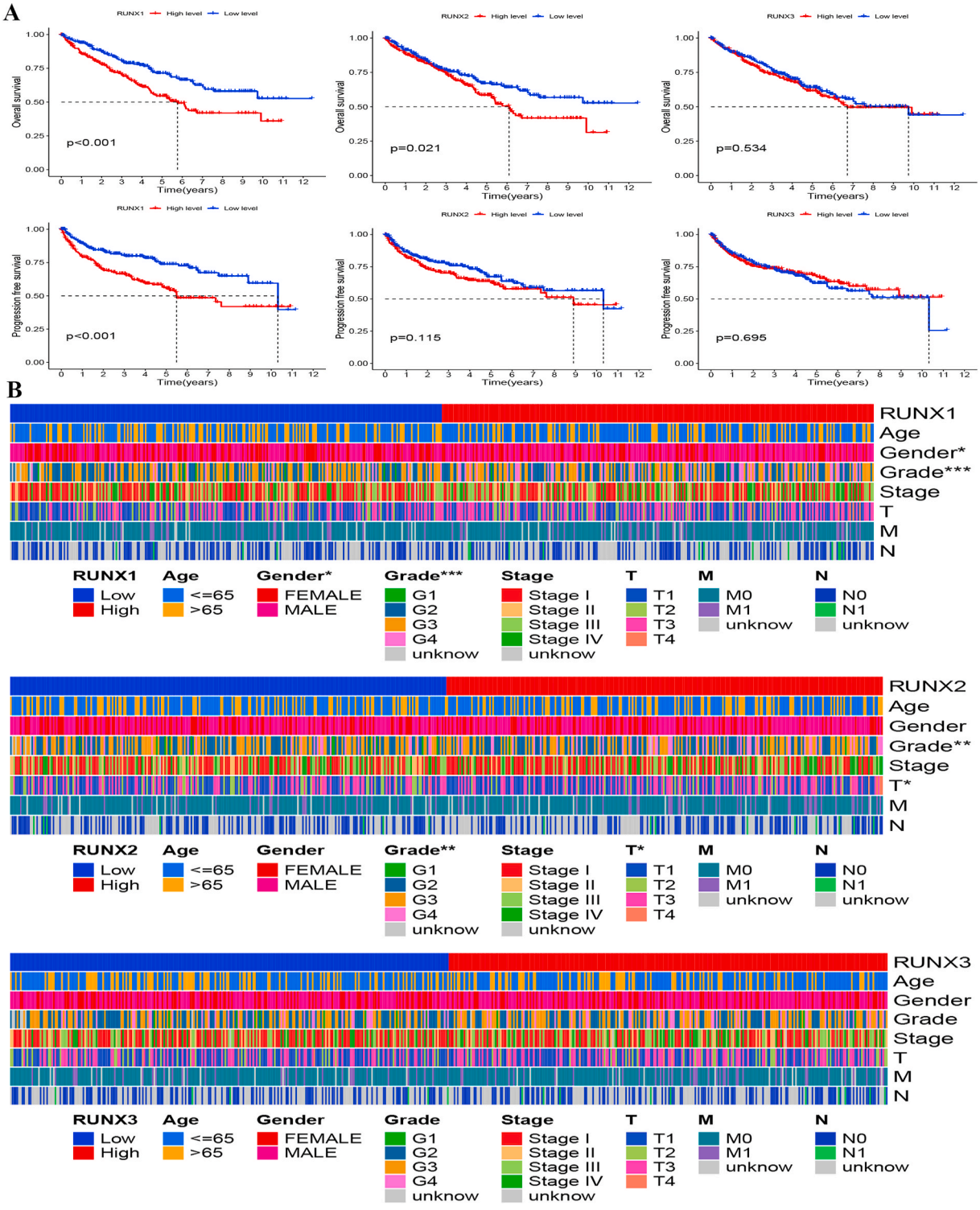

Fig. 2. RUNX family genes expression affects the prognosis of KIRC patients. (A) RUNX family genes were correlated with OS and PFS in KIRC patients. (B) Correlation analysis between RUNX family and different pathological features in KIRC.

processing [20]. In this database , we investigated the prognostic roles of the RUNX family in patients with KIRC.

2.10. TISIDB database and immunology analysis

TISIDB provides information on gene and tumor immune infiltration interactions [21], which assists in the analysis of the immunological value of the RUNX family using the TISIDB database.

2.11. Statistical analysis

The statistical software R (version 4.2.1.) was used for statistical analysis. The Student's t-test was used for comparison between groups. $P < 0.05$ was considered to have statistical significance (*p < 0.05, **p < 0.01,***p < 0.001, ****p < 0.001).

3. Results

3.1. RUNX family expression in human KIRC patients

Firstly, we confirmed the transcription expression levels of RUNX genes in human cancers using the UALCAN project (Fig. S1A). Next, we analyzed the TCGA-KIRC dataset and observed that three RUNX members were significantly increased in KIRC patients

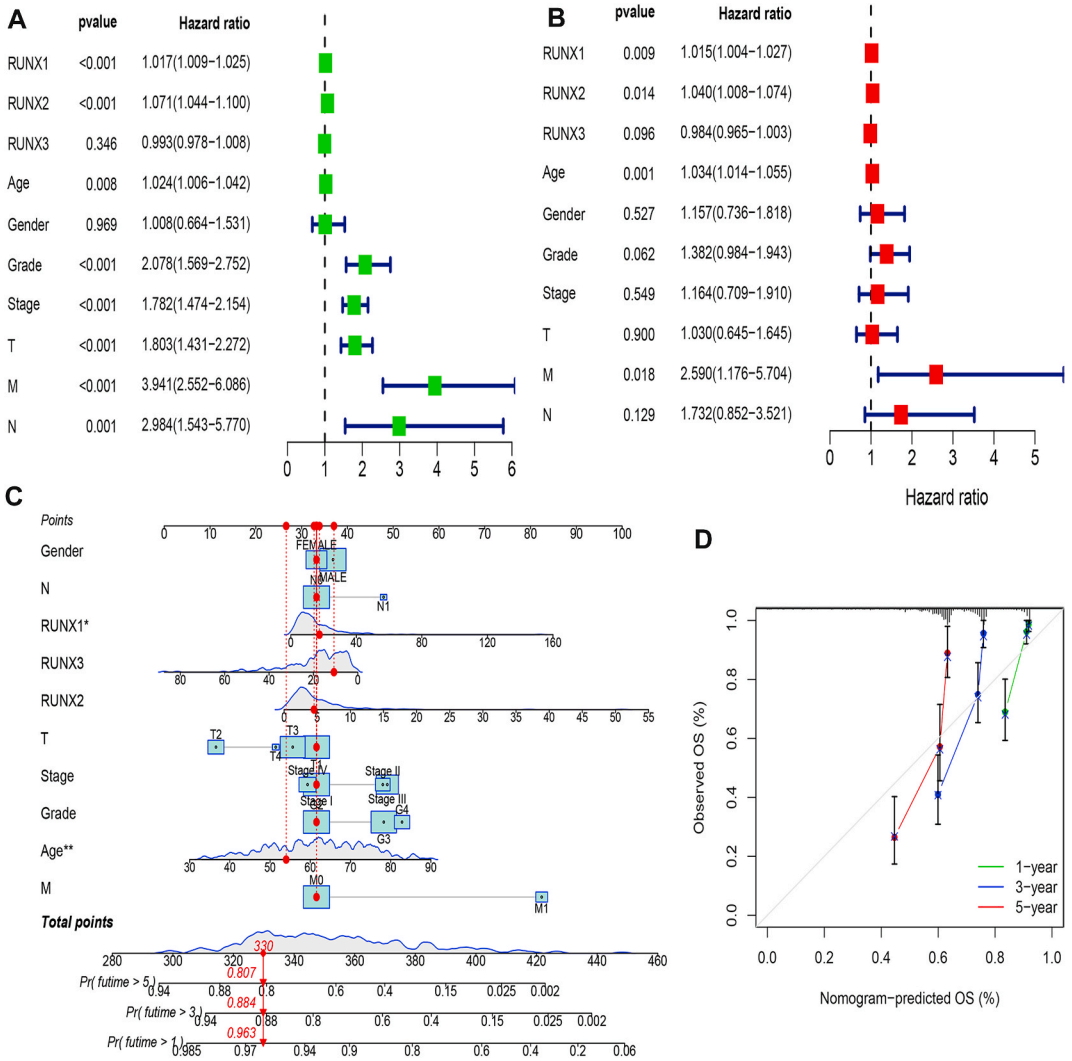

Fig. 3. The relationship RUNX family genes and independent risks for KIRC patients. Univariate (A) and multivariate (B) Cox regression analysis showed that **RUNX family genes** was an independent risk factor correlated with the survival in KIRC. (C) A prognostic nomogram with NXPH4 and clinical variables was constructed. (D)The 1-, 3- and 5-year calibration plots demonstrated the performance of the nomogram.

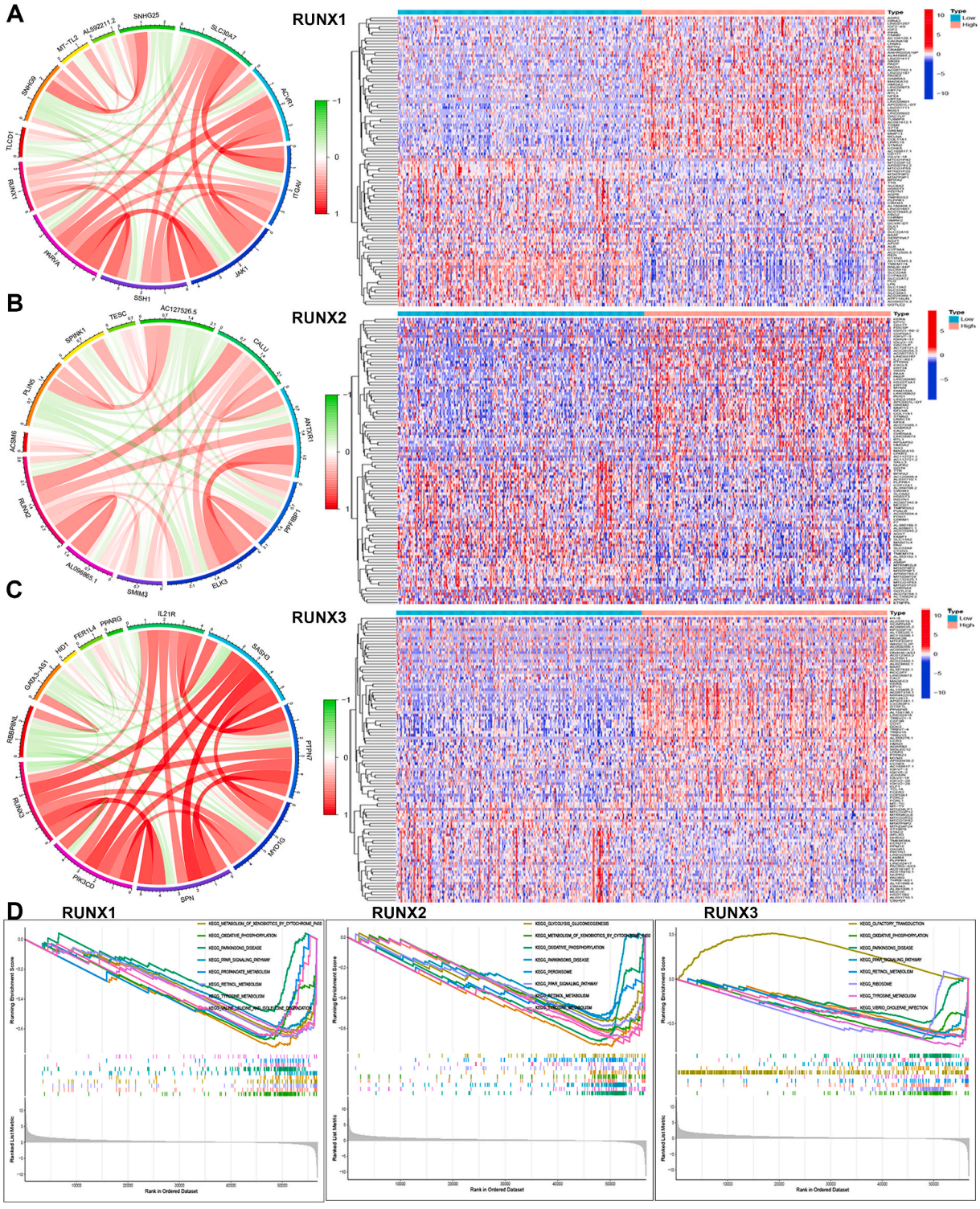

Fig. 4. Gene co-expression analysis and GSEA of RUNX family. (A) The RUNX1 of patients with KIRC co-expressed the gene and the top 50 DEGs. (B) The RUNX2 of patients with KIRC co-expressed the gene and the top 50 DEGs. (C)The RUNX3 of patients with KIRC co-expressed the gene and the top 50 DEGs. (D) GSEA was used to identify signaling pathways.

(Fig. 1A and B). And We also assessed the mRNA levels of RUNX family factors in cancer cell lines (Fig. 1C) and the expression of RUNXs in renal cancer by RT-qPCR (Fig. 1D). Moreover, we observed that the promoter methylation levels of RUNX1 and RUNX3 are different between normal and KIRC samples, whereas RUNX2 did not show such differences (Fig. S1B).

To confirm the above results, we collected tissue specimens and clinical data from 60 renal cancer patients. According to our study results, all three RUNX genes showed increased mRNA expression in renal cancer (Fig. 1D and Table 2). Then we further investigated the protein expression of these genes in renal cell carcinoma (RCC) through the HPA database. Compared to normal tissues, RUNX1 and RUNX2 exhibited increased protein levels in renal cancer, while the protein level of RUNX3 did not present an obvious difference (Fig. S1C).

3.2. Prognostic analysis of the three RUNX genes in KIRC patients

To assess the prognostic value possessed by the RUNX family in KIRC patients, we conducted prognostic analyses using the GEPIA database. The results indicated that high expression of RUNX1 and RUNX2 was associated with an unfavorable prognosis in KIRC patients, but RUNX3 expression was not statistically different (Fig. 2A). Later, correlation analysis was performed to explore the association between RUNX family members and pathological features in KIRC. These analyses revealed that RUNX1 exhibited a clear relevance to gender and tumor grade, and RUNX2 was clearly associated with tumor grade (Fig. 2B).

3.3. RUNX1 and RUNX2 were independent risk factors for KIRC patients

We performed univariate and multivariate Cox regression analyses to confirm factors that could independently predict patient prognosis. These analyses revealed that several clinicopathological features and RUNX family were associated with patient survival.

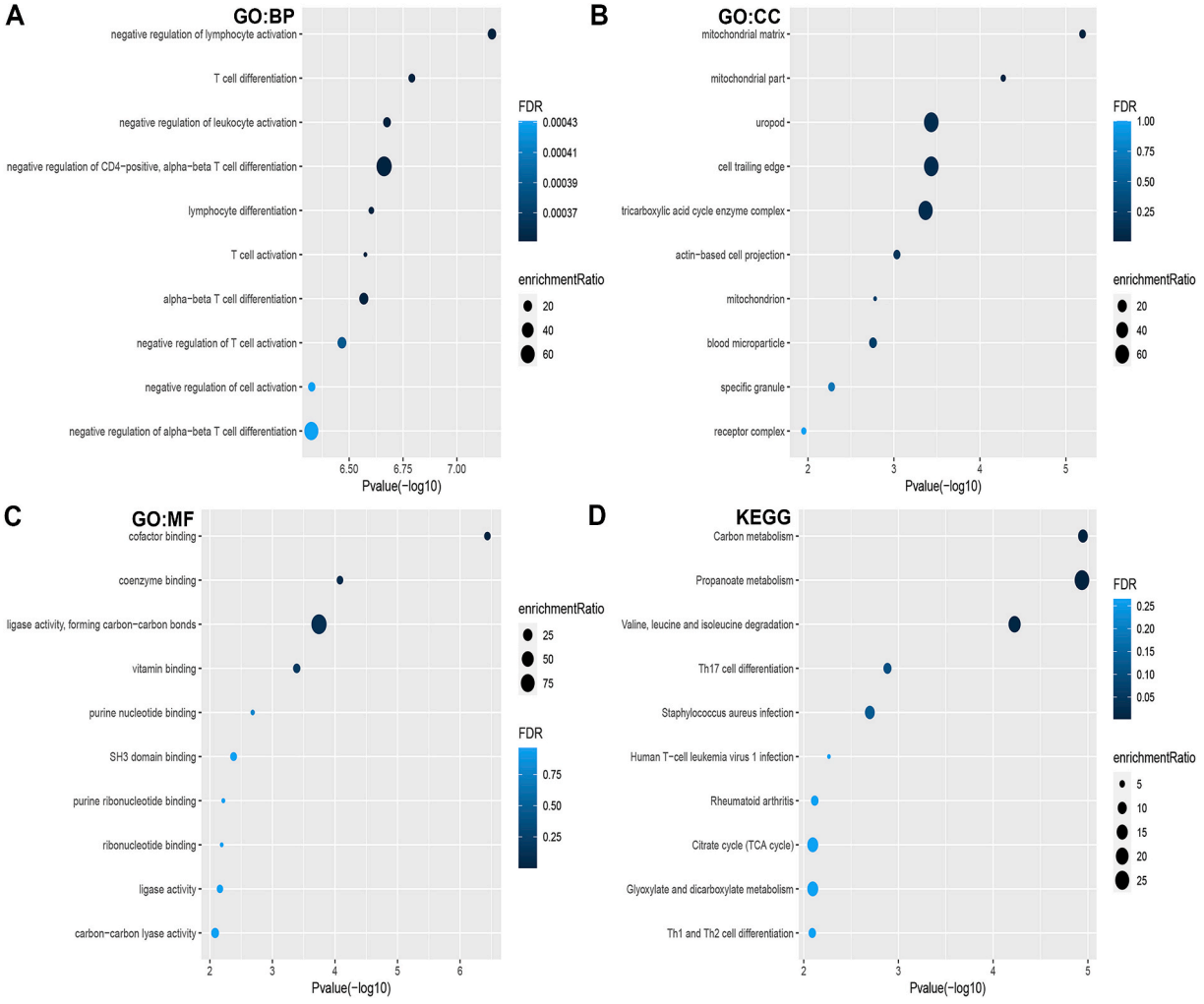

Fig. 5. Enrichment analysis of three RUNX genes and co-expression genes in KIRC patients. (A) BP. (B) CC. (C) MF. (D) KEGG.

Univariate analysis result indicated that a significant association of age, grade, clinical stage, TMN stage, RUNX1, and RUNX2 with patient outcomes. Multivariate analysis showed that age and M stage, RUNX1 and RUNX2 were significantly associated with patient prognosis (Fig. 3A and B). To further assess the potential clinical value possessed by the RUNX family, we established a nomogram combining clinicopathological features and RUNX family members. As shown in Fig. 3C, RUNX1 expression and age variables are

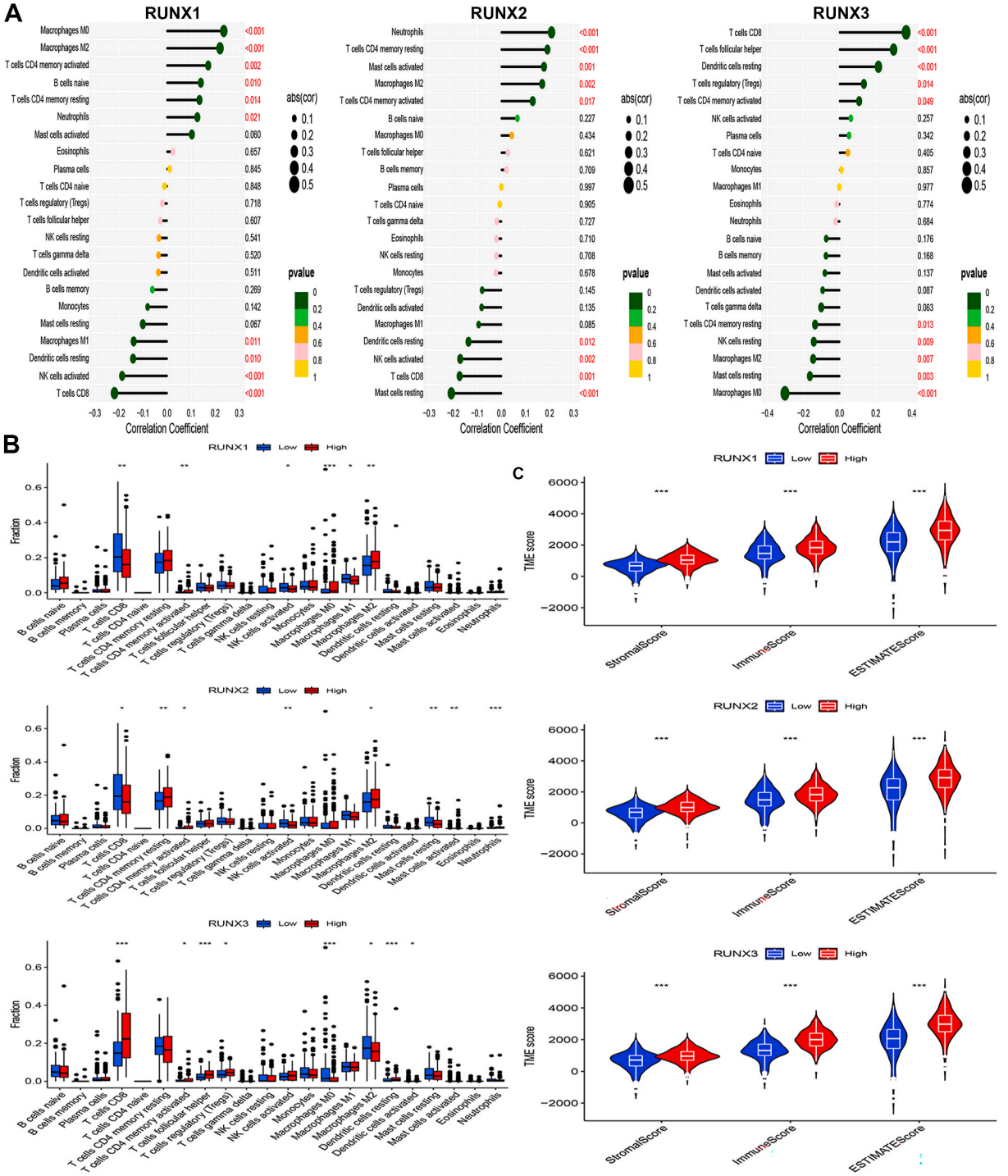

Fig. 6. The relationship between the RUNXs expression and immune infiltration levels in TIMER. (A) The relationship between the expression of three RUNX family members and immune cell infiltration in KIRC. (B) The relationship between RUNXs copy number variations and immune infiltrates in KIRC.

Table 3
The correlation between RUNXs expression and immune cells related gene markers in KIRC (TIMER database).

Cell type	Gene mark	RUNX1				RUNX2				RUNX3			
		None		Purity		None		Purity		None		Purity	
		Cor	P	Cor	P	Cor	P	Cor	P	Cor	P	Cor	P
B cell	CD19	0.355	***	0.281	***	0.314	***	0.271	***	0.45	***	0.422	***
	CD20	0.022	0.605	−0.01	0.827	0.05	0.25	0.045	0.333	0.045	0.305	0.013	0.779
	CD38	0.267	***	0.251	***	0.339	***	0.309	***	0.607	***	0.568	***
$CD8^+$ T Cell	CD8A	0.208	***	0.141	**	0.255	***	0.223	***	0.677	***	0.626	***
	CD8B	0.165	***	0.1	*	0.22	***	0.191	***	0.659	***	0.623	***
Tfh	CXCR5	0.427	***	0.36	***	0.378	***	0.341	***	0.486	***	0.447	***
	ICOS	0.3	***	0.246	***	0.365	***	0.342	***	0.697	***	0.665	***
	BCL-6	0.397	***	0.43	***	0.351	***	0.387	***	0.12	***	0.111	*
Th1	IL12RB2	0.077	0.0757	0.042	0.366	0.191	***	0.151	***	0.249	***	0.197	***
	IL27RA	0.347	***	0.314	***	0.37	***	0.352	***	0.255	***	0.214	***
	TBX21	0.125	**	0.082	0.0799	0.257	***	0.236	***	0.536	***	0.502	***
Th2	CCR3	0.156	***	0.109	*	0.244	***	0.213	***	0.375	***	0.344	***
	STAT6	0.032	0.461	0.066	0.154	0.13	***	0.169	***	0.229	***	0.249	***
	GATA-3	0.185	***	0.106	*	0.172	***	0.131	***	0.349	***	0.339	***
Th9	TGFBR2	0.061	0.16	0.054	0.248	0.141	***	0.127	***	0.13	***	0.088	0.0576
	IRF4	0.362	***	0.307	***	0.396	***	0.386	***	0.666	***	0.628	***
	PU.1	0.381	***	0.306	***	0.358	***	0.313	***	0.655	***	0.649	***
Th17	IL-21R	0.52	***	0.455	***	0.484	***	0.437	***	0.619	***	0.586	***
	IL-23R	0.236	***	0.19	***	0.296	***	0.266	***	0.217	***	0.175	***
	STAT3	0.331	***	0.296	***	0.31	***	0.283	***	0.298	***	0.251	***
Th22	CCR10	0.266	***	0.216	***	0.181	***	0.154	***	0.125	**	0.094	*
	AHR	0.228	***	0.211	***	0.338	***	0.336	***	0.241	***	0.183	***
Treg	FOXP3	0.409	***	0.345	***	0.388	***	0.377	***	0.559	***	0.52	***
	CCR8	0.347	***	0.294	***	0.417	***	0.408	***	0.56	***	0.51	***
	CD25	0.551	***	0.529	***	0.54	***	0.528	***	0.358	***	0.32	***
T cell exhaustion	PD-1	0.187	***	0.125	***	0.225	***	0.198	***	0.694	***	0,699	***
	CTLA4	0.258	***	0.187	***	0.338	***	0.318	***	0.616	***	0.589	***
Macrophage	CD68	0.325	***	0.319	***	0.323	***	0.342	***	0.375	***	0.359	***
	CD11b	0.343	***	0.303	***	0.401	***	0.371	***	0.529	***	0.489	***
M1	NOS2	0.073	0.0941	0.041	0.374	0.108	*	0.069	0.136	0.19	***	0.161	***
	ROS1	0.237	***	0.218	***	0.235	***	0.219	***	0.018	***	−0.05	0.287
M2	ARG1	−0.007	0.872	0.026	0.58	0.033	0.447	0.05	0.286	0.076	0.0784	0.046	0.324
	MRC1	0.245	***	0.225	***	0.28	***	0.252	***	0.192	***	0.143	**
TAM	HLA-G	0.105	*	0.048	0.305	−0.021	0.63	−0.047	0.31	0.471	***	0.437	***
	CD80	0.413	***	0.335	***	0.444	***	0.43	***	0.547	***	0.535	***
	CD86	0.379	***	0.334	***	0.429	***	0.405	***	0.587	***	0.563	***
Monocyte	CD14	0.428	***	0.364	***	0.371	***	0.326	***	0.42	***	0.378	***
	CD16	0.439	***	0.402	***	0.466	***	0.439	***	0.544	***	0.513	***
NK	XCL1	0.201	***	0.123	***	0.219	***	0.187	***	0.512	***	0.469	***
	KIR3DL1	−0.026	0.545	−0.022	0.645	0	0.995	0.016	0.729	0.194	***	0.179	***
	CD7	0.204	***	0.113	*	0.213	***	0.172	***	0.638	***	0.592	***
Neutrophil	CD15	0.332	***	0.3	***	0.359	***	0.325	***	0.336	***	0.298	***
	MPO	0.23	***	0.218	***	0.274	***	0.267	***	0.278	***	0.241	***
DC	CD1C	0.066	0.127	−0.019	0.686	0.166	***	0.114	*	0.319	***	0.278	***
	CD141	0.422	***	0.387	***	0.285	***	0.256	***	0.133	**	0.041	0.382

$P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

KIRC, kidney renal clear cell carcinoma; Tfh, follicular helper T cell; Th, T helper cell; Treg, regulatory T cell; TAM, tumor associated macrophage; NK, natural killer cell; DC, dendritic cell; None, correlation without adjustment; Purity, correlation adjusted for tumor purity; Cor, R value of Spearman's correlation.

valuable predictors of prognosis in KIRC patients (Fig. 3D).

3.4. Biological function analysis of the RUNX family in KIRC patients

To investigate the potential functions of three RUNX genes, we performed correlation analysis on the TCGA-KIRC dataset to identify genes which are related with the RUNX family significantly. We obtained the top 10 co-expressed genes presenting an obviously positive or negative relevance to the RUNX family gene, and the top 50 up-regulated and 50 down-regulated genes based on RUNXs expression (Fig. 4A–C). In addition, GSEA was performed under the assistance of the TCGA-KIRC dataset, thereby determining the signaling pathways of differentially activated RUNX family related genes in KIRC. According to the study results, we observed that these pathways are closely related to cellular metabolism (Fig. 4D). Then, RUNXs and these co-expressed genes were analyzed for GO and KEGG enrichment in the WebGestalt database, indicating that these genes were enriched in energy metabolism and immune regulation (Fig. 5A–D).

3.5. RUNX genes and immune cells infiltration in KIRC patients

Firstly, we calculated the proportion of 22 infiltrating immune cell types in KIRC patients. We found that RUNX family expression correlated with several of these immune cells, and its dysregulation was strongly associated with different distributions of immune cells (Fig. 6A and B). In addition, we observed that the group with high RUNX expression presented higher immune scores and stromal scores compared to the group with low expression (Fig. 6C). In the TIMER 2.0 database, high expression of the three RUNX genes was associated with several immune cells types in KIRC patients, including B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells (Fig. S2A). In Fig. S2B, RUNX genes' copy number variations (CNV) are significantly correlated with infiltration levels of several immune cells. Additionally, in the TISIDB database, we validated the association of the RUNX family with immune-infiltrating cells in KIRC patients and the results showed that the association is obvious (Fig. S3A). The inflammatory (C3) subtype represented the largest proportion of all subtypes, suggesting that most KIRC patients with RUNXs expression were in the C3 immune subtype (Fig. S3B).

3.6. The three RUNX genes and representative immune markers in KIRC patients

After purity-related adjustment, the expression of RUNX genes was associated with most of the immunomarker sets of various immune cells in patients with KIRC (Table 3). Interestingly, we observed that the RUNX genes are related to macrophage, TAM, and monocyte marker groups. Noticeably, the expression of Treg marker genes was also significantly correlated with the three RUNX genes in patients with KIRC, including FOXP3, CCR8, and CD25 (Table 4).

We performed a correlation analysis between known immune checkpoint inhibitors (ICIs) and RUNX family. These genes were positively correlated with most ICIs, such as CD276, LAIR1, CD44, CD86 and TNFRSF8, among others (Fig. 7A). Compared with the

Table 4
The correlation between RUNXs expression and immune cells related gene markers in KIRC (GEPIA database).

Cell type	Gene mark	RUNX1				RUNX2				RUNX3			
		None		Purity		None		Purity		None		Purity	
		Cor	P	Cor	P	Cor	P	Cor	P	Cor	P	Cor	P
Tfh	CXCR5	0.18	***	0.19	0.1	0.078	0.076	0.33	**	0.15	***	0.37	**
	ICOS	0.13	**	0.51	***	0.13	**	0.71	***	0.67	***	0.66	***
	BCL-6	0.3	***	0.29	*	0.21	***	0.31	**	0.092	*	0.032	0.79
Treg	FOXP3	0.2	***	0.29	*	0.12	**	0.33	**	0.48	***	0.65	***
	CCR8	0.18	***	0.27	*	0.16	***	0.5	***	0.46	***	0.64	***
	CD25	0.34	***	0.52	***	0.33	***	0.7	***	0.18	***	0.66	***
T cell exhaustion	PD-1	0.049	0.26	0.078	0.51	0.049	0.26	0.34	**	0.56	***	0.58	***
	CTLA4	0.13	**	0.47	***	0.17	***	0.6	***	0.59	***	0.48	***
Macrophage	CD68	0.19	***	0.36	**	0.18	***	0.45	***	0.27	***	0.91	***
	CD11b	0.053	0.23	0.65	***	0.061	0.16	0.76	***	0.14	**	0.89	***
TAM	HLA-G	0.032	0.47	0.3	*	−0.053	0.23	0.24	*	0.5	***	0.81	***
	CD80	0.27	***	0.73	***	0.3	***	0.66	***	0.49	***	0.4	***
	CD86	0.28	***	0.8	***	0.24	***	0.81	***	0.52	***	0.68	***
Monocyte	CD14	0.29	***	0.49	***	0.25	***	0.65	***	0.32	***	0.8	***
	CD16	0.32	***	0.6	***	0.27	***	0.56	***	0.47	***	0.94	***
	CD7	0.052	0.23	0.4	***	−0.0079	0.86	0.48	***	0.06	0.17	0.88	***
Neutrophil	CD15	0.27	***	0.71	***	0.2	***	0.75	***	0.33	***	0.44	***
	MPO	0.043	0.33	0.59	***	0.059	0.18	0.48	***	0.021	0.63	0.31	**
DC	CD1C	0.019	0.66	0.42	***	−0.017	0.7	0.62	***	0.27	***	0.38	***
	CD141	0.27	***	0.44	***	0.13	**	0.42	***	0.076	0.081	0.38	***

$P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
KIRC, kidney renal clear cell carcinoma; Treg, regulatory T cell; TAM, tumor associated macrophage; NK, natural killer cell; DC, dendritic cell; None, correlation without adjustment; Purity, correlation adjusted for tumor purity; Cor, R value of Spearman's correlation.

RUNX1 and RUNX2 high expression groups, the RUNX1 and RUNX2 low expression groups showed higher IPS, whereas the opposite was observed for RUNX3 expression (Fig. 7B). Immunohistochemistry of 15 KIRC patients showed that there were significant differences in the expression of CD4, CD25, Foxp3 and RUNX1 between tumor tissue and adjacent tumor tissue (Fig. 8A and B). In addition, we evaluated the effect of Tregs infiltration level and RUNXs expression on clinical outcome in KIRC patients using Cox proportional hazards models. There was a significant correlation between Tregs infiltration and RUNXs expression in the prognosis of KIRC patients (Figs. S4A–S4B). After adjusting for age, we observed worse survival in the higher-infiltration level group as patients aged (Fig. S4C).

4. Discussion

RUNX family members are dysregulated in a variety of malignant tumors [6,22,23]. The biological and immunological roles of RUNXs in KIRC patients have not been defined. We hope that our bioinformatics analysis will conduce to existing research, and optimize treatment strategies to enhance the prognosis for renal cancer patients.

The three RUNX genes have important functions in the differentiation of various immune cell subsets. The development of T

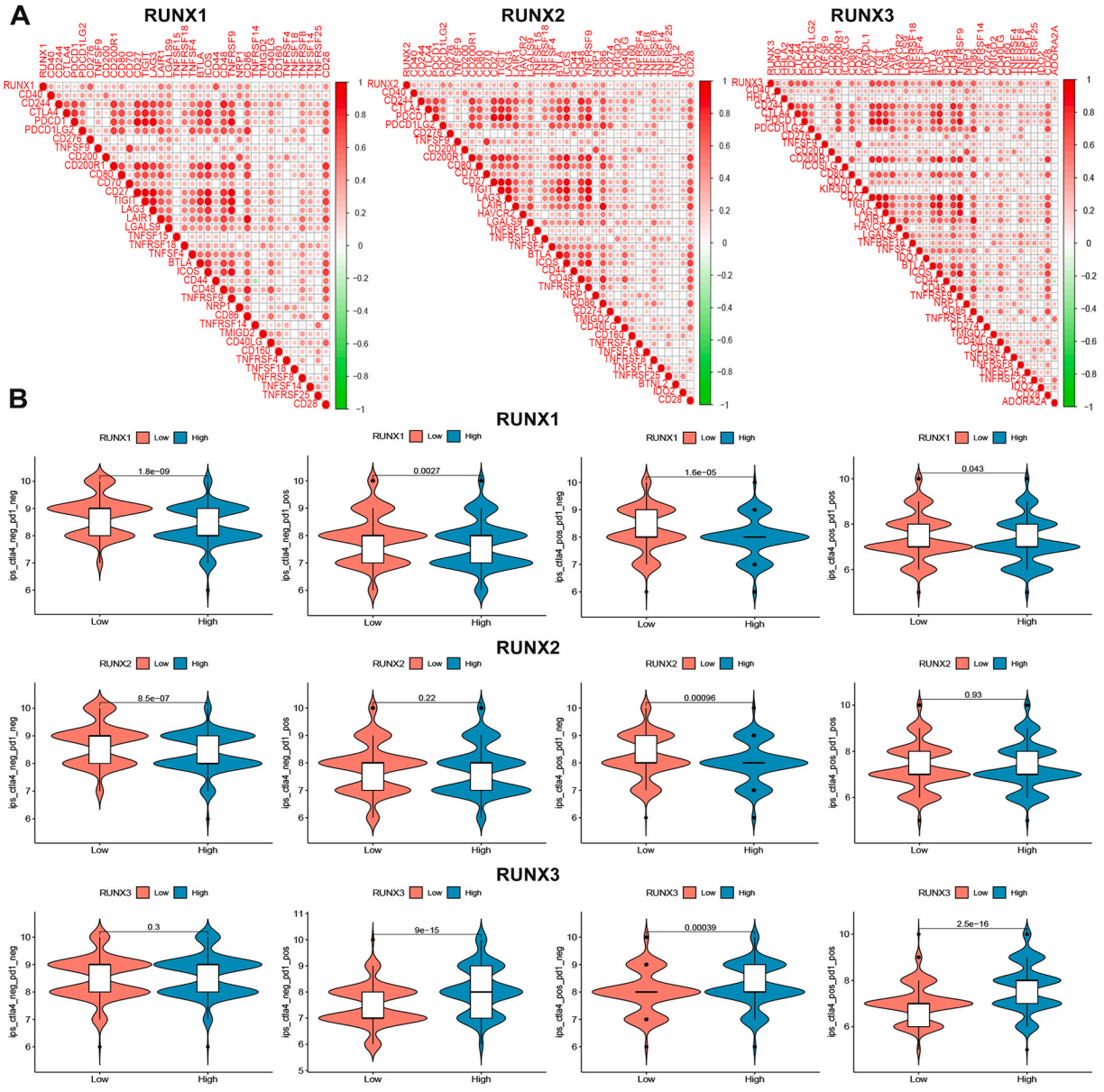

Fig. 7. Correlations between RUNXs expression and immune infiltration levels in TISIDB. (A) The heat map showed the correlation between RUNXs expression and immune infiltration levels. (B) The correlation between RUNXs expression and KIRC immune subtypes.

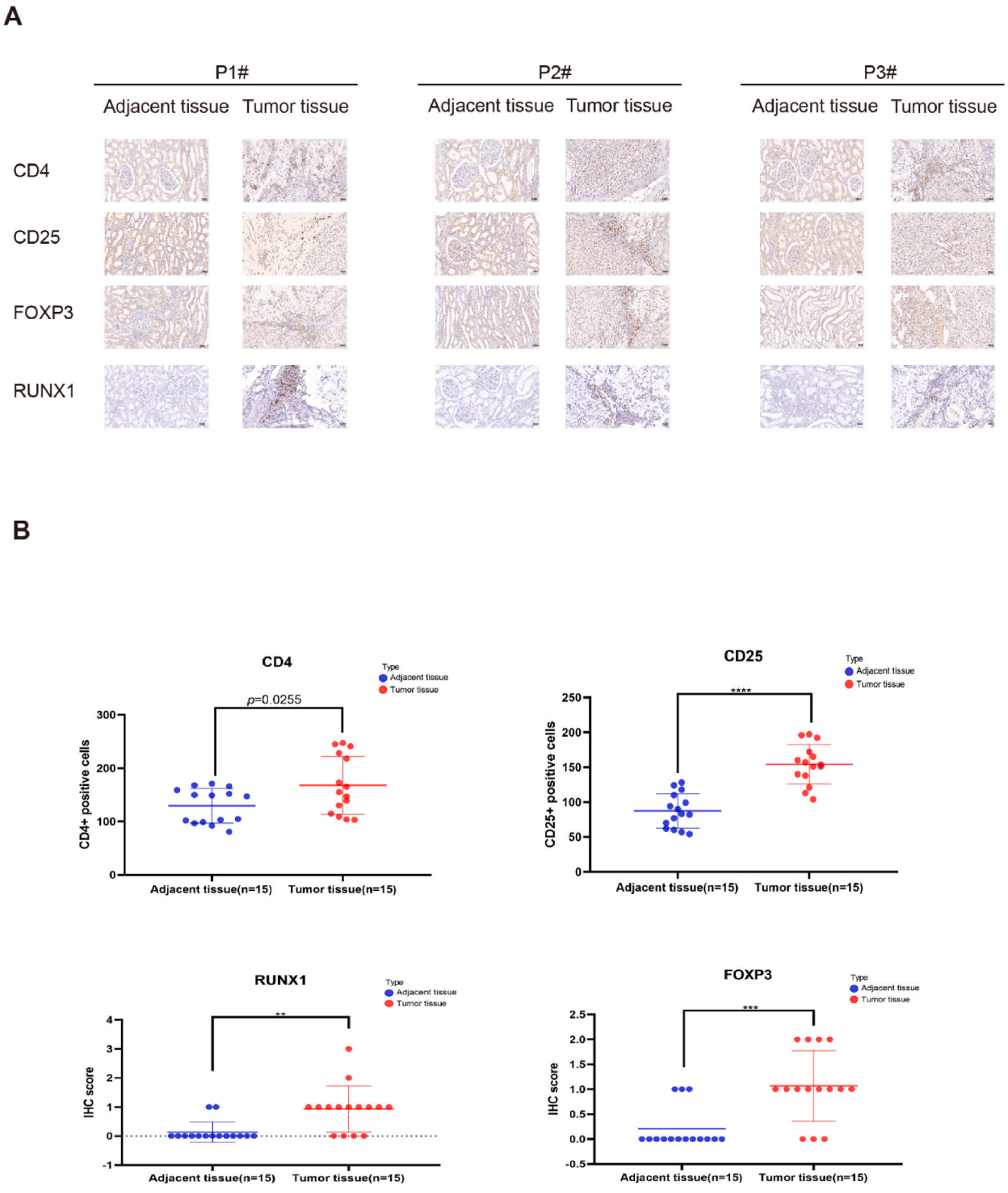

Fig. 8. The expression of RUNX1, CD4, CD25 and Foxp3 in KIRC tissues. (A) IHC analysis of RUNX1, CD4, CD25 and Foxp3 in tumor tissues and tumor-adjacent tissues from the same KIRC patient. (B) Statistical analysis showed that the levels of RUNX1, CD4, CD25 and Foxp3 were higher in tumor tissues than in tumor-adjacent tissues from the same patient (n = 15, *p < 0.05, **p < 0.01,***p < 0.001, ****p < 0.001).

lymphocyte in the thymus requires the involvement of RUNX1 and RUNX3, while RUNX2 and RUNX3 are important for effector B cell growth and differentiation in bone marrow [24]. Additionally, these transcription factors are important in the differentiation of natural killer and memory cytotoxic T lymphocytes [25]. However, the abnormal expression of three transcription factors may weaken

the differentiation ability of these immune cells, thereby increasing the risk of dedifferentiation and tumor transformation. The RUNX transcription factor shows its regulatory function in many human cancers, and its function depends on the tumor environment. Moreover, the three RUNX genes are expressed interdependently, and their crosstalk can result in different or even opposite functions [3]. Hence, it is reasonable to suggest the impact of three RUNX family members expression on KIRC patients' TIIC function.

RUNX1 is overexpressed in many epithelial tumors and shows a complex role in tumor initiation. It exhibits dual characteristics, tumor suppressor and oncogene, in a lot of solid tumors [26–28]. In previous studies, we found that RUNX1 expression is closely related to the infiltration of cancer-associated fibroblasts [13]. Although the exact role of RUNX1 in renal cancer remains unclear, increased RUNX1 expression is linked to the development and prognosis of RCC [29,30]. Our results indicate that RUNX1 expression may have predictive value in patients with KIRC, and was closely related to patients' clinical characteristics, including mRNA and promoter methylation levels. Integrating the infiltrating level of TIICs in the tumor with the clinicopathological features of the patient can be used to predict the response to immunotherapy [31,32]. There was positive correlation between RUNX1 and TIICs, like Tfh, Tregs, macrophages, and monocyte markers, suggesting the relevance of RUNX1 to the infiltration of TIICs in patients with KIRC.

RUNX2 is closely associated with osteoblast differentiation, but its dysregulation may contribute to the progression of several human cancers, including bone tumors and bone metastases [3]. In the study by Pratap et al., overexpression of RUNX2 was found to promote breast cancer cell growth [33]. Furthermore, RUNX2 expression is up-regulated in advanced prostate cancer, which may promote the growth of tumor cells and bone metastases [34]. These results showed that upregulated RUNX2 contributes to the development of KIRC. RUNX2's mRNA expression showed a strong relevance to patients' clinical characteristics, but the promoter methylation level of RUNX2 has no significant difference. The progress of cancer can be inevitably affected by the TME, and RUNX 2 may exhibit an unfavorable role in KIRC prognosis. Moreover, RUNX 2 expression is related to the expression of TIIC markers, such as Tfh and Tregs T cells, macrophages, and monocytes.

RUNX3 has been shown to inhibit the development of several solid tumors [35]. However, recent studies have indicated that RUNX3 promotes proliferation and tumorigenesis in several cancers, and its carcinogenic effect has attracted increasing attention [8]. In addition, RUNX3 is highly expressed in pancreatic cancer, which may contribute to tumor growth and metastasis [36]. While overexpression of RUNX3 has been reported to inhibit the invasive ability of RCC. Considering the complex role of RUNX3 in various tumors, its role in maintaining immune cells and regulating inflammation has received increasing attention [37]. According to our study, RUNX3 promoter methylation increased in KIRC patients and was closely associated with patients' clinical characteristics. Some studies suggest that higher levels of RUNX3 methylation may be an adverse prognostic factor in patients with KIRC [38,39]. According to two databases and tissue verification, RUNX3 showed up-regulated mRNA expression in KIPC patients, but protein expression was not remarkably different. These results may be related to genetic and epigenetic changes in the RUNX3 gene and insufficient sample size. Moreover, our finding indicates that RUNX3 is related to the expression of multiple TIIC markers, like CD8 +, Thf, Th9, and Treg T cells, macrophages, TAM, and monocytes.

Tregs are widely expressed in infiltrating tumors and are involved in tumor progression and angiogenesis [40–42]. The RUNX1 and RUNX3 expression can regulate the differentiation of Tregs and the production of the proinflammatory cytokine, IL-17, which mediates its immunosuppression and cytotoxicity [43]. The overexpression of RUNX3 has been implicated in activating Treg-mediated immunosuppression in breast cancer [37]. Moreover, RUNX2 is involved in inducing Drp 1 activation, promoting T-cell extravasation, and homing in acute lymphoblastic leukemia [44]. Our study paid attention to evaluating the way Treg infiltration level and RUNXs expression affected KIRC patients' prognosis. The survival outcome of patients with high infiltration levels becomes worse with age. However, our analysis was based on publicly available data and a few samples from patients with KIRC, and we need further experiments to verify these bioinformatics analysis hypotheses. Our findings demonstrate the importance of RUNX1 and RUNX2 as potential prognostic biomarkers and that the expression of the three RUNX genes reflects TIICs in KIRC patients.

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Data availability statement

Datasets adopted in the study could be obtained publicly: TCGA (https://portal.gdc.cancer.gov/), Oncomine (https://www.oncomine.org/), Ualcan (ualcan.path.uab.edu/analysis.html), HPA (https://www.proteinatlas.org), LinkedOmics (www.linkedomics.org/login.php), and Kyoto encyclopedia of genes and genomes (https://www.genome.jp/kegg/), gene ontology (http://geneontology.org/) and WebGestalt (www.webgestalt.org/option.php), TIMER (https://cistrome.shinyapps.io/timer/), GEPIA (http://gepia.cancer-pku.cn/), and TISIDB (cis.hku.hk/TISIDB/index.php).

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

All data related to the results of this study are available within the article.

CRediT authorship contribution statement

Zhiwei Jiang: Conceptualization. **Chao Jiang:** Writing – review & editing. **Xiangyu Teng:** Formal analysis. **Yidong Hou:** Validation. **Shuxin Dai:** Resources. **Chang Liu:** Investigation. **Zhouting Tuo:** Software, Conceptualization. **Liangkuan Bi:** Writing – original draft, Formal analysis. **Chao Yang:** Writing – review & editing. **Jinyou Wang:** Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29870.

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