

# Upregulation of ARNTL2 Predicts Poor Survival for Clear Cell Renal Cell Carcinoma and Explores its Associations with PD-L1 and Immune Infiltration

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## Research Article

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# Abstract

**Background:** Aryl hydrocarbon receptor nuclear translocator like 2 (ARNTL2) pertains to the PAS superfamily. Emerging evidences have demonstrated the carcinogenic roles of transcription factor ARNTL2 in human malignancies, while its roles in ccRCC remain elusive. We sought to explore the comprehensive roles of ARNTL2 in ccRCC and place major emphasis on its correlations with tumor immunity.

**Methods:** The available data from GEO, TCGA and GTEx database were combined with our ccRCC patient tissues to verify the upregulation of ARNTL2, Kaplan–Meier survival curve analysis, Cox regression analyses (including univariate and multivariate) were utilized to evaluate the prognostic values of ARNTL2, the potential biological mechanisms of ARNTL2 were analyzed by using GSEA method. The ssGSEA and xCell algorithm were employed to assess the correlations of ARNTL2 expression with tumor immune microenvironment (TIME), The spearman analyses were applied to investigate the relationships between ARNTL2 expression and the tumor mutational burden (TMB), PD-L1 expression and microsatellite instability (MSI) in pan-cancer.

**Results:** ARNTL2 was overexpressed in ccRCC and increased ARNTL2 expression strongly linked to advanced clinical stage and unfavorable overall survival. ARNTL2 was recognized as an independent prognostic marker through cox regression analyses. A prognostic nomogram was subsequently constructed to predict 1-, 3- and 5-year overall survival via integrating ARNTL2 expression with other clinicopathologic variables. GSEA analysis revealed that the focal adhesion, T cell receptor, cell cycle and JAK-STAT signaling pathway were remarkably enriched in high ARNTL2 samples. xCell analysis suggested that high expression of ARNTL2 exhibited an immune infiltration status similar to CD8+ inflamed ccRCC subtype, which characterized by a high infiltration level of CD8+ T cell and elevated expression level of the immune escape biomarkers such as PD-L1, PD-L2, PD1 and CTLA4. Further pan-cancer analysis indicated that ARNTL2 was tightly linked to TMB, MSI, PD-L1 expression, tumor immunity and poor OS in diverse cancer types.

**Conclusions:** ARNTL2 is an independent adverse predictor of ccRCC patient survival and tightly linked to TMB, MSI, PD-L1 expression and immunity.

## Background

Kidney cancer has emerged as top ten cancer killers worldwide recent years, with more than 431,000 new cases and 179,000 deaths annually, among these, clear cell renal cell carcinoma (ccRCC) represents the most common histology of kidney cancer[1, 2]. Despite substantial advancements have been made in the modalities of diagnosis and treatment in the last decades, ccRCC still threatens the health and quality of life of many patients around the globe, especially these metastatic patients that benefit few from currently available treatments[3]. There is increasing evidence that the interactions between cancer cells and the immune system plays a vital role in the carcinogenesis of ccRCC[4, 5], meanwhile, currently

mainstream immunotherapies, such as monoclonal antibodies targeting PD-L1 and/or CTLA4, are becoming the standard paradigm in advanced ccRCC treatment[6]. However, there are still many patients benefit few from immunotherapies since lacking sensitivity to these immunotherapy agents. Therefore, discovering effective prognostic biomarkers and highly specific tumor immune related therapeutic targets are of paramount importance for the treatment of ccRCC patients.

Aryl hydrocarbon receptor nuclear translocator like 2 (ARNTL2) pertain to the PAS superfamily and can encode a transcription factor, whose protein structure is remarkably similar to hypoxia-inducible factors, such as HIF1alpha, ARNTL2 was reported to play important roles in circadian and hypoxia process[7]. Emerging evidences have demonstrated the carcinogenesis roles of ARNTL2 in human malignancies such as breast carcinoma and colorectal adenocarcinoma [8, 9], there are also two recent researches have showed that ARNTL2 correlated with the poor survival and immune infiltration level of lung adenocarcinoma[10, 11]. However, the potential roles of ARNTL2 in ccRCC have not been investigated thus far.

In this study, we demonstrated that ARNTL2 was strikingly elevated in ccRCC through integrated analyses of RNA-seq data from TCGA and GEO database and validated in human ccRCC clinical samples, highly expressed ARNTL2 correlated with advanced clinical tumor stage and poor OS. ARNTL2 can also serve as an independent predictor of ccRCC patient survival. The potential biological functions and mechanisms of ARNTL2 were also explored through GSEA analysis. The ssGSEA and xCell algorithm were subsequently combined to analyze the associations between overexpression of ARNTL2 and TIME in ccRCC. Additionally, we also comprehensively investigated the correlations between ARNTL2 and MSI, TMB, PD-L1 expression, TIME and OS of pan-cancer. We sought to explore the comprehensive roles of ARNTL2 in ccRCC and place major emphasis on its correlations with tumor immunity.

## Materials And Methods

### Data acquisition and analysis

The transcriptome and clinical data of the ccRCC (also namely KIRC) patients were downloaded from the Genomic Data Commons (GDC) data portal of TCGA database (<https://portal.gdc.cancer.gov/>), including 539 ccRCC tissues and 72 normal tissues. The data of GSE15641, GSE46699 and GSE53757 datasets from GEO database (<https://www.ncbi.nlm.nih.gov/geo/>) were downloaded and used to further validate the expression level of ARNTL2. We also downloaded the genetic mutation data from TCGA and visualized using the package “maftools” in R software[12]. In the section involving the pan-cancer analysis of ARNTL2, the transcriptome and clinical data of thirty-three cancer types were also obtained through GDC portal of TCGA, while data of normal tissues were obtained in GTEx database (<https://gtexportal.org/home/datasets>) V8 version.

### Human clinical samples and qRT-PCR analysis

Twenty pairs ccRCC and adjacent nontumorous samples were gathered from patients who had received radical nephrectomy (from 2011 to 2013) at first affiliated hospital of Zhejiang University, informed consents were acquired from every patient and this study was approved by Institutional Ethics Committee in the hospital. All patients' clinical information was shown in Supplementary Table 1. Total RNA was extracted with TRIzol agentia (Takara) and with the help of PrimeScript RT Reagent Kit (Takara), RNA was then reverse transcribed into cDNA. The ARNTL2 mRNA relative expression was detected by RT-qPCR, through utilizing the ABI 7500 fast real-time PCR System (Applied Biosystems) and SYBR Premix Ex Taq (Takara). GAPDH was used as endogenous normalization reference to quantify the relative mRNA expression of ARNTL2. All primers are listed: ARNTL2 F 5'-ACTTGGTGCTGGTAGTATTGGA-3'; ARNTL2 R 5'-TGTTGGACTCGAATCATCAAGG-3'; GAPDH F 5'-CTGGGCTA

CACTGAGCACC-3'; GAPDH R 5'- AAGTGGTCGTTGAGGGCAATG-3'.

### **Univariate and multivariate cox regression analysis and the construction of nomogram model**

Cox regression analyses including univariate and multivariate analysis were employed to identify independent overall survival-related factors, the forest plot was used to show the P value, HR and 95% CI of each variable through 'forest plot' R package. In order to contribute to predict the OS possibility of ccRCC individuals, a nomogram was subsequently developed based upon the results of multivariate Cox proportional hazards through utilizing the 'rms' R package. The Kaplan-Meier plotter analysis (<http://kmplot.com/analysis/>) and prognoscan analysis based upon GEO database (<http://dna00.bio.kyutech.ac.jp/PrognoScan/index.html/>) were utilized to externally validate the prognostic values of ARNTL2 in ccRCC and other cancer types, respectively.

### **Gene set enrichment analysis (GSEA)**

To investigate the underlying mechanisms and biological functions of ARNTL2, we used the "Cluster Profiler" (version: 3.18.0) package to conduct the GSEA analysis[13] to explore the KEGG signaling pathway analysis of ARNTL2 in ccRCC, in the enrichment results, adjust p value <0.01 and q-values<0.01 were recognized as a significantly meaningful pathway.

### **Tumor immune microenvironment analysis**

To perform a reliable assessment of immune infiltration, we preliminarily assess the associations of the expression of ARNTL2 and its top three co-expressed genes with various tumor infiltrated immune cell types by using the ssGSEA algorithm of the GSVA package[14]. The xCell algorithm[15] was subsequently employed to calculate the relative proportions of diverse tumor-infiltrating immune cells in every cancer sample, and the results were implemented by R packages "immunedeconv" and "pheatmap", xCell algorithm was also applied to explore the correlations between ARNTL2 and tumor-immune infiltration cell across thirty-three cancer types. To further estimate the relationships of ARNTL2 expression with the tumor immune microenvironment (TIME) in pan-cancer, we also extract the expression values of seven

most common immune checkpoint-related genes (PD1, PD-L1, CTLA4, TIM3, LAG3, PD-L2 and TIGIT) and assessed the correlations of their expression with ARNTL2 across 33 cancer types.

### **The association of ARNTL2 with tumor mutational burden (TMB), microsatellite instability (MSI)**

In order to investigate the correlations between ARNTL2 expression and TMB/MSI, we downloaded and analyzed the TMB data from the article [16] published by Vesteyinn Thorsson et al. in 2018 and obtained MSI data from the article [17] published by Russell Bonneville et al. in 2017. We utilized Spearman's correlation analysis to evaluate the associations of ARNTL2 expression with TMB/MSI by using R software v4.0.3.

### **Statistical analysis**

Statistics in this study were conducted with the R version 4.0.3, SPSS 24.0 and GraphPad Prism 8.0. Wilcoxon test and Kruskal-Wallis test were respectively utilized to implement the intergroup comparisons of two and three groups. Spearman correlation analyses were implemented to assess the correlations of ARNTL2 expression with TMB, MSI, mismatch repair genes and PD-L1 expression. For Kaplan–Meier curves, hazard ratio (HR) and p-values were yielded by univariate Cox proportional hazards regression. The predictive model of ARNTL2 was estimated with the receiver operating characteristic curves (ROC) via using pROC package of R software.  $p < 0.05$  denoted statistical significance.

## **Results**

### **Elevated expression levels of ARNTL2 in ccRCC**

We preliminarily investigated the expression level of ARNTL2 in pan-cancer by analyzing TCGA and GTEx database. ARNTL2 was observed to be upregulated in various cancer types, including ccRCC (Fig. 1a, b), a paired line graph of 72 pairs of ccRCC samples and matching nontumorous samples indicated that most ccRCC tissues possessed higher ARNTL2 expression compared to matching normal tissues (Fig. 1c). Three independent datasets from GEO database were utilized to externally illustrate the expression of ARNTL2 in ccRCC, which also demonstrate the highly expressed level of ARNTL2 in ccRCC (Fig. 1d-e), and increased ARNTL2 expression significantly associated with advanced ccRCC clinical stage (Fig. 1f, Table 1) and tumor histologic grade (Table 1). Additionally, we demonstrated that most ccRCC tissues possessed higher ARNTL2 expression compared to adjacent nontumorous in 20 pairs ccRCC tissues via qRT-PCR method (Fig. 1g). These results revealed that ARNTL2 was significantly elevated in ccRCC.

Table 1  
Clinicopathological correlation of ARNTL2 expression in human ccRCC.

Characteristic	Low expression of ARNTL2	High expression of ARNTL2	p <sup>a</sup>
n	269	270	
Age, n (%)			0.518
<=60	130 (24.1%)	139 (25.8%)	
>60	139 (25.8%)	131 (24.3%)	
Gender, n (%)			0.903
Female	94 (17.4%)	92 (17.1%)	
Male	175 (32.5%)	178 (33%)	
T stage, n (%)			0.027
T1-T2	183 (33.9%)	166 (30.8%)	
T3-T4	86 (16%)	95 (19.3%)	
N stage, n (%)			0.034
N0	119 (46.3%)	122 (47.5%)	
N1	3 (1.2%)	13 (5.1%)	
M stage, n (%)			0.118
M0	220 (43.5%)	208 (41.1%)	
M1	32 (6.3%)	46 (9.1%)	
Pathologic stage, n (%)			0.031
Stage I-II	174 (32.5%)	157 (29.3%)	
Stage III-IV	95 (17.7%)	110 (20.6%)	
Histologic grade, n (%)			0.012
G1-3	233 (43.9%)	223 (42%)	
G4	31 (5.8%)	44 (8.3%)	
<sup>a</sup> p-values were derived with chi-square test.			

### Multivariate cox regression analysis of ARNTL2 and construction of nomogram model

We then evaluated the prognostic values of ARNTL2 in ccRCC, as plotted in Fig. 2a and b, elevated expression of ARNTL2 indicated poor OS in both TCGA (HR = 1.67, P = 0.001) and Kaplan-Meier plotter

(HR = 1.91, P = 2.2e-05) database. The cox analyses were also applied to explore associations between ARNTL2 expression and OS in ccRCC, univariate analysis indicated that ARNTL2 expression (HR = 1.45853, p = 0.00029), age (HR = 1.02888, p = 1e-05), pathological TNM stage (HR = 1.86653, p < 0.0001) and tumor grade (HR = 2.29073, p < 0.0001) are significantly correlated with OS of ccRCC (Fig. 2c). Multivariate analysis, depicted as a forest boxplot in Fig. 2d, revealed that ARNTL2 expression (p = 0.01016), as well as the age (p = 1e-05), pathological TNM stage (p < 0.0001) and tumor grade (p = 0.00083) were independent predictor of ccRCC patient OS. ARNTL2 expression also showed preferable predictive ability as the ROC curve exhibited that the AUC of ARNTL2 expression for predicting OS was 0.773 (Fig. 2e). Furthermore, we developed a nomogram model by integrating ARNTL2 and other independent prognostic variables base on the multivariate cox regression analysis results (Fig. 2f), the nomogram can contribute to quantitatively assess ccRCC patients 1-y, 3-y and 5-y survival probability.

### **Mutation patterns of SMGs in different ARNTL2 expression level of ccRCC**

In order to investigate mutation patterns of significantly mutated genes (SMGs) in different ARNTL2 expression level of ccRCC, we downloaded and analyzed the genetic mutation data of ccRCC patients from TCGA database. We identified 30 SMGs in ccRCC cohort, among these, VHL, PBRM1, TTN, SETD2, BAP1, MUC16 were the top six frequently mutated genes in ccRCC (Fig. 3a). Subsequently, we divided the ccRCC patients with genetic mutation data into two groups based upon the median expression value of ARNTL2, namely ARNTL2 high and ARNTL2 low group. As shown in Fig. 3b-g, we explore the mutation patterns of top six SMGs in two ARNTL2 groups. Significantly higher mutation frequency of PBRM1 was observed in ARNTL2 low expression group (P < 0.001) (Fig. 3b), while other SMGs were found no differences in two ARNTL2 groups (P > 0.05) (Fig. 3c-g).

## **Gsea Analysis Of Arntl2 In Ccrcc**

To investigate the potential functions and mechanisms of ARNTL2 in ccRCC, we utilized GSEA analysis to explore ARNTL2-related pathways in ccRCC carcinogenesis, GSEA analysis contribute to reveal significantly enriched KEGG pathways in highly expressed ARNTL2 samples with a high accuracy. The results revealed that renal cell carcinoma (NES = 1.96614, P-adjust < 0.001), focal adhesion (NES = 2.06698, P-adjust < 0.001), Toll-like receptor signaling pathway (NES = 1.965199, P-adjust < 0.001), JAK-STAT signaling pathway (NES = 1.999494, P-adjust < 0.001), T cell receptor signaling pathway (NES = 2.036177, P-adjust < 0.001) and cell cycle pathways (NES = 1.551655, P-adjust < 0.005) (Fig. 4a-f, Table 2) were significantly enriched in upregulated ARNTL2 samples. These results indicated that the immune response and cell cycle related pathways were strongly correlated with abnormal expression of ARNTL2 in ccRCC.

Table 2  
Gene set enrichment analysis (GSEA) of ARNTL2 in ccRCC.

GeneSet name	NES*	p-adjust	q-values
KEGG_FOCAL_ADHESION	2.06698	0.000503	0.000288
KEGG_JAK_STAT_SIGNALING_PATHWAY	1.999494	0.000503	0.000288
KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY	2.036177	0.000503	0.000288
KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	1.965199	0.000503	0.000288
KEGG_RENAL_CELL_CARCINOMA	1.96614	0.000503	0.000288
KEGG_CELL_CYCLE	1.551655	0.002661	0.001522
*NES: Normalized Enrichment Score.			

### Significant correlations between the expression of ARNTL2 and its co-expressed genes and tumor infiltrating immune cells in ccRCC

In order to further explore the potential functions of ARNTL2 in the ccRCC carcinogenesis, we employed R software “stat” package to identify ARNTL2 positively co-expressed genes, and the strongly co-expressed genes (spearman analysis,  $r > 0.70$ ,  $p < 0.001$ ) were selected to further analysis, as shown in Fig. 5a-5b, the actin related protein 2/3 complex subunit 2 (ARPC2), guanine nucleotide binding protein beta polypeptide 4 (GNB4) and capping-protein muscle Z line alpha 1 (CAPZA1) were the only three positively co-expressed genes with spearman coefficient higher than 0.7 ( $p < 0.001$ ). Subsequently, we applied ssGSEA algorithm in R package GSVA to preliminarily estimate the relationships between the expression of ARNTL2 and its top 3 co-expressed genes and tumor infiltrating immune cell types, as shown in Fig. 5c, ARNTL2 and its co-expressed genes ARPC2, GNB4, CAPZA1 were remarkably associated with the infiltrating level of T helper cells, macrophages, T cells, B cells and dendritic cells (DC) ( $P < 0.001$ ), while showed weak associations with neutrophils and mast cells ( $P < 0.01$ ). Further correlation analysis demonstrated that ARNTL2 expression significantly associated with the enrichment of T cells ( $r = 0.440$ ,  $P < 0.001$ ), T helper cells ( $r = 0.580$ ,  $P < 0.001$ ), Macrophages ( $r = 0.460$ ,  $P < 0.001$ ), B cells ( $r = 0.350$ ,  $P < 0.001$ ) and DC ( $r = 0.300$ ,  $P < 0.001$ ) (Fig. 5d). In summary, these results indicated that ARNTL2 might be participated in the immune response in ccRCC TIME.

The xCell algorithm was further employed to calculate the fraction of diverse tumor-infiltrating immune cells in the TIME of high and low ARNTL2 expression ccRCC samples. Distinct infiltrating levels of immune cells were observed in two ARNTL2 groups (Fig. 6a). With the rise of the ARNTL2 expression, the immune score ( $P < 0.001$ ), microenvironment score ( $P < 0.001$ ) and stroma score ( $P < 0.05$ ) in ccRCC TIME were enhanced (Fig. 6b), high expression level of ARNTL2 tightly linked to the infiltrating levels of CD8 + T cell, CD4 + memory T cell, Myeloid dendritic cell, macrophage and CD4 + Th2 T cell, while CD4 + Th1 T cell, B naive cell and NK T cell were downregulated in highly ARNTL2 expression group (Fig. 6c). Additionally, the immune checkpoint-related genes such as PD-L1, PD-L2, CTLA4, PD-1, TIM3, LAG3 and

TIGIT were also tightly related to the expression level of ARNTL2 (Fig. 6d). These results implied that ARNTL2 might be related with T cell exhaustion and immune evasion in ccRCC.

### **Associations of ARNTL2 with PD-L1, TMB, MSI and mismatch repair genes**

We observed that in addition to ccRCC, ARNTL2 was also highly expressed in various tumor types compared to their corresponding normal tissues (Fig. 1a), we subsequently desired to assess whether ARNTL2 has a universal role in immune response in pan-cancer. Considering the GSEA results indicated the potential KEGG pathway of ARNTL2 in regulation of T cell receptor signaling pathway (Fig. 4e), we firstly explore the correlations between PD-L1 and ARNTL2 expression in pan-cancer, as shown in Fig. 7a, ARNTL2 expression remarkably correlated with the expression level of PD-L1 in various cancer types, including ccRCC. Meanwhile, ARNTL2 expression showed positive associations with the TMB of Uterine Carcinosarcoma (UCS), Sarcoma (SARC), Colon adenocarcinoma (COAD), Pancreatic adenocarcinoma (PAAD), Brain Lower Grade Glioma (LGG), Stomach adenocarcinoma (STAD), Breast invasive carcinoma (BRCA), Bladder Urothelial Carcinoma (BLCA), Lung adenocarcinoma (LUAD) and Ovarian serous cystadenocarcinoma (OV) ( $P < 0.01$ ), while possessed negative associations with the TMB of Uveal Melanoma (UVM), Esophageal carcinoma (ESCA) and Lung squamous cell carcinoma (LUSC) ( $P < 0.01$ ) (Fig. 7b). For MSI, another important prognostic indicator besides the PD-L1 and TMB in cancer immunotherapy, ARNTL2 also showed positive associations with the MSI of Mesothelioma (MESO), Adrenocortical carcinoma (ACC), Testicular Germ Cell Tumors (TGCT), SARC, Rectum adenocarcinoma (READ), UVM, UCS, Acute Myeloid Leukemia (LAML), Stomach adenocarcinoma (STAD) and OV ( $P < 0.01$ ), while negatively associated with the MSI of Lymphoid Neoplasm Diffuse Large B-cell Lymphoma (DLBC),Cholangiocarcinoma

(CHOL), HNSC, LUAD, and Pheochromocytoma and Paraganglioma (PCPG) and GBM ( $P < 0.01$ ) (Fig. 7c). Furthermore, we also explore the relationships between ARNTL2 expression and mismatch repair genes such as MLH1, MSH2, MSH6 and PMS2, the results demonstrated that ARNTL2 tightly related with the expression of mismatch repair genes, especially the expression of MSH2 and MSH6 (Fig. 7d). In summary, ARNTL2 expression showed significant correlations with the level of three vital prognostic indicators (PD-L1, TMB and MSI) for cancer immunotherapy in various cancer types.

## **Associations Of Arntl2 With Time In Pan-cancer**

To further investigate the influence of ARNTL2 on TIME of pan-cancer, xCell algorithm was employed to explore the links of ARNTL2 expression with cancer- infiltrating cells in pan-cancer. The results showed that ARNTL2 was tightly related to the enrichment of immune cells, especially the Th2 CD4 + T cell, CD4 + memory T cell, Macrophages and (Fig. 8a), while remarkably correlated with the decrease of NK T cell and CD4 + central memory cell. Immune checkpoint-related genes had been demonstrated to be involved in immune escape in carcinogenesis, among these, PD-1, PD-L1, PD-L2, CTLA4, LAG3, TIM3, TIGIT were the seven most common immune checkpoint related genes, our results also indicated that ARNTL2

correlated with the expression level of these immune escape markers in various cancers, including ccRCC (Fig. 8b). These results further illustrated the vital role of ARNTL2 in the TIME of pan-cancer.

### **Overexpression of ARNTL2 predicted unfavorable overall survival in multiple cancer types**

The relationships between ARNTL2 expression and OS of cancer patients were also analyzed both in TCGA and GEO database, as shown in Fig. 9a-9h, highly expressed ARNTL2 was tightly associated with the dismal OS in LUAD (HR = 1.62,  $P < 0.01$ ) (Fig. 9a), MESO (HR = 2.77,  $P < 0.001$ ) (Fig. 9b), Glioblastoma multiforme and Lower Grade Glioma (GBMLGG) (HR = 4.83,  $P < 0.001$ ) (Fig. 9c), LGG (HR = 2.10,  $P < 0.001$ ) (Fig. 9d), UVM (HR = 4.63,  $P = 0.003$ ) (Fig. 9e), UCEC (HR = 1.66,  $P = 0.016$ ) (Fig. 9f), PAAD (HR = 1.87,  $P = 0.004$ ) (Fig. 9g) and LIHC (HR = 2.11,  $P = 0.004$ ) (Fig. 9h) in TCGA database. ARNTL2 also showed poor OS in Glioma (HR = 3.25,  $P < 0.001$ ) (Fig. 9i), Astrocytoma (HR = 1.73,  $P = 0.004$ ) (Fig. 9j), Lung Adenocarcinoma (HR = 1.36,  $P < 0.01$ ) (Fig. 9k) and AML (HR = 2.38,  $P < 0.01$ ) (Fig. 9l) in GEO database. In summary, highly expressed ARNTL2 showed unfavorable OS and can serve as an adverse prognostic biomarker in multiple prevailing cancers.

## **Discussion**

Although substantial advancements have been made in therapeutic strategies such as surgical excision, chemotherapy and targeted therapies of ccRCC in recent years, ccRCC still influences the health and quality of life of many patients around the world[3]. Immunotherapy has emerged as the last chance for advanced ccRCC patients, especially for patients who cannot tolerate chemotherapy, while most patients unable to obtain durable benefits from currently immunotherapy represented by anti-PD-1 antibodies[18, 19]. It is urgent to identify effective biomarkers and immune-related therapeutic targets to improve the patients' survival. In this study, we firstly discovered that the upregulated ARNTL2 correlated with unfavorable OS and immune infiltration in ccRCC, we also comprehensively investigated the associations between ARNTL2 expression and PD-L1 expression, TMB, MSI, immune infiltration and adverse prognosis in pan-cancer.

ARNTL2 is a member of PAS protein superfamily, located on chromosome 12p11, encodes a transcription factor, whose structure similar to HIF1alpha[7]. The potential roles of ARNTL2 has been explored in several human malignancies. Mazzocchi et al. discovered that ARNTL2 was upregulated in colorectal cancer specimens and could serve as an independent predictor of colorectal cancer patient survival[9]. Brady et al. demonstrated that highly expressed transcription factor ARNTL2 associated with dismal survival of lung adenocarcinoma patients[20]. However, the clinical values and potential functions of ARNTL2 in ccRCC remains elusive. In this study, we carried out a systematical analysis through integrating available data from GEO and TCGA database and validated the overexpression of ARNTL2 in human ccRCC clinical samples, overexpressed ARNTL2 correlated with advanced clinical tumor stages and grades, we also found that upregulation of ARNTL2 predicted poor overall survival and could serve as an independent prognostic variable in ccRCC. Additionally, in order to contribute to clinical decision

making, a nomogram was subsequently developed based upon the results of multivariate Cox analysis including the expression level of ARNTL2 in ccRCC.

Recent studies have recognized PBRM1 as the second-most common mutated gene behind VHL in ccRCC[21, 22]. In the present study, we downloaded and analyzed the genetic mutation information of ccRCC patients from TCGA database, PBRM1 also ranked second among 30 significantly mutated genes (SMGs) in ccRCC, the enrolled patients with genetic mutation data were subsequently separated into low-ARNTL2 and high-ARNTL2 group, we further analyzed the SMGs between this two ARNTL2 groups. Interestingly, PBRM1 mutation occurred significantly higher in ARNTL2 low group compared to ARNTL2 high group, while there were no differences of other SMGs between two groups. Miao et al. reported that PBRM1-mutated ccRCC patients could benefit more from immune checkpoint inhibitor (ICI) therapy[5], Braun et al. demonstrated that PBRM1 mutations could serve as a marker of ICI response in ccRCC in a randomized clinical trial[23]. These results implied that low ARNTL2 expression ccRCC patients with high PBRM1 mutation rates might obtain more clinical benefits from ICI therapy. Furthermore, two recent studies have revealed that ARNTL2 correlated with prognosis and immune infiltration of lung adenocarcinoma[10, 11]. Which also indicated ARNTL2 might influence the TIME of cancer. Thus, we subsequently explored the possible functions and mechanisms of ARNTL2 through GSEA analysis, the results indicated that ARNTL2 might participated in the signaling pathways including T cell receptor signaling pathway, renal cell carcinoma, focal adhesion, JAK-STAT signaling pathway and cell cycle pathway. JAK-STAT signaling pathway has been implicated in carcinogenesis and immune infiltration of ccRCC[24, 25], Fang et al. reported simvastatin could inhibit the growth and metastasis of renal cancer cell growth through JAK2/STAT3, ERK and AKT/mTOR pathway [26]. Miao et al discovered that the alterations of JAK-STAT and immune signaling pathways in PBRM1-deficient renal cancer cells[5]. These findings revealed that ARNTL2 might influence the TIME of ccRCC via activating JAK-STAT signaling pathway.

To explore the potential roles of ARNTL2 in the TIME of ccRCC, we initially employed ssGSEA algorithm to assess the associations of the expression of ARNTL2 and its top three co-expressed genes with various tumor infiltrated immune cell types in ccRCC, the results revealed that ARNTL2 and its co-expressed genes remarkably linked to the enrichment of T cells, T helper cells and macrophages in ccRCC. Furthermore, by using the xCell algorithm analysis, we found distinct immune score, microenvironment score, stroma score, infiltrating immune cell types and immune-evasion marker genes such as PD-1, PD-L1 expression between ARNTL2 high and low group. Notably, high ARNTL2 correlated with high infiltration level of CD8 + T cell, macrophage, DC and Th2 CD4 + T cell, as well as overexpression of immune escape-related genes such as PD-1, PD-L1, PD-L2, CTLA4. While low ARNTL2 expression group possessed high infiltration level of CD4 + Th1 T cell and NK T cells. Clark et al. utilized the xCell algorithm and combined molecular characteristics stratified ccRCC into four immune subtypes, among these four subtypes, CD8 + inflamed subtype correlated with the most worse prognosis and were characterized by a highly infiltrated level of CD8 + T cell, exceptionally overexpressed immune-escape genes such as PD1, PD-L1, PD-L2, and CTLA4 [27]. Braun et al. also illustrated that high infiltration level

of CD8 + T cell predicted poor prognosis of patients with ccRCC [28]. These findings indicated that ARNTL2 might involve in the formation of CD8 + inflamed ccRCC subtype and immune escape.

Previous studies have evidenced that the cancer cells could evade anti-tumor immunity and drive tumor development by overexpressing PD-L1 to interact with immune cells [29, 30]. We applied a correlation analysis across 33 cancer types and found that significantly positive associations between ARNTL2 and PD-L1 expression, as well as the other immune-checkpoint related genes expression. In addition, we also utilized xCell algorithm to investigate the connections of ARNTL2 expression with the infiltrating level of immune cell across 33 cancer types, the results revealed that ARNTL2 positively associated with Th2 CD4 + T cell and CD4 + memory T cell, while negatively correlated with Th1 CD4 + T cell and NK T cell across diverse cancer types. Accumulating studies have revealed that Th2 leading inflammation was implicated in the development of cancer and facilitated tumor immune escape[31–33]. The ratio of Th2/Th1 in the TIME of cancer could act as an independent prognostic biomarker of cancer patients' survival[34]. These findings indicated that ARNTL2 might implicated in Th1/2-polarizing in cancer. Furthermore, we also found that elevated ARNTL2 expression correlated with dismal prognosis in a variety of cancer types besides ccRCC through analyzing the clinical data from TCGA and GEO database. In short, ARNTL2 might be an effective prognostic marker and a potential target for cancer immunotherapy.

Nevertheless, several limitations also merit attentions. Firstly, the associations between the TIME and ARNTL2 were analyzed only based on TCGA database due to paucity of applicable data in our own cohort, although we illustrated the correlations through multiple methods. Further reliable clinical trials with larger samples are needed to be implemented in the future. Additionally, the underlying regulatory mechanisms (intrinsic? extrinsic? or both?) of ARNTL2 in cancer still need a series of basic studies and clinical trials to be elucidated.

## Conclusions

To conclude, our study revealed that the elevated expression of ARNTL2 correlated with unfavorable prognosis, PD-L1 expression, TMB, MSI and immune infiltration in diverse prevailing cancer types, including ccRCC. ARNTL2 could also serve as an independent prognostic marker for ccRCC. Moreover, the interactions between ARNTL2 and focal adhesion pathway, JAK-STAT signaling pathway, cell cycle pathway, Toll-like receptor pathway and T cell receptor signaling pathway might involve in the carcinogenesis and regulation of TIME of ccRCC. High expression of ARNTL2 exhibited immune infiltration status similar to CD8 + inflamed ccRCC subtype, which characterized by a highly infiltrated level of CD8 + T cell, exceptionally overexpressed immune-escape genes such as PD1, PD-L1, PD-L2, and CTLA4. Further deep studies based on basic experiments in vivo and in vitro and large samples randomized clinical trials are warranted to verify these findings.

## Abbreviations

GEO, Gene Expression Omnibus; ARNTL2, Aryl hydrocarbon receptor nuclear translocator-like 2; TCGA, The Cancer Genome Atlas; GSEA, Gene set enrichment analysis; GTEX, Genotype-Tissue Expression; TIME: Tumor immune microenvironment; TMB, tumor mutational burden; OS: Overall survival; MSI, microsatellite instability; ccRCC, Clear cell renal cell carcinoma; PD-L1, Programmed cell death ligand 1; CTLA4, cytotoxic T-lymphocyte antigen 4; LAG3, lymphocyte-activation gene 3; PD1, programmed cell death 1; TIM3, T cell immunoglobulin domain and mucin domain 3; PD-L2, Programmed death ligand 2; TIGIT, T cell immunoglobulin and ITIM domain; ROC, receiver operating characteristic curve; SMGs, significantly mutated genes; ARPC2, actin related protein 2/3 complex subunit 2; GNB4, guanine nucleotide binding protein beta polypeptide 4; CAPZA1, capping protein muscle Z line alpha 1; UCS, Uterine Carcinosarcoma; SARC, Sarcoma; COAD, Colon adenocarcinoma; PAAD, Pancreatic adenocarcinoma; BRCA, Breast invasive carcinoma; LGG, Brain Lower Grade Glioma; BLCA, Bladder Urothelial Carcinoma; OV, Ovarian serous cystadenocarcinoma; STAD, Stomach adenocarcinoma; LUAD, Lung adenocarcinoma; UVM, Uveal Melanoma; ESCA, Esophageal carcinoma; MSH2, mutS homolog 2; LUSC, Lung squamous cell carcinoma; MESO, Mesothelioma; ACC, Adrenocortical carcinoma; MLH1, MutL Homolog 1; TGCT, Testicular Germ Cell Tumors; READ, Rectum adenocarcinoma; LAML, Acute Myeloid Leukemia; STAD, Stomach adenocarcinoma; MSH6, mutS homolog 6; DLBC, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; CHOL, Cholangiocarcinoma; HNSC, Head and Neck squamous cell carcinoma; PCPG, Pheochromocytoma and Paraganglioma; GBMLGG, Glioblastoma multiforme and Lower Grade Glioma; PMS2, post meiotic segregation increased 2.

## **Declarations**

### **Acknowledgments**

Not applicable.

### **Authors' contributions**

SW, XM, YY drafted and wrote the manuscript. SW, JS, ZY performed the data collecting. SW, JL conducted the bioinformatics analysis. JK, XW, BX searched the related literatures, XZ, BL and LX revised the manuscript. All authors approved the final manuscript.

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### **Availability of data and materials**

The datasets exhibited in present study can be discovered in online repositories, further inquiries can be directed to the corresponding author.

## Ethics approval and consent to participate

The human participants in this study were approved by First Affiliated Hospital, School of Medicine, Zhejiang University, they also provided written informed consent to participate in this research.

## Consent for publication

All authors agreed on the manuscript.

## Competing interests

The authors declare no potential conflicts of interest.

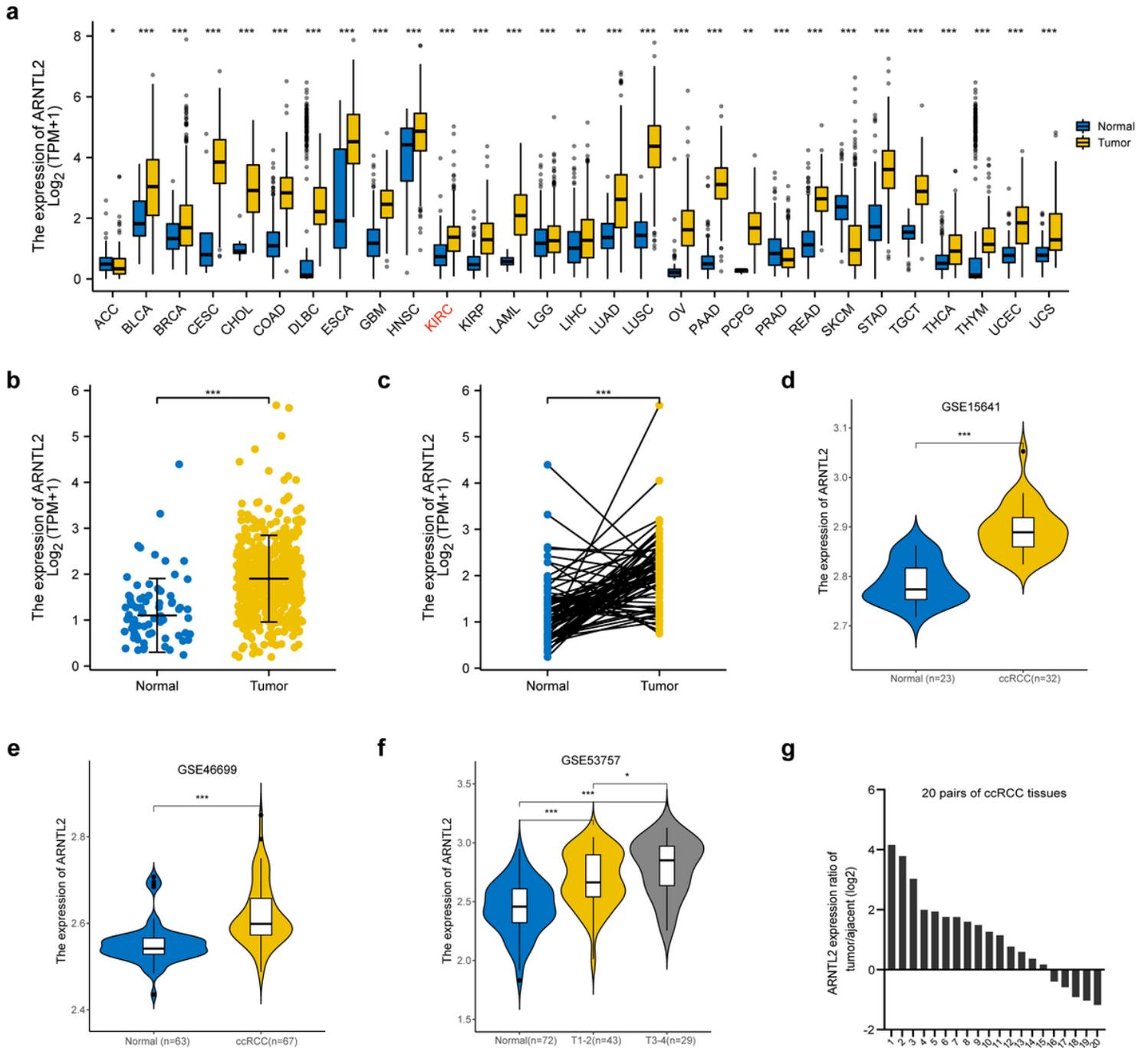
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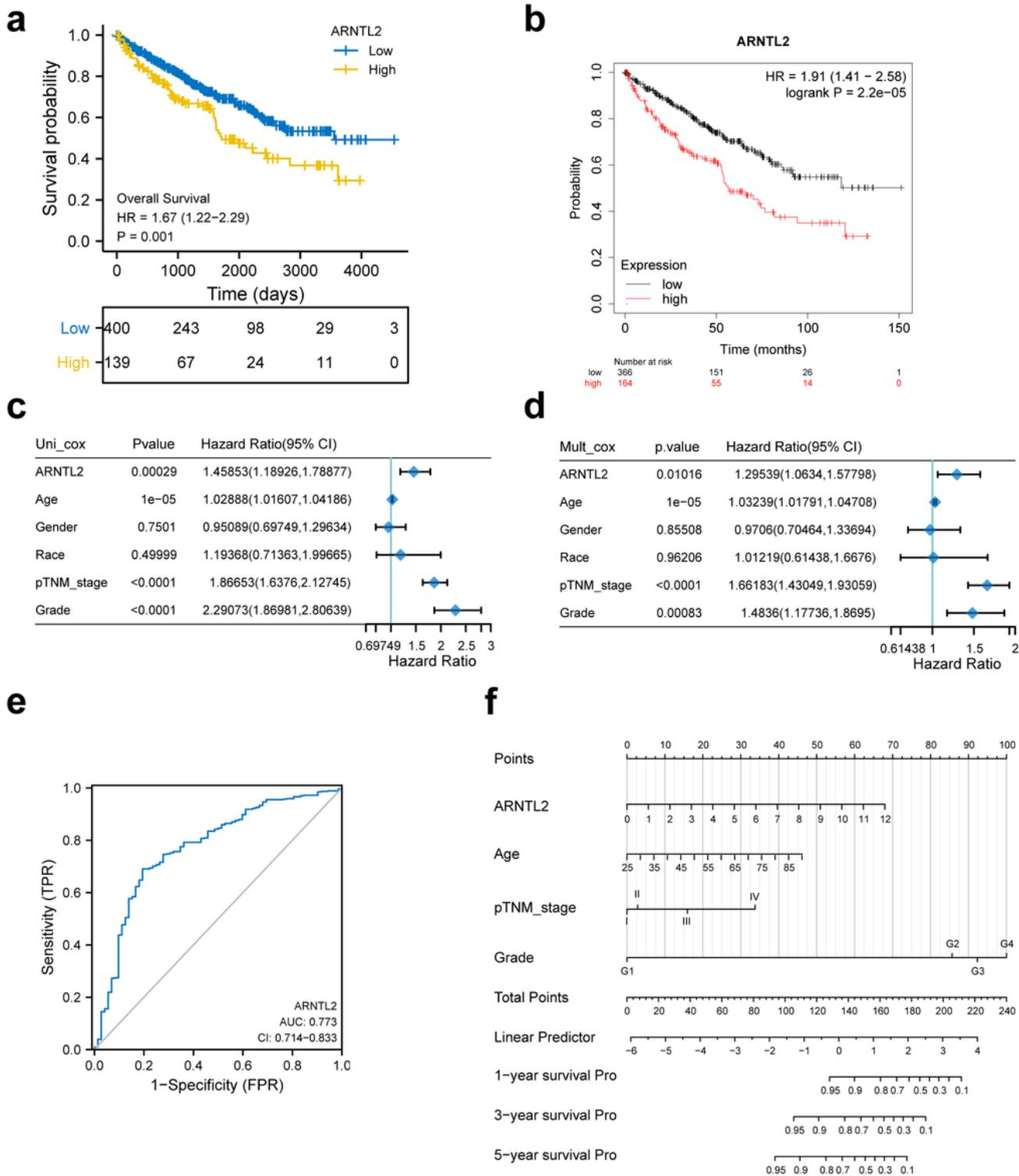
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## Figures



**Figure 1**

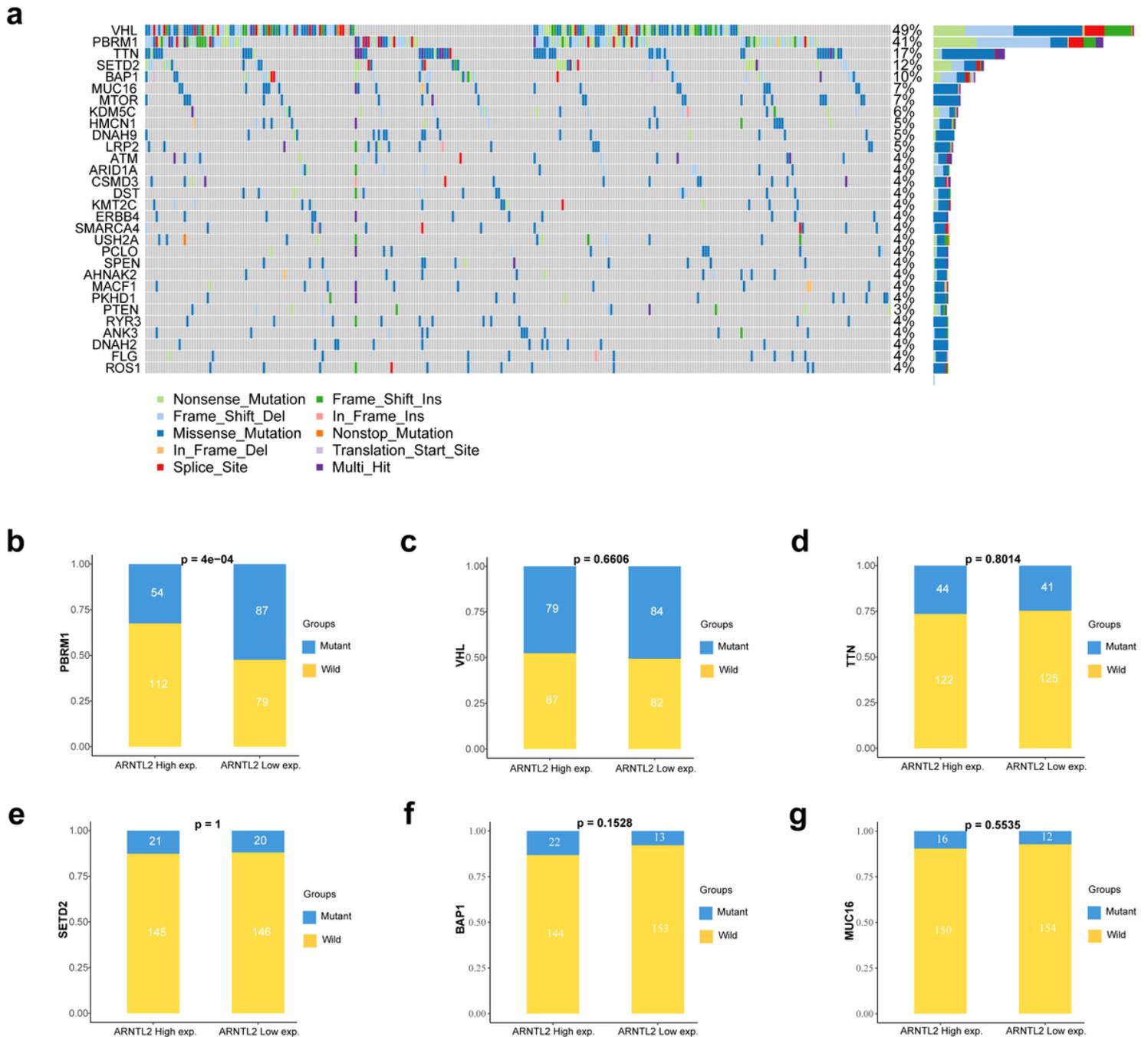
Elevated ARNTL2 expressed in ccRCC. a The expression of ARNTL2 in diverse cancer types in TCGA and GTEx dataset. ARNTL2 was overexpressed in ccRCC tissues compared to normal kidney tissues in b non-paired (N = 72; T = 539) and c paired samples (N = 72; T = 72). The violin plots visualized the relative expression of ARNTL2 in ccRCC in d GSE15641, e GSE46699 and f GSE53757 dataset. g The relative expression levels of ARNTL2 in 20 pairs of ccRCC and adjacent normal tissues. \* $p < 0.05$ ; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 2**

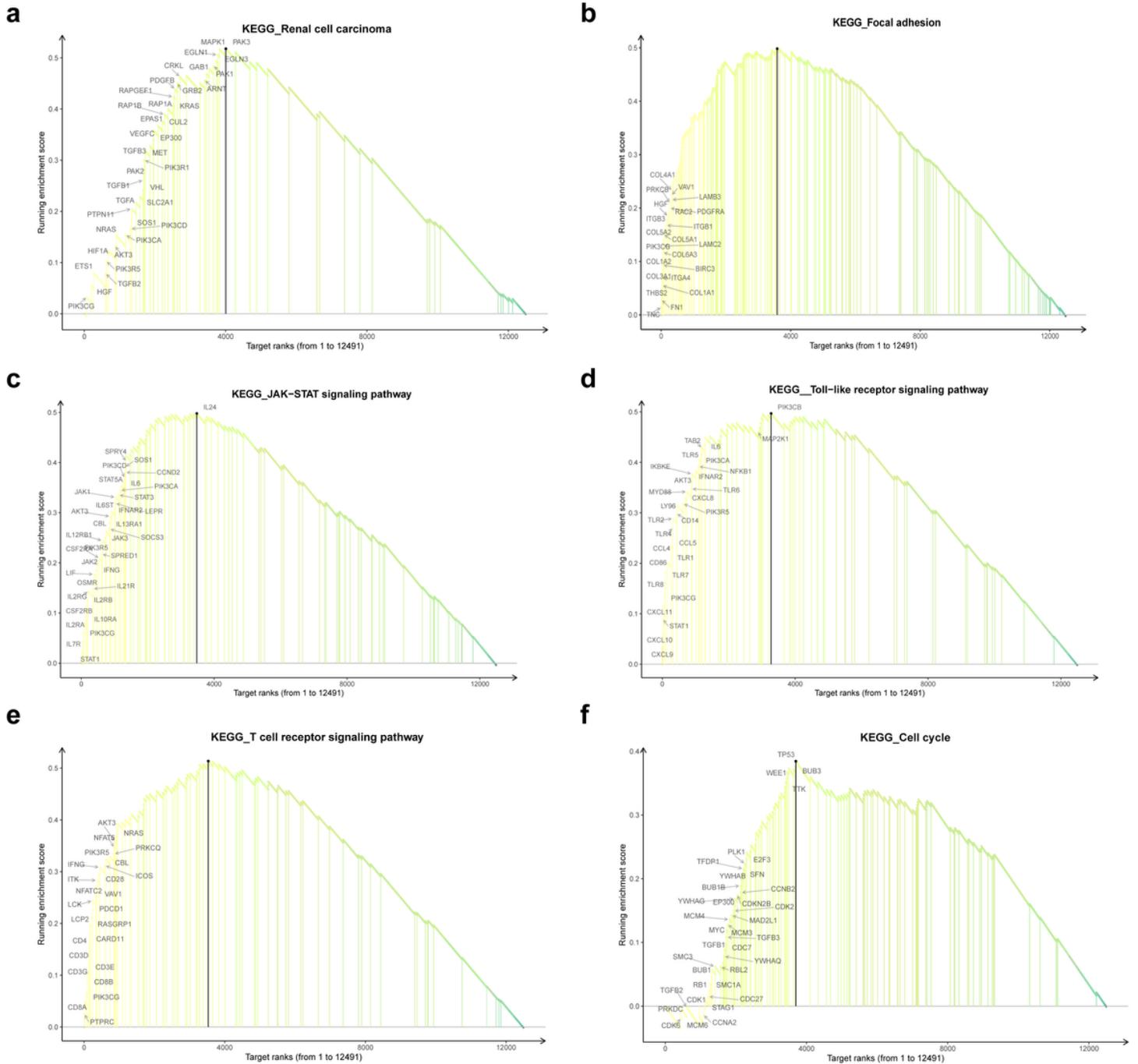
High ARNTL2 expression was an independent predictor of dismal prognosis in ccRCC. a The Kaplan-Meier survival curves of the high and low ARNTL2 expression ccRCC patients in TCGA database. b The Kaplan-Meier survival curves of the high and low ARNTL2 expression ccRCC patients in Kaplan-Meier Plotter database. c Univariate and d multivariate Cox regression analysis of ARNTL2 and other

clinicopathologic variables with overall survival in ccRCC patients. e ROC curve of ARNTL2 in ccRCC. f Nomogram was constructed to predict the 1-year, 3-year and 5-year overall survival of ccRCC patients.



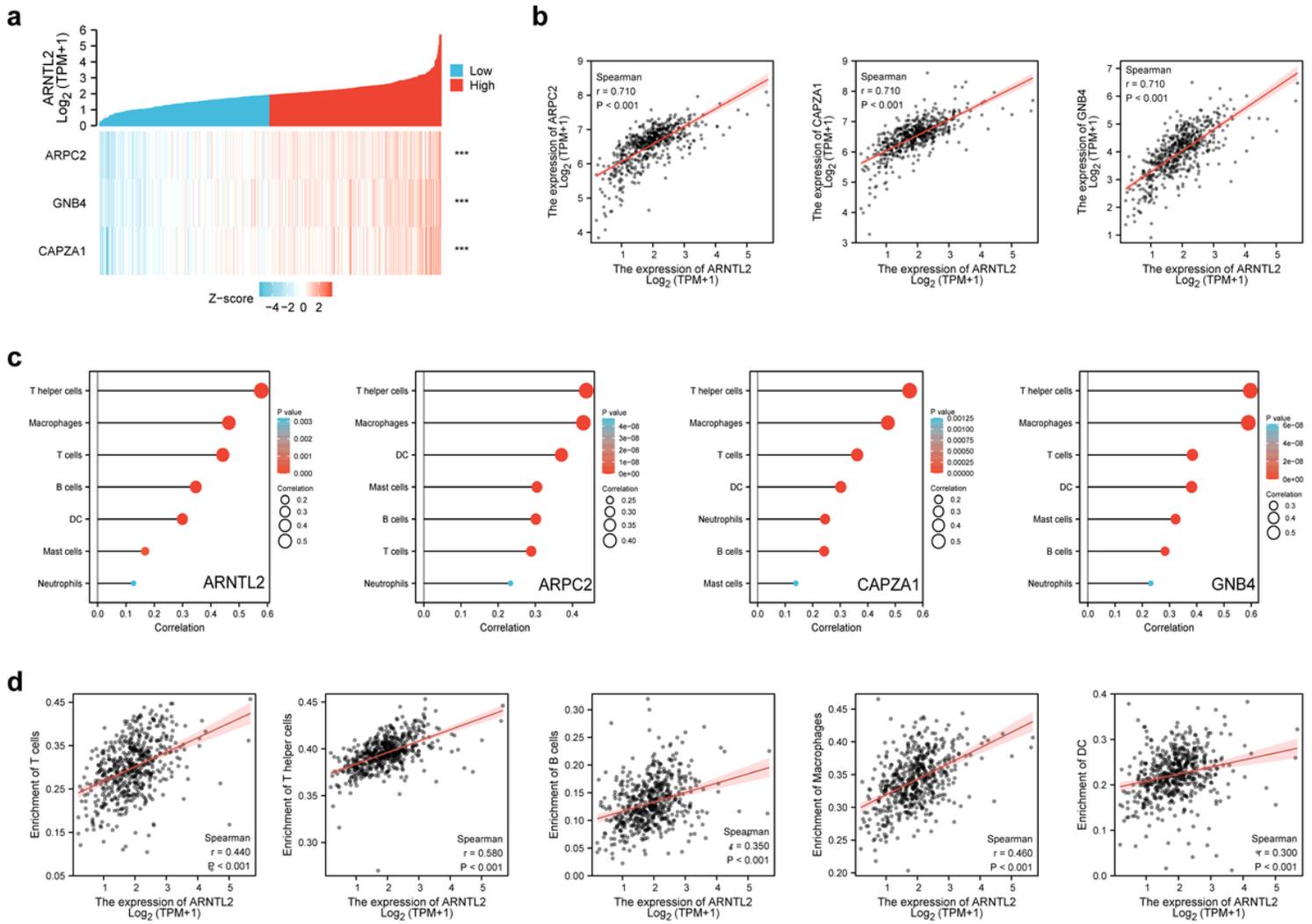
**Figure 3**

The divergences of significantly mutated genes (SMGs) in ccRCC base on ARNTL2 expression. a The mutational landscape of SMGs in ccRCC. The divergence of b PBRM1, c VHL, d TTN, e SETD2, f BAP1, g MUC16 mutation between ARNTL2 high and low ccRCC samples.



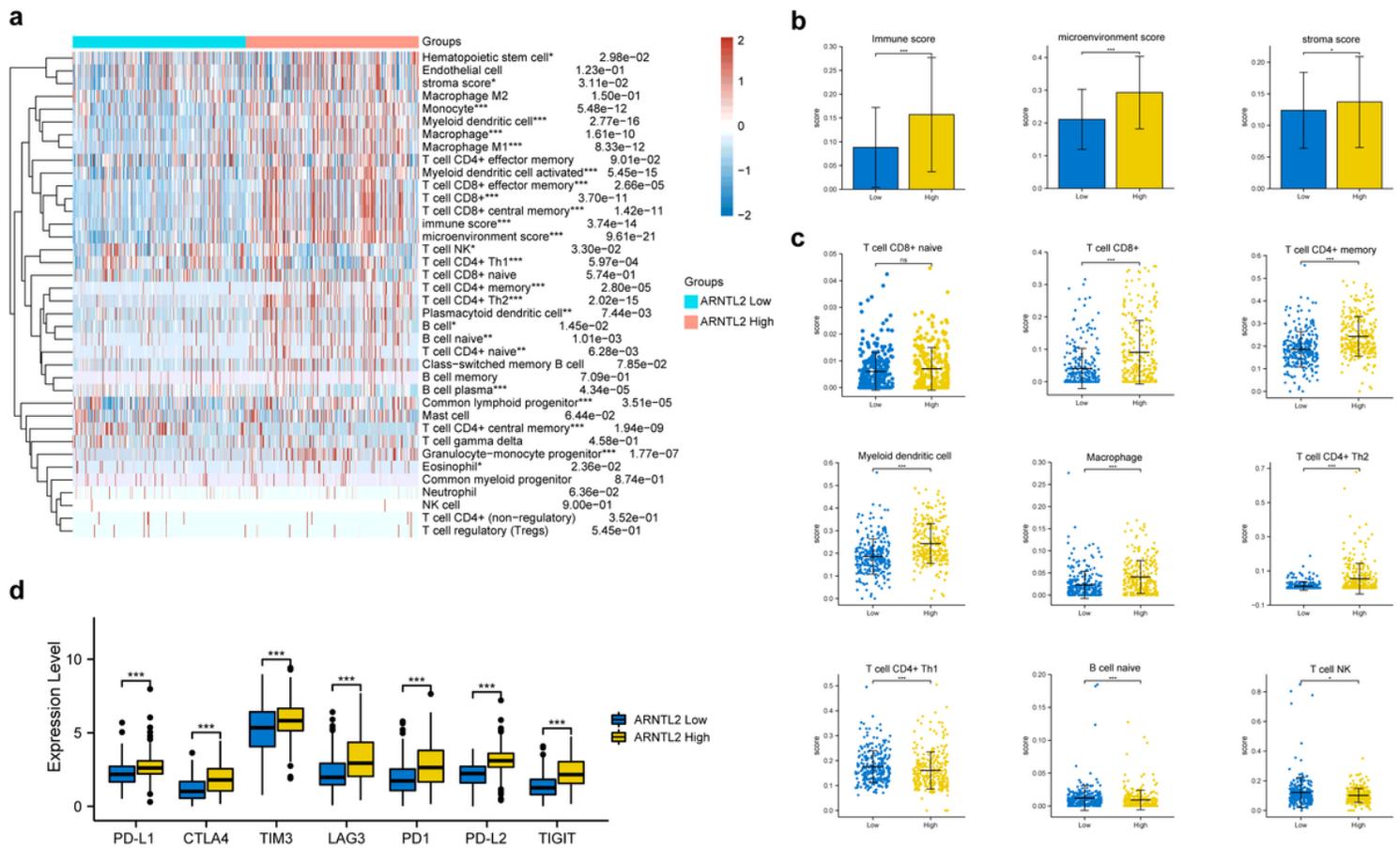
**Figure 4**

GSEA analyses of ARNTL2 in ccRCC. a Renal cell carcinoma. b Focal adhesion. c JAK-STAT signaling pathway. d Toll-like receptor signaling pathway. e T cell receptor signaling pathway. f Cell cycle.



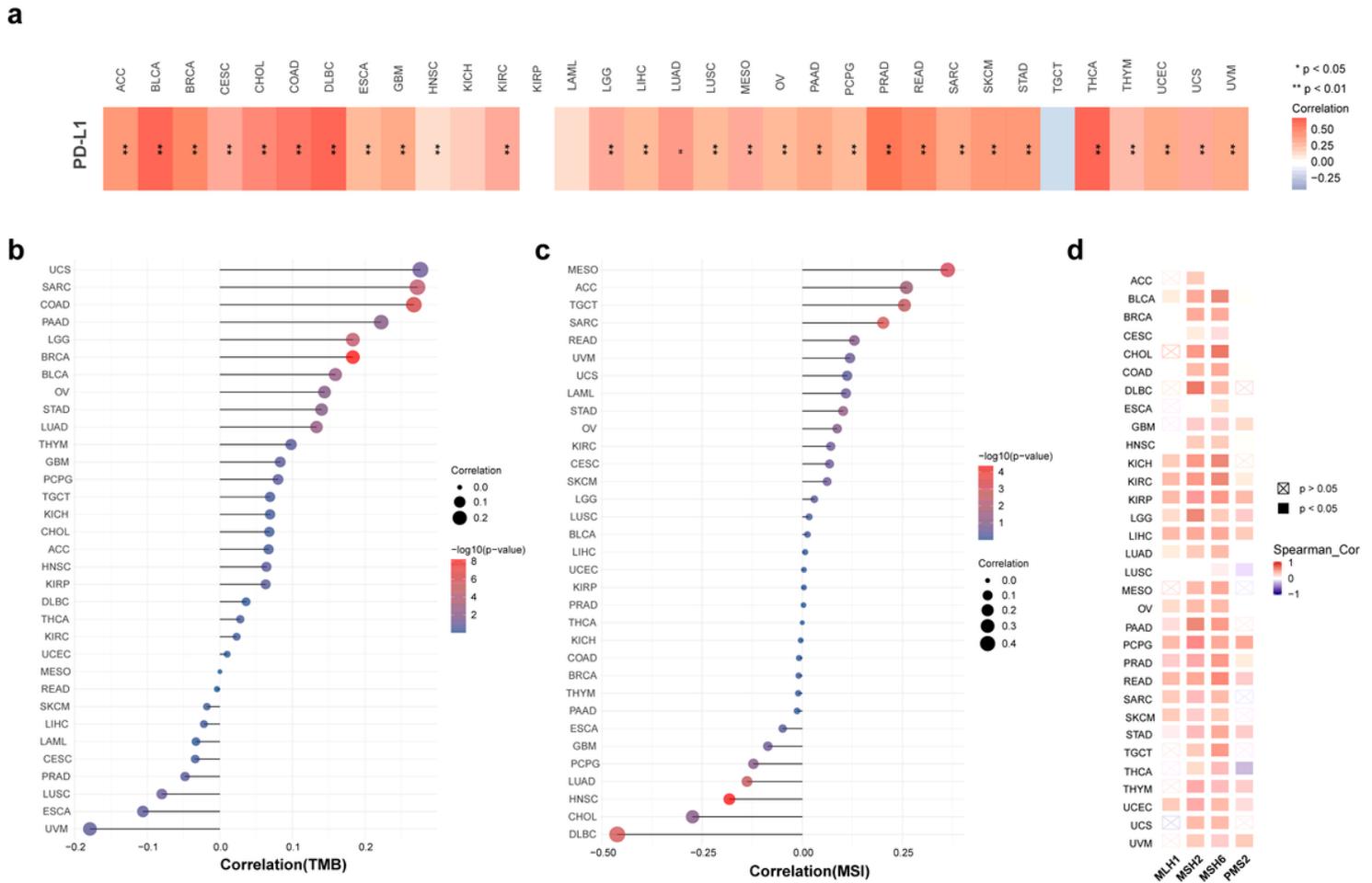
**Figure 5**

Significant correlations between ARNTL2 and its co-expressed genes and the immune infiltration levels in ccRCC. **a** The heatmap visualized the expression patterns of three most co-expressed genes of ARNTL2 in ccRCC. **b** ARNTL2 was remarkably linked to the expression of ARPC2, CAPZA1 and GNB4 in ccRCC. **c** Associations of ARNTL2, ARPC2, CAPZA1 and GNB4 expression with cancer-infiltrating immune cell types in ccRCC via ssGSEA analysis. **d** ARNTL2 was significantly linked to the expression level of T cells, T helper cells, B cells, Macrophages and DC in ccRCC.



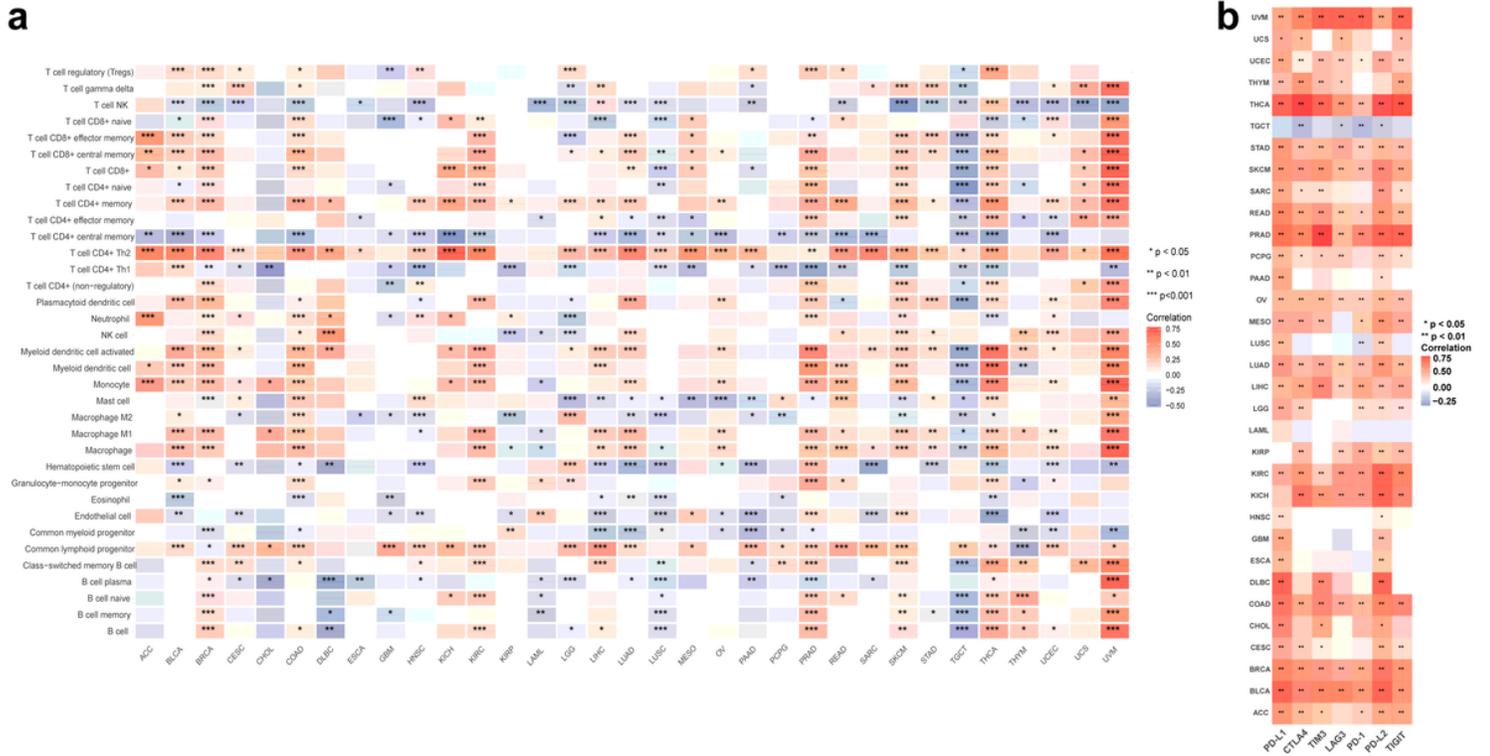
**Figure 6**

Expression patterns of immune cells and evasion markers in high and low ARNTL2 groups. a The heatmap showed significant differences of tumor immune microenvironment between two groups based upon ARNTL2 expression in ccRCC. b High ARNTL2 expression correlated with higher immune score, microenvironment score and stroma score compared to low ARNTL2 group. c The relative expression level of CD8+ naive T cell, CD8+ cell, CD4+ memory cell, myeloid dendritic cell, macrophage, Th2 CD4+ T cell, Th1 CD4+ T cell, naive B cell, NK T cell. d The relative expression level of immune evasion-markers in low and high ARNTL2 expression group.



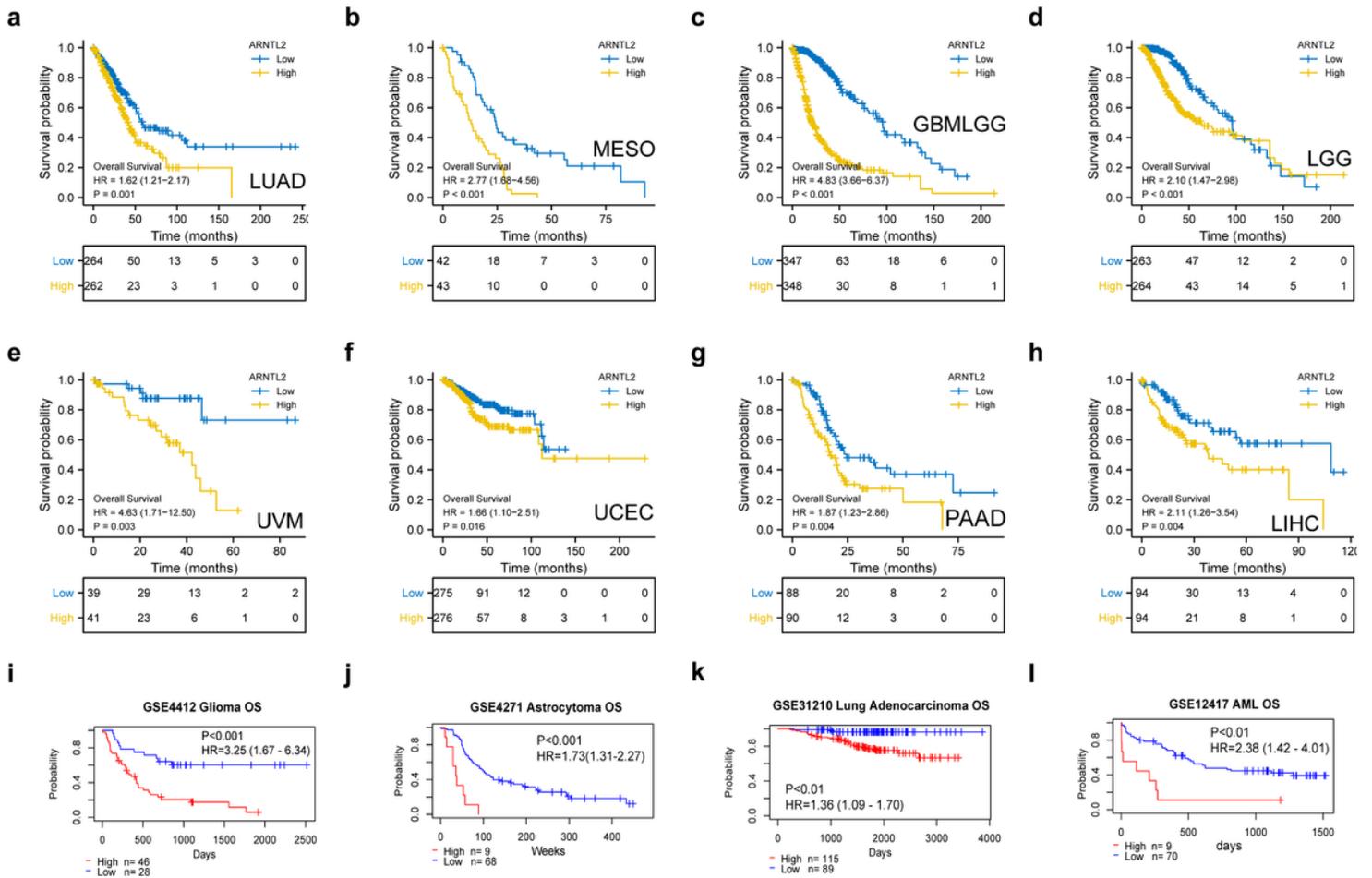
**Figure 7**

The associations of ARNTL2 with PD-L1, TMB, MSI and mismatch repair proteins in pan-cancer. ARNTL2 correlated with a PD-L1 expression, b TMB, c MSI and d mismatch repair proteins expression in diverse cancer types.



**Figure 8**

Relationship between ARNTL2 expression and tumor microenvironment in pan-cancer. a The correlations of ARNTL2 expression with 35 immune infiltrate cell types across 33 cancer types via xCell algorithm. b Correlations between immune checkpoint-related genes across 33 cancer types.



**Figure 9**

ARNTL2 expression correlated with the OS of patients in pan-cancer. Highly expressed ARNTL2 correlated with dismal OS of a Lung adenocarcinoma (LUAD), b Mesothelioma (MESO), c Glioma (GBMLGG), d Brain Lower Grade Glioma (LGG), e Uveal Melanoma (UVM), f Uterine Corpus Endometrial Carcinoma (UCEC), g Pancreatic adenocarcinoma (PAAD), h Liver hepatocellular carcinoma (LIHC) in TCGA database. Overexpressed ARNTL2 associated with worse OS of i Glioma (GSE4412), j Astrocytoma (GSE4271), k Lung adenocarcinoma (GSE31210) and l Acute Myeloid Leukemia (GSE12417) in GEO database.

## Supplementary Files

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- [SupplementaryTable1.docx](#)