Original Article

A comprehensive analysis of the prognostic and immunological role of *ANK3* in pan-cancer

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Background: Cancer is a common cause of death around the world. Immunotherapy plays a significant role in cancer treatment but still has limitations. The ankyrin-3 (*ANK3*) gene has been shown to have a variety of biological roles and has also been shown to be closely linked to individual cancers.

Methods: We systematically investigated the role of *ANK3* in pan-cancer, particularly in relation to immunity. We collected data from a number of databases, including the The University of ALabama at Birmingham CANcer data analysis Portal (UALCAN), tumor-immune system interactions (TISIDB), cBioPortal, Tumor Immune Estimation Resource (TIMER), Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), BioGRID, and SangerBox databases. R (version 3.6.3) was used for the statistical analysis and data visualization. The expression of *ANK3* in tumors and its effects on patient prognosis, immune infiltration, neoantigens, the microenvironment, immune checkpoints (ICs), the tumor mutation burden, microsatellite instability (MSI), methylation, mismatch repair (MMR) genes, and cancer-associated fibroblasts were investigated. A gene set enrichment analysis (GSEA) was also conducted.

Results: The *ANK3* gene was differentially expressed at the messenger RNA (mRNA) and protein levels in various human tumors. The prognosis of patients with different types of malignancies was correlated with the level of *ANK3* expression. The immunological microenvironment was also linked to *ANK3* expression, especially in colon adenocarcinoma (COAD), kidney renal clear cell carcinoma (KIRC), and liver hepatocellular carcinoma (LIHC). *ANK3* was also associated with ICs, immune neoantigens, MSI, the tumor mutation load, MMR genes, and DNA methylation. Finally, we found the key pathway related to the *ANK3* gene through the enrichment analysis.

Conclusions: *ANK3* could serve as a new biomarker specific to prognosis and immunotherapy in various cancers. Our findings could contribute to the development of novel strategies for treating malignancies.

Keywords: Ankyrin-3 (*ANK3*); pan-cancer; prognosis; immune; bioinformatics

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Introduction

Cancer is a common cause of death in both China and developed countries. It was estimated that there were approximately 19.3 million cancer new cases worldwide in 2020, resulting in approximately 9.9 million cancer-related deaths (1,2). The aging population and the cumulative effects of risk factor exposure constitute new challenges in cancer prevention. In recent years, biological therapies involving targeted drugs and immunotherapy have developed rapidly, surpassing traditional methods, such as surgery, radiotherapy, and chemotherapy, and becoming the mainstream trend in the development of oncology treatments. Immunotherapy, especially immune checkpoint (IC) inhibitor therapy, is a hot topic of research nowadays. Following continuous advancements in research on cytotoxic T lymphocyte-associated antigen 4 and programmed cell death receptor 1 (PD-1), immune-blocking agents have been approved for marketing and are have been successful in treating some tumors (3). However, as IC inhibitor therapy is still ineffective or works poorly in a large number of cancers, it is imperative that new IC research be conducted. Encouragingly, databases, including The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx), which can be used to study the expression, prognosis, and various biological processes of pan-cancer by exploring new immunotherapeutic target loci, have developed rapidly in recent times (4,5). Ankyrin (ANK) proteins have multiple biological actions and exhibit conservative properties during phylogeny (6).

ANK proteins participate in a number of cellular processes, such as intercellular junctions, signal transduction, cell cycle regulation, vesicular transportation, cytoskeletal integrity, inflammatory responses, and transcriptional regulation (7). Ankyrin-3 (*ANK3*) is an immune-specific gene product of Ankyrin 1 and Ankyrin 2, and was originally found in axon initiation segments and the Ranvier nodes of neurons in the central nervous system and peripheral nervous system (8). In recent years, researchers have identified many relevant transcript variants to encode various isoforms (9). Researchers have reported 270 human ANK proteins (10), and *ANK3* has a variety of important physiological functions. Recent study has examined the relevance of *ANK3* to the androgen receptor signaling pathway and its effect on the prognosis of breast cancer (BC) patients (11).

Nevertheless, most research on *ANK3* has been limited to certain cancer types. In fact, to date, no studies have conducted systematic pan-cancer analyses of *ANK3*. This study comprehensively analyzed *ANK3* from a biological perspective by mining data from different databases to comprehensively investigate the expression patterns of *ANK3* in normal and cancer tissues. In addition, this study also explored its prognostic role in various tumors and examined the relationship between *ANK3* expression in pan-cancer tissues with six tumor-infiltrating immune cells (TIICs) and immunosuppressive molecules. The results of this study suggest that *ANK3* can be used as a biomarker to affect tumor progression and prognosis, regulate tumor immunity, and improve the effect of immunotherapy, providing a reference for clinical treatment of pan-cancer. We present this article in accordance with the REMARK reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2379/rc).

Highlight box

Key findings

- Ankyrin-3 (ANK3) could serve as a new biomarker specific to prognosis and immunotherapy in various cancers. Our findings could contribute to the development of novel strategies for treating malignancies.

What is known and what is new?

- ANK3 plays an important role in the occurrence and progression of various cancers.
- Diagnosis, prognosis and immunological value of ANK3 in pan-cancer.

What is the implication, and what should change now?

- This study comprehensively explored the potential action mechanism of ANK3 in human tumors, while also supporting the findings of other experimental investigations.

Methods

ANK3 messenger RNA (mRNA) and protein expression analysis

A Kruskal-Wallis analysis of *ANK3* expression was performed to compare *ANK3* mRNA expression in 31 normal tissues and 21 cancer tissues. Data from TCGA database (12) were used to compare the differences in *ANK3* expression. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). We also analyzed *ANK3* expression in GTEx. The University of ALabama at Birmingham CANcer data analysis Portal (UALCAN) database was used to detect *ANK3* expressions in Clinical

Proteomic Tumor Analysis Consortium samples (13).

ANK3 mRNA expression was detected by quantitative real-time-polymerase chain reaction (qRT-PCR)

RNA was obtained by adding Trizol reagent to HK-2 (normal kidney cell lines), 786-O (renal cancer cell lines), and ACHN cells (renal cancer cell lines) according to the manufacturer's instructions. Complementary DNA was obtained by a reverse transcription kit. Finally, qRT-PCR was performed with SYBR green reagent using the $2^{-\Delta\Delta Ct}$ method. The primer sequences for *ANK3* were 'ACACCTTGAACAGAAGCTCCTA' (forward) and 'CGTCCACCATAAAGCTAACCAG' (reverse). The primer sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 'TCAAGAAGGTGGTGAAGCAGG' (forward) and 'TCAAAGGTGGAGGAGTGGGT' (reverse).

ANK3 protein expression was detected by Western blot analysis

The cells were lysed with lysis buffer as described in previous study (14), and protein samples were separated by 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The cells were incubated overnight with the first *ANK3* antibody (Ab134317, Abcam, 1:1000). After washing with phosphate buffered saline, the cells were incubated with the secondary antibody (1:5000) for one hour at room temperature. The protein bands were visualized with enhanced chemiluminescence reagents, and GAPDH was used as a housekeeping control protein.

Gene mutation analysis

A pan-cancer analysis of *ANK3* was conducted using the cBioPortal database to observe the mutation frequency and type, and the copy number alterations (CNAs) in TCGA tumors (15). The locations and sample sizes of the *ANK3* mutations were also analyzed. Finally, the CNAs in the pan-cancer tissues were studied by Genomic Identification of Significant Targets in Cancer (GISTIC).

Survival analysis

Gene Expression Profiling Interactive Analysis 2 (GEPIA2) was used to analyze the correlation between *ANK3* gene expression and patient prognosis in different tumors (16). A Kaplan-Meier (K-M) analysis was conducted to examine overall survival (OS), disease-specific survival (DSS), and the progression-free interval (PFI). The final results are presented in a forest map and survival curve and were determined using the Cox regression test and the K-M test, respectively.

Immunological correlation analysis

Tumor Immune Estimation Resource (TIMER) database has a large number of samples from TCGA database that can be used for immune analyses (17). We used this database to investigate the correlation between *ANK3* expression and the degree of tumor immune infiltration (TII) in colon adenocarcinoma (COAD), kidney renal clear cell carcinoma (KIRC), and liver hepatocellular carcinoma (LIHC). We present the results as immune scores, stromal scores, and estimate scores. When an immune score or a stromal score is high, the proportion of immune or matrix components is high. The estimate score represents the combination ratio of the two components in the tumor microenvironment (TME). Tumor-associated fibroblasts (TAFs) were also selected for immune-infiltration assessment based on different algorithms, such as CIBERSORT, XCELL, MCPCOUNTER, and EPIC. Subsequently, we analyzed the correlation between *ANK3* and immunosuppressive agents, immune agonists, and major histocompatibility complex (MHC) molecules through the immunomodulator module of tumor-immune system interactions (TISIDB) (18). At the same time, we also used the subtype module of the TISIDB database to investigate the correlation between *ANK3* expression and immune typing and molecular typing.

Correlation between ANK3 expression and IC genes, immune neoantigens (INAs), the tumor mutation burden (TMB), microsatellite instability (MSI), mismatch repair (MMR) genes, and DNA methylation

A Pearson correlation analysis was conducted to study the correlation between 47 IC-related genes of 33 cancer types and *ANK3* in Sangerbox. We also studied the association between gene expression and the neoantigen number in Sangerbox. We also analyzed the effect of *ANK3* expression on the TMB and MSI, and the correlation between *ANK3* expression and methylation, MMR genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*) and DNA methylation.

Gene set enrichment analysis (GSEA)

GSEA was used to evaluate the tendency of genes from a predefined gene set to be distributed in a list of genes ranked by phenotypic correlation, and thus to determine their contribution to the phenotype. First, we conducted a GSEA to divide *ANK3* data into high expression and low expression groups. Next, a Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was conducted to identify important pathways related to the *ANK3* genes (The parameters with significant effects were set as |NES|>1, NOM P < 0:05, and FDR q<0.05).

Analysis of ANK3-related gene

The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database was used to establish the *ANK3* gene co-expression network (19). BioGRID was used to create an *ANK3* interactive network with a layout set to concentric circles.

Statistical analysis

The Bioinformatics analysis section filters the entire data set by removing missing and duplicated data and performs all statistical analysis and visualization using R software (version 3.6.3). For experimental data, continuous variable *t*-test was used to compare the two groups, and P<0.05 was considered statistically significant.

Results

Analysis of ANK3 expression in normal and cancer tissues

We analyzed the differences in *ANK3* expression in two groups of tissues, and found that *ANK3* was highly expressed in brain, heart, muscle and nerve tissues (*Figure 1A*, P<0.001). *ANK3* expression also differed significantly in different cell lines (*Figure 1B*, P<0.001). According to TCGA database analysis, *ANK3* was expressed in colon cancer (COAD), glioma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), renal suspicious cell carcinoma (KICH), renal clear cell carcinoma (KIRC), renal papillary cell carcinoma (KIRP), hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), rectal adenocarcinoma (READ), and thyroid cancer (THCA); however, the expression was only significantly upregulated in cholangiocarcinoma (CHOL) (*Figure 1C*, P<0.001). According to TCGA-integrated GTEx data, relative to the normal tissue samples, the *ANK3* genes was significantly upregulated in breast invasive carcinoma (BRCA), CHOL, diffuse large B-cell lymphoma (DLBC), esophageal cancer (CSCA), LUSC, pancreatic adenocarcinoma (PAAD), prostate adenocarcinoma (PRAD), gastric cancer (STAD), and thymic adenocarcinoma (THYM). Conversely, it was obviously downregulated in colon cancer (COAD), glioblastoma multiforme (GBM), HNSC, KICH, KIRC, acute myeloid leukemia (LAML), low-grade glioma (LGG) of the brain, LIHC, ovarian plasmacytoid cystic adenocarcinoma (OV), READ, cutaneous melanoma (SKCM), testicular cancer (TGCT), THCA, endometrial cancer (UCEC) and uterine sarcoma (UCS) (*Figure 1D*). According to the paired-sample analysis, *ANK3* was obviously upregulated in CHOL, PRAD, and obviously downregulated in COAD, KICH, READ and THCA (*Figure 1E*). Thus, it can be concluded from the analysis that *ANK3* expression is heterogeneous in various tissues and tumors.

ANK3 mRNA and protein expression levels were examined by qRT-PCR and Western blot

The results of the qRT-PCR analysis showed that the expression level of *ANK3* mRNA in the HK-2 (normal kidney) cell line was lower than that in the 786-O (renal cancer) cell line and the ACHN (renal cancer) cell line (P<0.01) (*Figure 2A*). In addition, the Western bot analysis revealed that the expression level of the *ANK3* protein in the HK-2 (normal kidney) cell line was also lower than that in the 786-O (renal cancer) cell line and the ACHN (renal cancer) cell line (*Figure 2B*).

Analysis of genetic alterations in the ANK3 gene in different tumor tissues

The characteristics exhibited by the *ANK3* gene in cancers were further explored through a TCGA pan-cancer mapping study of data from the cBioPortal database. The results revealed 764 (7%) of 10,953 patients had alterations in the *ANK3* gene. Among them, skin melanoma (SKCM), UCEC, STAD, and uterine sarcoma (UCS) had higher gene variation rates that were all greater than 10% (*Figure 3A*). In this study, we found that the major types of genetic alterations in *ANK3* were amplifications, profound deletions, nonsense mutations, and missense mutations (*Figure 3B*). *Figure 3C* displays the location, type, and sample number of the mutations in *ANK3* gene alterations. Notably, *ANK3*

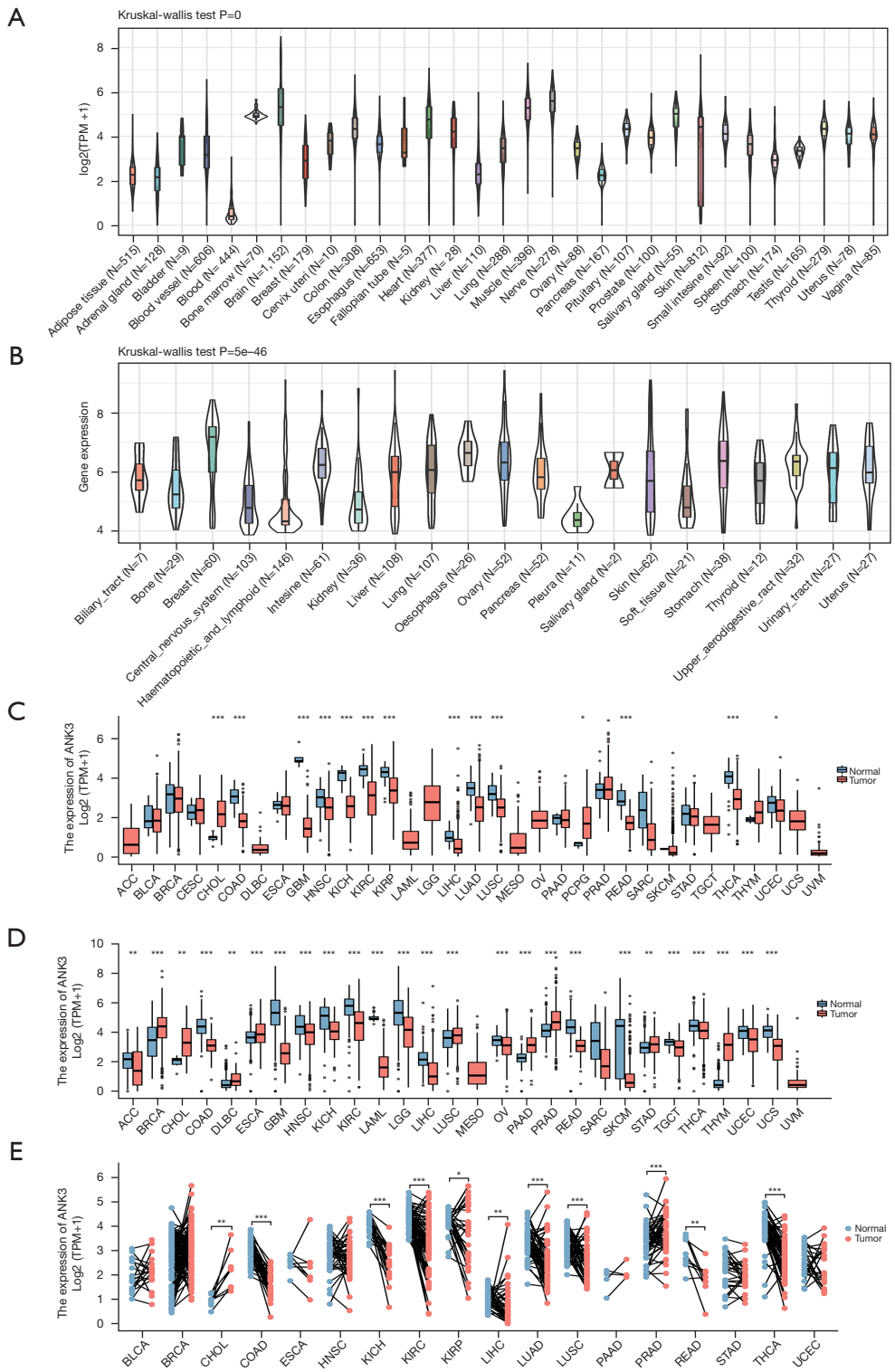


Figure 1 Expression of *ANK3* in normal and tumor tissues of patients with pan-cancer. (A) *ANK3* expression in normal tissues. (B) *ANK3* expression in tumor tissues. (C) *ANK3* expression was analyzed using TCGA data. (D) Integration of TCGA and GTEx data. (E) Paired-sample analysis of *ANK3* expression. *, P<0.1; **, P<0.01; ***, P<0.001. TPM, transcripts per million; ANK3, ankyrin-3; TCGA, The Cancer Genome Atlas; GTEx, Genotype-Tissue Expression.

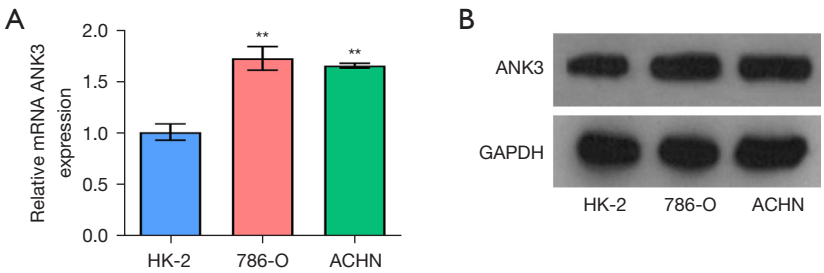


Figure 2 Relative expression of *ANK3* in human normal kidney and ccRCC cell lines. (A) Relative *ANK3* mRNA expression in human normal kidney and ccRCC cell lines. **, P<0.01. (B) Relative expression of *ANK3* protein in human normal kidney and ccRCC cell lines. mRNA, messenger RNA; *ANK3*, ankyrin-3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ccRCC, clear cell renal cell carcinoma.

fusion mutations were the predominant mutation type, with K4140Nfs*15 alterations found in UCEC, K4140Nfs*15 alterations found in STAD, and W4141Mfs*12 alterations found in BRCA. The genomic co-occurrence analysis revealed that several genes, including *TTN*, *MUC16*, *XRCC6P1*, *RYR2*, *LRP1B*, *DNAH5*, *CSMD3*, *SYNE1*, and *CSMD1*, were more common in the *ANK3* alteration group (*Figure 3D*). In addition, *ANK3* CNAs on display from GISTIC include a variety of types, such as deep deletions, and diploidy, resulting in changes in gene expression (*Figure 3E*).

Protein expression analysis of ANK3 based on the CPTAC data set

In terms of protein levels, the CPTAC dataset was used for analysis. According to the results, the *ANK3* protein was downregulated in BC, clear cell RCC, colon cancer, hepatocellular carcinoma, ovarian cancer and pancreatic carcinoma (*Figure 4*).

ANK3 expression in pan-cancer and its relationship with OS

The data were divided into high expression and low expression groups based on *ANK3* expression. TCGA database was used to investigate the association between *ANK3* expression and tumor patient prognosis. As *Figure 5* shows, ACC (P<0.0001) and KIRC (P<0.0001) patients with low levels of *ANK3* expression had poor OS. We then examined the relationship between *ANK3* and DSS and PFI in 33 cancer types. We investigated how *ANK3* gene expression in TCGA affected pan-cancer patients' prognosis and generated K-M curves showing the DSS and PFI results (Figures S1,S2).

Analysis of the correlation between ANK3 expression and TII and TME in pan-cancerous tissues

Tumor-infiltrating lymphocytes can independently predict tumor-primary lymph node status and survival status. According to the TII analysis, *ANK3* expression predicted the TII rate of various tumor types (COAD, KIRC, and LIHC). In LIHC, *ANK3* expression was obviously positively correlated with B cells, cluster of differentiation (CD)4$^+$ T cells, CD8$^+$ T cells, dendritic cells, neutrophils, and macrophages (P<0.001) (*Figure 6A*). In COAD, KIRC, *ANK3* expression was significantly correlated with six tumor-infiltrating lymphocytes (B cells, CD4$^+$ T, CD8$^+$ T cells, dendritic cells, neutrophils and macrophages). Additionally, to explore the effect of *ANK3* on the tumor immune microenvironment (TIME) in tumor development, we calculated the immune and mesenchymal scores of individual tumor specimens using the R language package (*Figure 6B*). Among the 33 tumors, LGG (R=−0.372, P<0.001), TGCT (R=0.083, P<0.001) and THCA (R=−0.078, P<0.001) were tumors in which ANK3 expression was the most highly correlated with immune scores, and LGG (R=−0.372, P<0.001), LIHC (R=0.452, P<0.001), and SKCM (R=0.348, P<0.001) were the top three tumors in which *ANK3* expression was the most highly correlated with mesenchymal scores. In relation to the TME, *ANK3* expression was clearly and positively correlated with immune scores in SKCM and LIHC, and negatively correlated with immune scores in LGG. Notably, in LGG, *ANK3* expression was significantly and negatively correlated with mesenchymal scores and immune scores.

ANK3 expression in relation to immunomodulators

We also investigated *ANK3* expression in human cancers

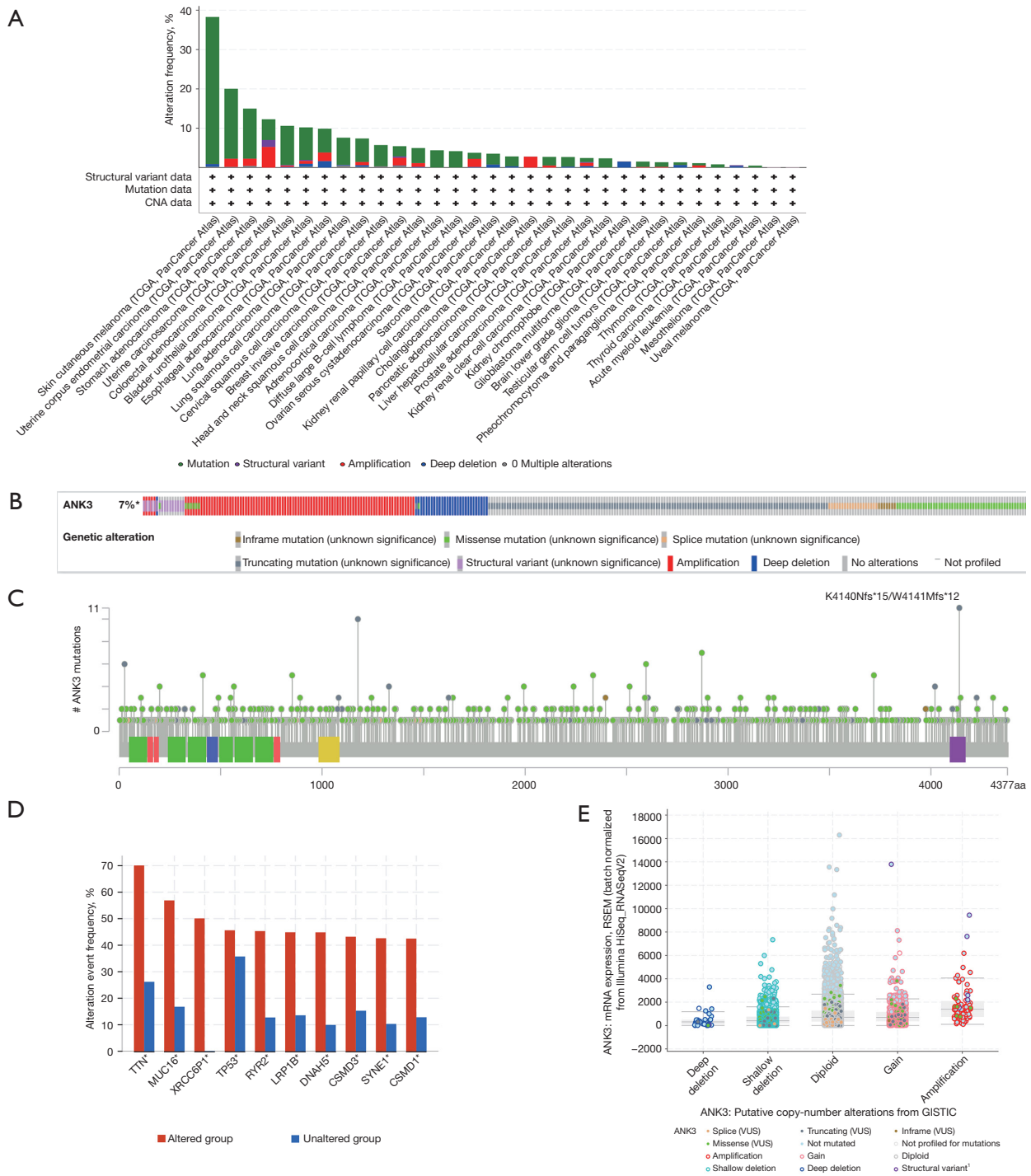


Figure 3 Characteristics of *ANK3* gene changes in pan-carcinoma tissues. (A) *ANK3* changed frequency with mutation type in various types of cancer. (B) Different change types in *ANK3* genes (the color represents the change type in the *ANK3* genes). (C) The type, location, and number of samples of *ANK3* mutations (Green: Ank_2: Ankyrin repeats (3 copies); Red: Ank: Ankyrin repeat; Blue: Ank_4: Ankyrin repeats (many copies); Yellow: ZU5: ZU5 domain). (D) *ANK3* gene mutations co-exist in tumors. *, P<0.05. (E) Types of associated alterations and possible CNAs of the *ANK3* gene in pan-carcinoma tissues. CNAs, copy number alterations; TCGA, The Cancer Genome Atlas; *ANK3*, ankyrin-3; RSEM, recurrent spinal epidural metastases; GISTIC, genomic identification of significant targets in cancer; VUS, variants of uncertain significance.

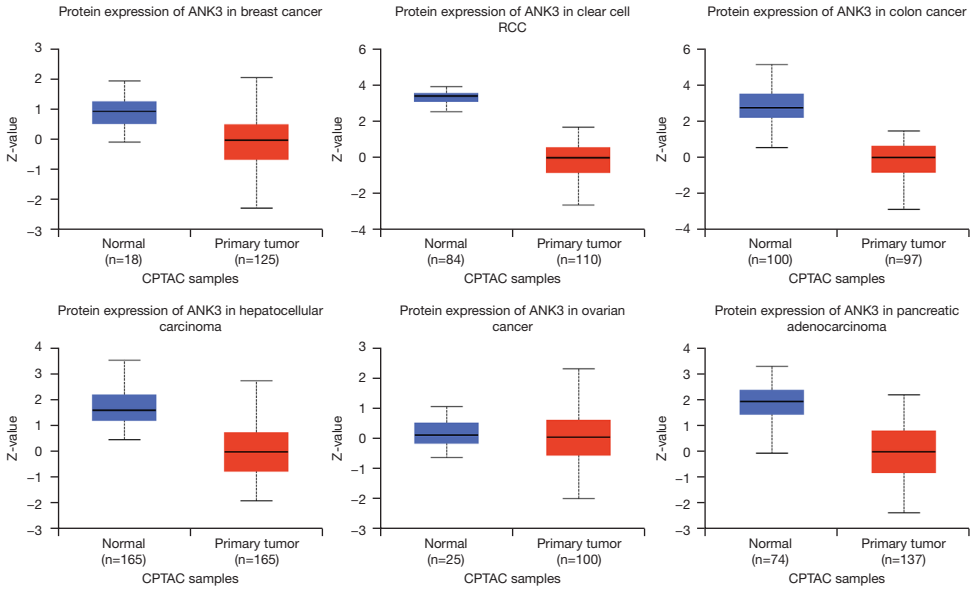


Figure 4 Protein expression of *ANK3*. The *ANK3* protein was downregulated in breast cancer, clear cell RCC, colon cancer, hepatocellular carcinoma, ovarian cancer and pancreatic carcinoma. CPTAC, Clinical Proteomic Tumor Analysis Consortium; *ANK3*, ankyrin-3; RCC, renal cell carcinoma.

in relation to immunomodulators. As *Figure 7A* shows, *ANK3* was positively correlated with several well-known immunosuppressive agents (including *CD274*, *KDR*, and *VTCN1*) in most cancers. *KDR* is associated with a variety of immune responses and has been shown to have a novel immunosuppressive locus (20). Our study confirmed the positive correlation between *ANK3* and *KDR* in various tumors. *Figure 7B* shows the correlation between *ANK3* expression and immune agonists in various cancers. *ANK3* was found to be positively correlated with TNFSF15 in KICH, PAAD, and UCS, and negatively correlated with CD276 in Cervical Cancer (CESC), Lower Grade Glioma (LGG), Thyroid Cancer (THCA), and Ocular melanomas (UVM). *ANK3* was also negatively correlated with the MHC molecules of *TAP1* and *TAPBP* in several cancer types, including CHOL, LGG, and TGCT (*Figure 7C*).

Analysis of the correlation between ANK3 expression and immune and molecular subtypes

This study analyzed the violin profiles and found that *ANK3* expression was significantly correlated with the immune and molecular subtypes. It also interpreted the mechanism of action of *ANK3*. The results of the immune subtype correlation analysis are shown in *Figure 8A*. *ANK3* expression was found to be correlated with Bladder Cancer (BLCA) (P=3.01e−03), BRCA (P=1.99e−09), KIRC (P=4.62e−08), LGG (P=1.3e−06), LIHC (P=2.93e−08), LUAD (P=1.9e−08), and PRAD (P=1.11e−04). *ANK3* expression differed significantly (P<0.001) in the cancer tissues of different molecular subtypes and was expressed in LGG (P<0.001), LUSC (P<0.001), OV (P<0.001), and UCEC (P<0.001). In ACC, *ANK3* was lowly expressed in the CIMP-high isoform, moderately expressed in the CIMP-medium isoform, and highly expressed in the CIMP-low isoform. In LGG, *ANK3* was highly expressed in the Codel, PA-like isoform, lowly expressed in the classic-like isoform, and moderately expressed in G-CIMP-high, G-CIMP-low, and mesenchymal-like isoforms. In LIHC, *ANK3* was highly expressed in iCluster 1, lowly expressed in iCluster 3, and moderately expressed in iCluster 2. In LUSC, *ANK3* was highly expressed in the classical, lowly expressed in the primitive, and moderately expressed in the basal and secretory. In OV, *ANK3* was highly expressed in differentiated and proliferative, lowly expressed in mesenchymal, and moderately expressed in immunoreactive. In UCEC, *ANK3* was highly expressed in CN_low and lowly expressed in CN_high (*Figure 8B*).

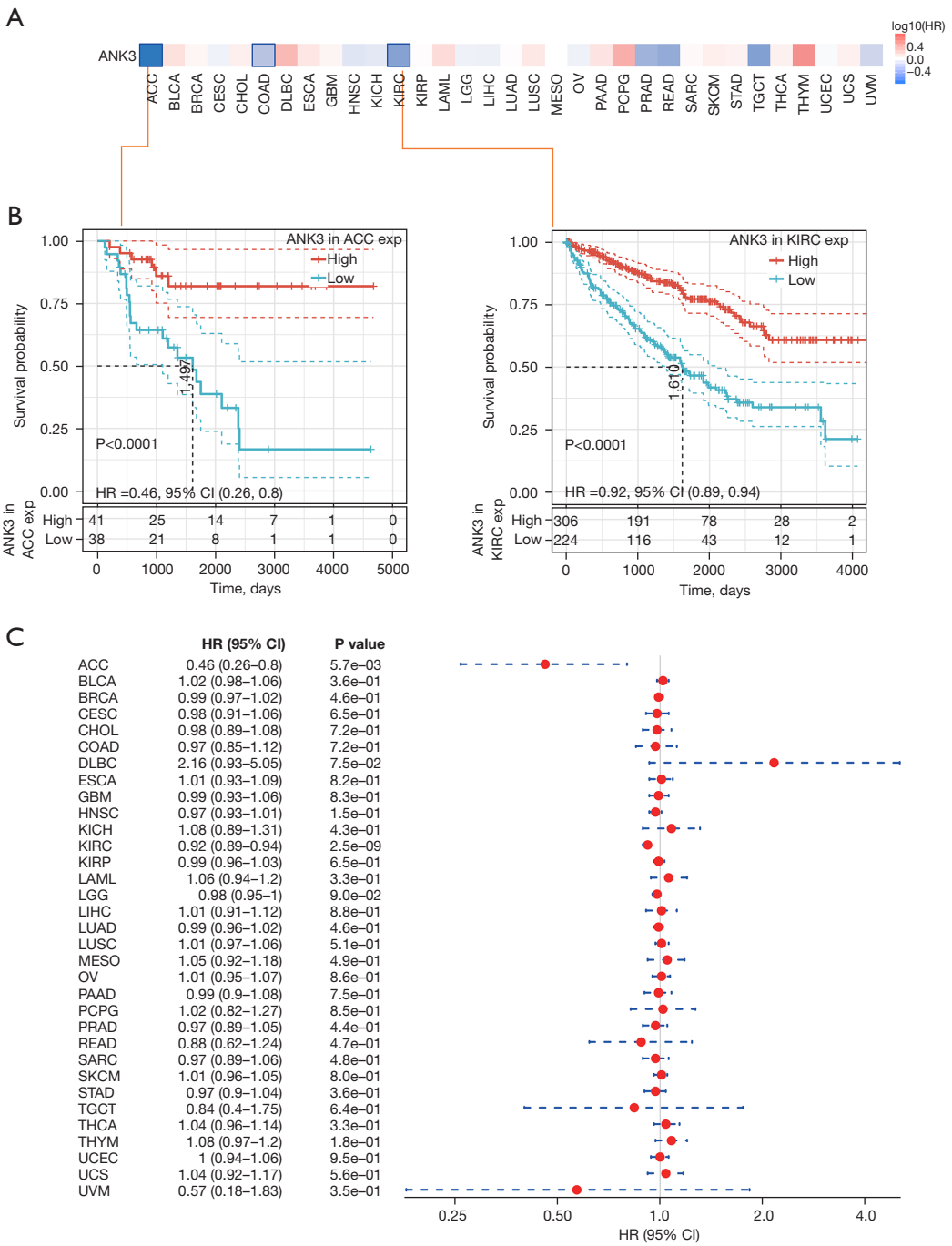


Figure 5 *ANK3* expression in pan-cancer and its relevance to OS. (A) The GEPIA2 tool was used to analyze *ANK3* gene expression in tumors in TCGA. (B) The K-M test was used to analyze the *ANK3*/OS correlation in ACC and KIRC. (C) Forest map based on prognosis of different types of cancer in TCGA. *ANK3*, ankyrin-3; HR, hazard ratio; ACC, adrenocortical carcinoma; CI, confidence interval; KIRC, kidney renal clear cell carcinoma; OS, overall survival; TCGA, The Cancer Genome Atlas; K-M, Kaplan-Meier.

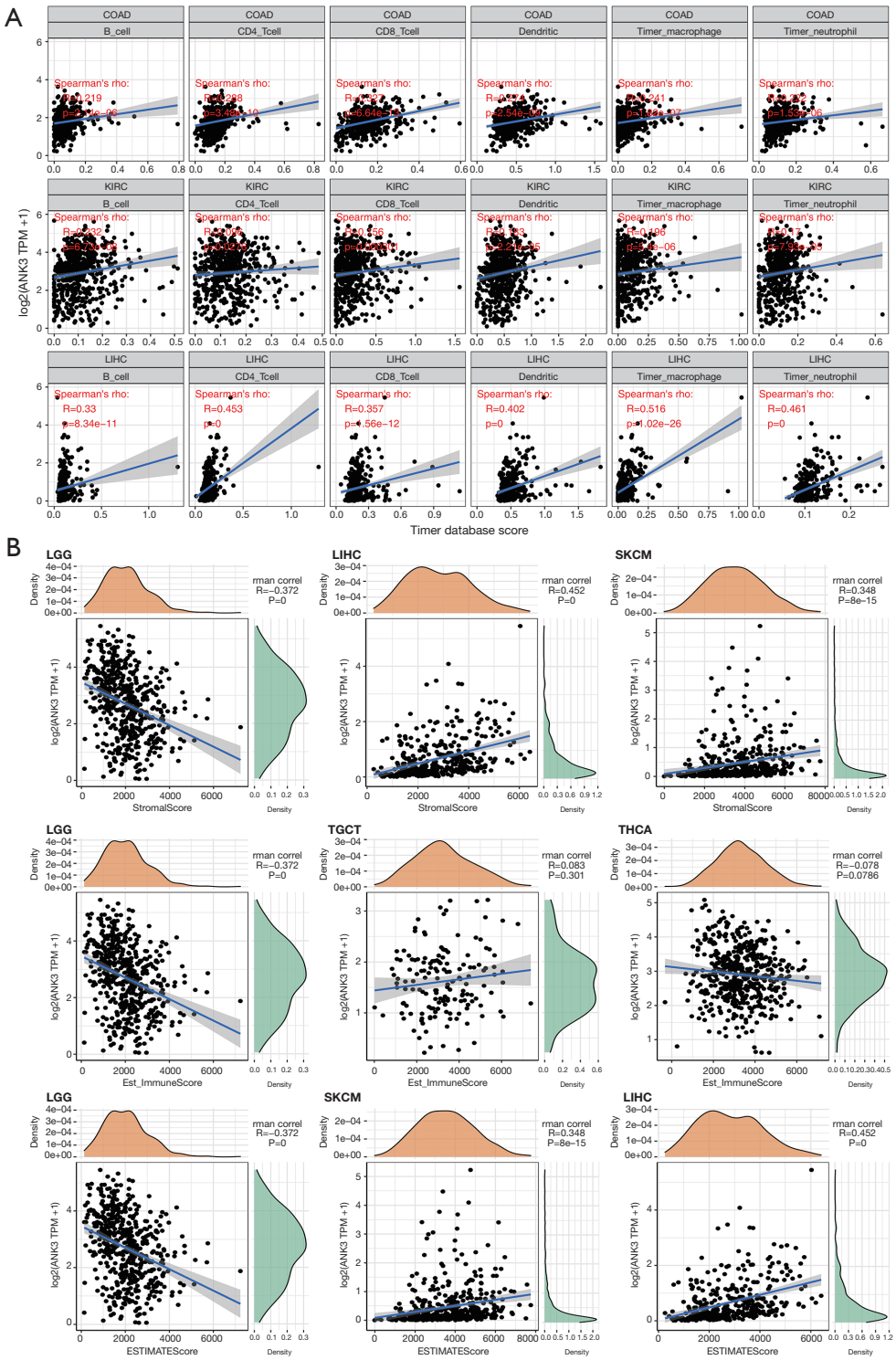


Figure 6 The relationship between *ANK3* expression and immune cell infiltration in tumor tissues and the link between *ANK3* expression and corresponding scores. (A) Analysis of the correlation between *ANK3* expression and immune cell infiltration in COAD, KIRC, and LIHC tissues. (B) Analysis of the correlation between *ANK3* expression and immune scores and interstitial scores. *ANK3*, ankyrin-3; TPM, transcripts per million; COAD, colon adenocarcinoma; KIRC, kidney renal clear cell carcinoma; LIHC, liver hepatocellular carcinoma; LGG, low-grade glioma; SKCM, cutaneous skin melanoma; TGCT, testicular germ cell tumors; THCA, thyroid cancer.

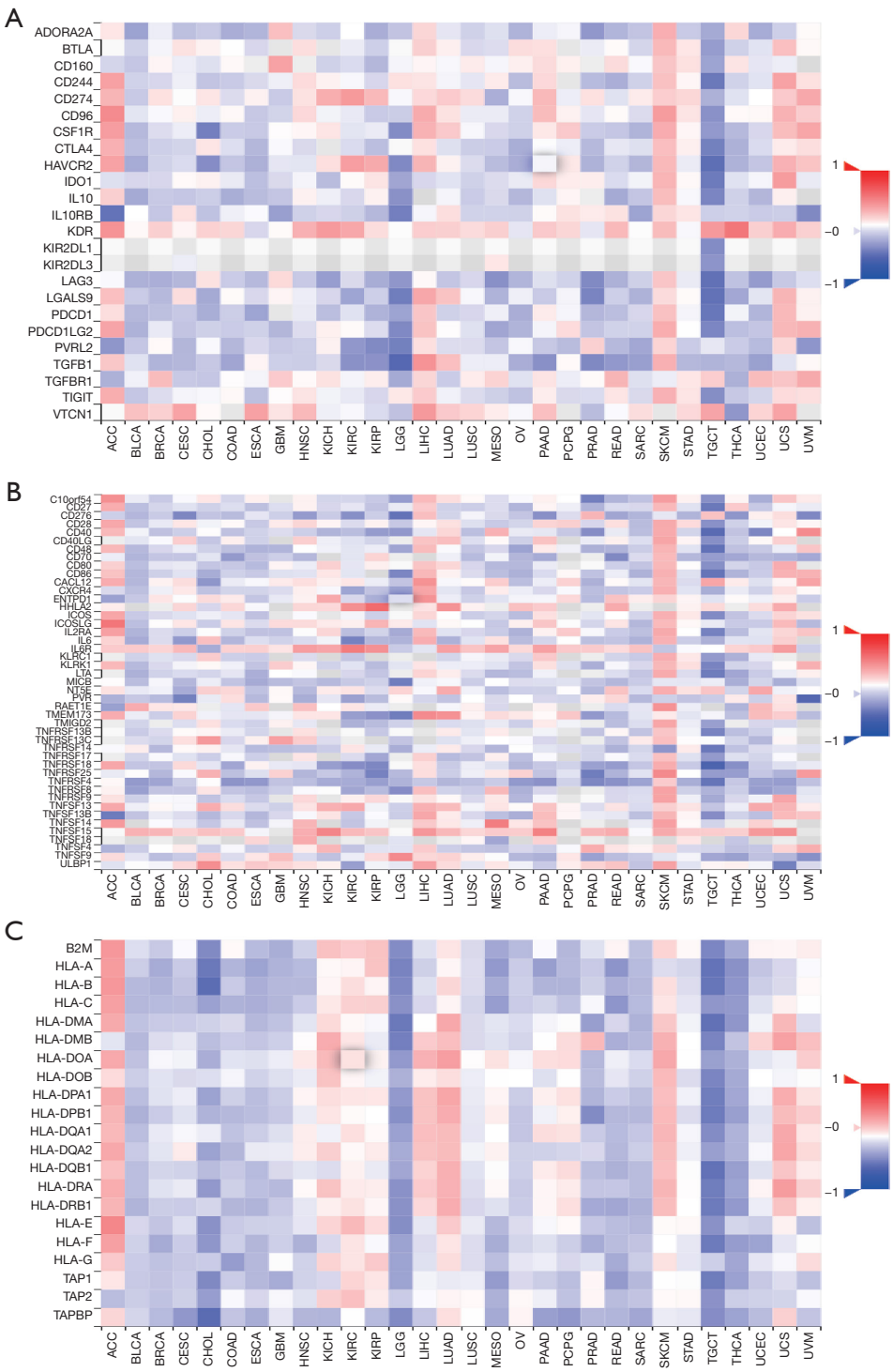


Figure 7 Correlation between *ANK3* and immunomodulators. (A) Immunosuppressive agents. (B) Immune agonists. (C) Correlation between MHC molecules and *ANK3* expression. Red and blue indicate positive and negative correlations, respectively. The color depth is in direct proportion to the correlation strength. *ANK3*, ankyrin-3; MHC, major histocompatibility complex.

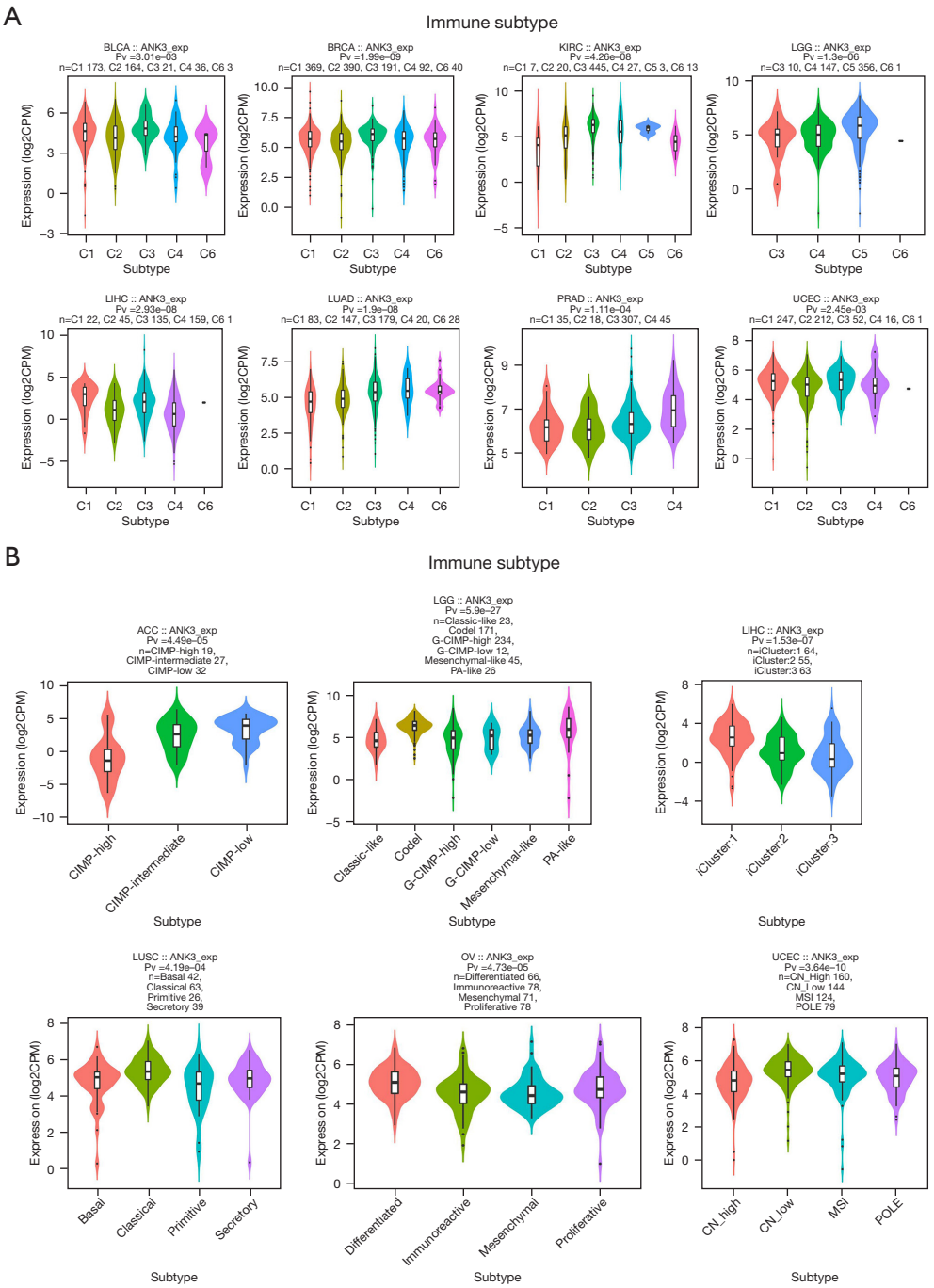


Figure 8 *ANK3* expression was analyzed using TISIDB to assess its correlation with immune and molecular subtypes across various cancers. (A) TISIDB was used to analyze the correlation between *ANK3* expression and the immune and molecular subtypes of pan-cancer [C1 (wound healing); C2 (interferon-γ dominant); C3 (inflammatory); C4 (lymphocyte depletion); C5 (immune resting); C6 (TGF-β dominated)]. (B) TISIDB was used to analyze the correlation between *ANK3* expression and pan-cancer molecular subtypes. BLCA, Bladder Cancer; *ANK3*, ankyrin-3; BRCA, breast invasive carcinoma; KIRC, kidney renal clear cell carcinoma; LGG, low-grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; PRAD, prostate adenocarcinoma; UCEC, uterine corpus endometrial carcinoma; ACC, adrenocortical carcinoma; LUSC, lung squamous cell carcinoma; OV, ovarian cancer; MSI, microsatellite instability; TGF, transforming growth factor.

Analysis of the correlation between ANK3 expression and immune neoantigens (INAs) and immune checkpoint genes (ICGs) in pan-cancer tissues

Figure 9A shows the *ANK3*/ICG relationship based on the expression data of over 40 common ICGs in different tumor types. The results revealed that the *ANK3*/ICG's correlation is negative in BLCA, LGG, and TGCT. This suggests that in some tumors, *ANK3* may regulate tumor immune patterns through the regulation of ICG expression. The number of neoantigens in different tumor types was counted separately, and their relationship with *ANK3* expression was analyzed (*Figure 9B*). The only significant negative correlation was found in LUAD (R=–0.23, P<0.01).

ANK3 expression is correlated with TMB and MSI in pan-cancer tissues

The TMB refers to the number of somatic mutations in the tumor genome after germline mutations are removed. It is also the sum of insertion, replacement, and deletion mutations in evaluated genes' coding regions in the tumor cell genome, and the total number of somatic mutations, which can also be expressed as non-synonymous mutations. Mutation types include single nucleotide variants (SNVs) and small insertions/deletions (INDELs). The TMB reflects the number of mutations in tumor cells that can be quantified. In *Figure 10A*, Spearman's rank correlation coefficients were used to analyze the correlation between the TMB and *ANK3* expression. Notably, *ANK3* was found to be positively correlated with the TMB in THYM, KIRP, and LAML. MSI refers to DNA MMR function that is abnormal in in pan-cancerous tissues compared to that in normal tissue; if microsatellite replication errors are not corrected and accumulate, they result in an altered microsatellite sequence length or base composition. The Spearman's rank correlation coefficient was also used to analyze the correlation between *ANK3* expression and MSI (*Figure 10B*). The results revealed a positive correlation between *ANK3* expression and MSI in COAD, KIRC, and LUSC, and a negative correlation between ANK3 expression and MSI in BRCA, HNSC, PRAD, SKCM, THCA, and UCS.

Effect of ANK3 on the expression of pan-cancer DNA MMR genes and methyltransferases

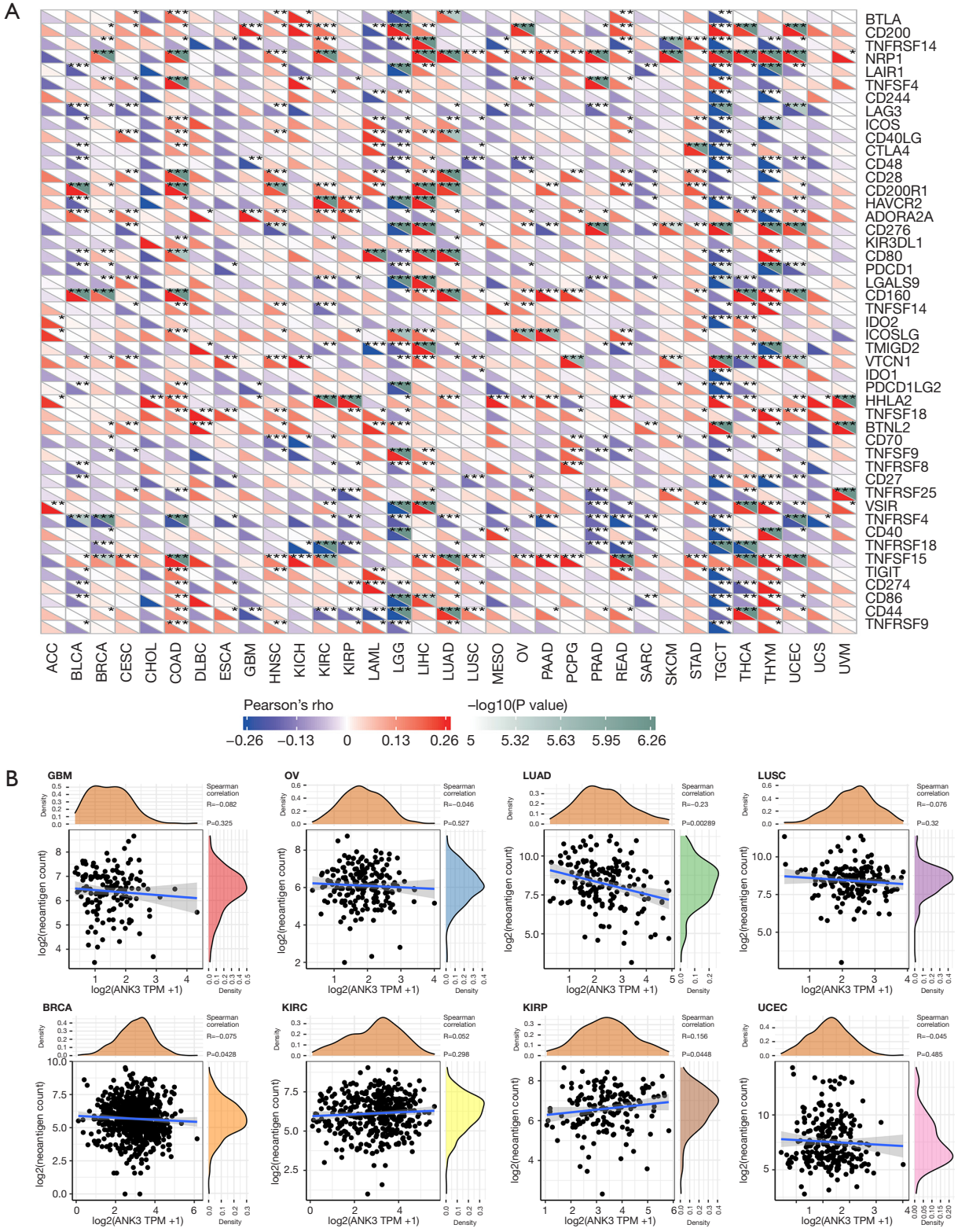
As *Figure 11A* shows, the MMR genes in most tumor tissues, such as OV, PAAD, PRAD, Rectal Cancer (READ), and STAD tumor tissues, were positively correlated with *ANK3* expression levels. DNA methylation is a widely studied epigenetic modification modality that, together with histone modifications and other modalities, effectively regulates gene expression and chromatin conformation. As *Figure 11B* shows, *ANK3* expression was significantly and positively correlated with the expression of the four methyltransferases in almost all tumors.

Analysis of the correlation between TAF ANK3 expression and immune invasion

TIICs, which essentially constitute the TME, remarkably affect the development, progression, and metastasis of tumors, and have generally been shown to be associated with a good prognosis (21). TAFs in the interstitial TME regulate the functions of various TIICs (22,23). In this article, we used the TIDE, XCELL, MCPCOUNTER, and EPIC algorithms to examine the correlation between immune cell infiltration levels and *ANK3* gene expression in various types of TCGA. According to the EPIC algorithm, in Melanoma (SKCM), SKCM-metastasis, and LIHC, *ANK3* expression was found to be positively correlated with the estimated infiltration values of cancer-associated fibroblasts (CAFs), and negatively correlated with KIRC (*Figure 12A*). *ANK3* expression in ACC was found to be correlated with the CAFs infiltration level according to the XCELL algorithm (cor=0.247, P=3.48e–02) (*Figure 12B*).

Enrichment analysis of ANK3 expression

Figure 13 shows samples with high and low expression levels of *ANK3* derived from the GSEA. According to the KEGG enrichment analysis, the high expression of *ANK3* is primarily associated with the neurotrophic factor, endometrial cancer signaling pathways, and the mammalian circadian pathway. The HALLMARK analysis showed that the high expression of *ANK3* was associated with the UV_RESPONSE_DN, HEDGEHOG, and HEME_METABOLISM signaling pathways. To further explore the mechanism of the *ANK3* gene in tumorigenesis, we screened out the targeting *ANK3* binding protein and genes related to *ANK3* expression. *Figure 14A*,*14B* shows the 10 *ANK3* protein expression-related genes. As *Figure 14C-14F* show, the GEPIA2 tool integrated all the tumor expression data from TCGA to identify multiple *ANK3* protein expression-associated genes. According



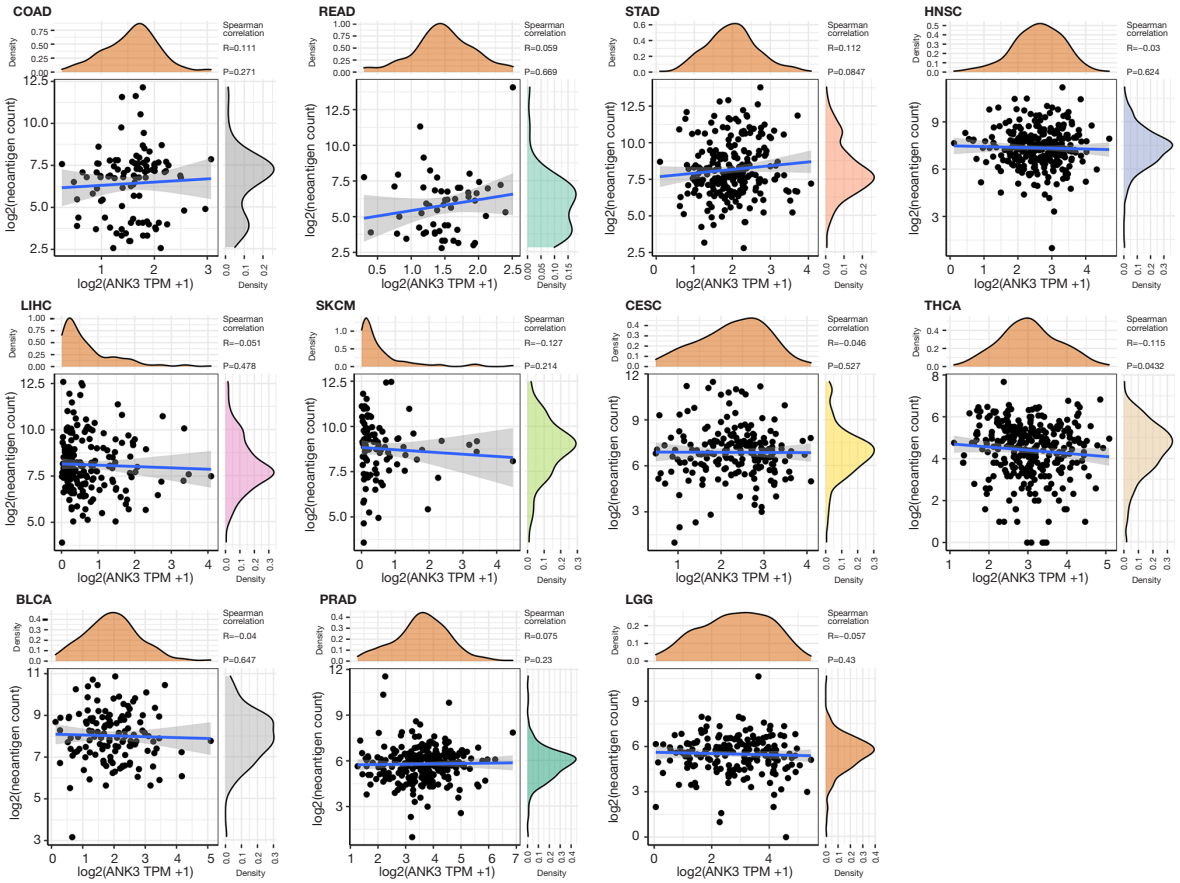


Figure 9 Analysis of the correlation between *ANK3* expression and INAs and ICGs in pan-cancer tissues. (A) Analysis of the correlation between *ANK3* and ICGs in pan-cancer tissues. (B) Analysis of the correlation between *ANK3* and INAs in the pan-cancer tissues of 19 tumors. *, P<0.05; **, P<0.01; ***, P<0.01. GBM, glioblastoma multiforme; *ANK3*, ankyrin-3; TPM, transcripts per million; OV, ovarian serous cystadenocarcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; BRCA, breast invasive carcinoma; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; UCEC, uterine corpus endometrial carcinoma; COAD, colon adenocarcinoma; READ, rectal adenocarcinoma; STAD, stomach adenocarcinoma; HNSC, head and neck squamous cell carcinoma; LIHC, liver hepatocellular carcinoma; SKCM, cutaneous skin melanoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; THCA, thyroid cancer; BLCA, bladder urothelial carcinoma; PRAD, prostate adenocarcinoma; LGG, low-grade glioma; INAs, immune neoantigens; ICGs, immune checkpoint genes.

to a further analysis, *ANK3* expression was positively associated with the expression of cervical intraepithelial neoplasia grade 1 (*CINNB1*) (R=0.063), murine sarcoma virus oncogene (*KRAS*) (R=0.0530), Parkinson's protein 2 (*PARK2*) (R=0.27), and RHO protein (*RHOB*) (R=0.2).

Discussion

In the past, surgery, supplemented by chemotherapy and radiotherapy, served as the primary method for treating tumors. In the last decade, following the emergence of immunotherapy, it has become possible to cure malignant tumors. However, tumors have complex ecosystems in which different cell types communicate with each other, and the efficacy of immunotherapy for patients with different tumor types or different patients with the same tumor types differs. Thus, new tumor immune markers and potential therapeutic targets need to be identified. The present study conducted a systematic and comprehensive investigation of *ANK3* expression and patient prognosis in pan-cancerous tissues to investigate its value in immunotherapy. Previous research examined the genotype of 24 single nucleotide

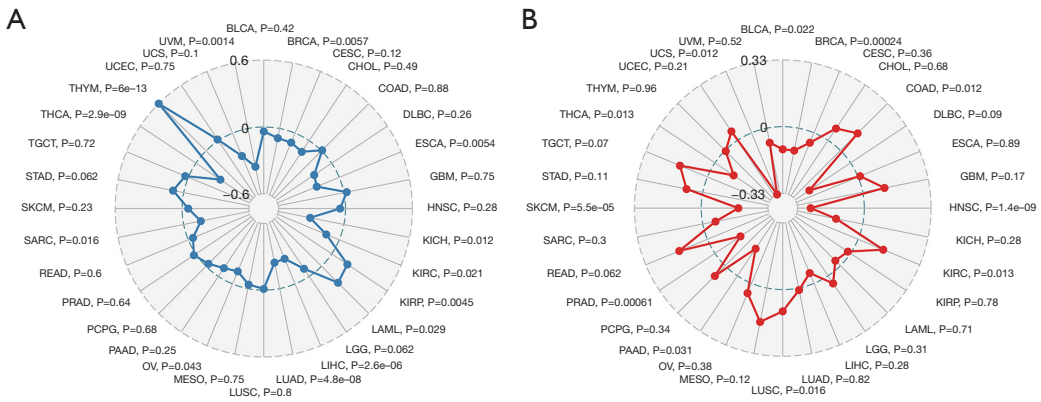


Figure 10 Analysis of the correlation between *ANK3* expression and TMB and MSI in pan-cancer tissues. (A) The Spearman's rank correlation coefficient was used to examine the *ANK3*/TMB correlation in cancer tissues. (B) Spearman's rank correlation coefficient was used to examine the *ANK3*/MSI correlation in pan-cancer tissues. *ANK3*, ankyrin-3; TMB, tumor mutation burden; MSI, microsatellite instability.

polymorphisms in 321 patients with social anxiety disorder and 804 control patients, and showed that genome-wide associated genetic variants in the bipolar disorder gene *ANK3* may affect the personality traits associated with anxiety (24). Subsequently, the other study (11) has shown its extensive involvement in tumor proliferation, invasion, and metastasis, and its different roles in tumors.

The present study analyzed the differential expression of *ANK3* in different tumor tissues and normal tissues and found that *ANK3* was significantly upregulated in CHOL, and PRAD, and significantly downregulated in COAD, HNSC, KICH, KIRC, and STAD tumor tissues. In this study, the mutation characteristics of *ANK3* gene in different tumor tissues were detected. The overall mutation rate was 7%, and the mutation types were deep deletions and diploidy. According to the literature, alterations in *ANK3* and type I collagen may increase the risk of developing ureteral primary malignant melanoma (25). The ectopic expression of *ANK3* significantly increased E-calcineurin expression and restricted the progression of papillary thyroid carcinoma (PTC), which suggests that *ANK3* has an anti-cancer action in PTC development, and serves as the inert maintainer of PTC (26). However, Previous studies suggest the function and mechanism of *ANK3* in tumor tissues are currently unknown, and need to be investigated further in future research.

By analyzing the influence of differential expression of *ANK3* protein on prognosis in normal and tumor tissues, we found that *ANK3* was down-regulated in KIRC, BC, rectal cancer, LIHC, OC, and pancreatic cancer, and low expression of *ANK3* was associated with adverse OS in ACC and KIRC patients, which counters other recent findings about its prognostic value in BC (11). We also found that the high expression of the *ANK3* protein was correlated with progesterone receptor positivity, and was independently associated with a good prognosis. It may be that BC patients have various molecular types and genetic backgrounds. Thus, studies with larger sample sizes need to be conducted in the future to validate these findings. Our analysis showed that while *ANK3* was lowly expressed in most tumor tissues, it indicates a poor prognosis in some tumors. A recent study showed that the upregulation of *ANK3* in neuroblastoma patients with poor OS, as it affects the cell motility of *PTK2* signaling and the *MAPK* pathway related to matrix metalloproteinase activity.

TME can be understood as the occurrence, development, metastasis of tumors and the internal and external environment of tumor cells. Tumor immune infiltrating cells crucially affect tumor appreciation and metastasis (27). Thus, we investigated the relevance of *ANK3* expression to the TME in pan-cancer and its TII to identify how ANK3 regulates the TME. This study first examined if ANK3 exhibited a clear association with immune cell infiltration, and found an obvious association between ANK3 expression and the TII rate in COAD, KIRC, and LIHC. These findings suggest that *ANK3* may affect cancer progression by affecting TII in malignant tumors. According to recent findings, the interaction between innate immune cells (innate lymphoid-like cells, macrophages, neutrophils, dendritic cells, suppressor cells from bone marrow, and

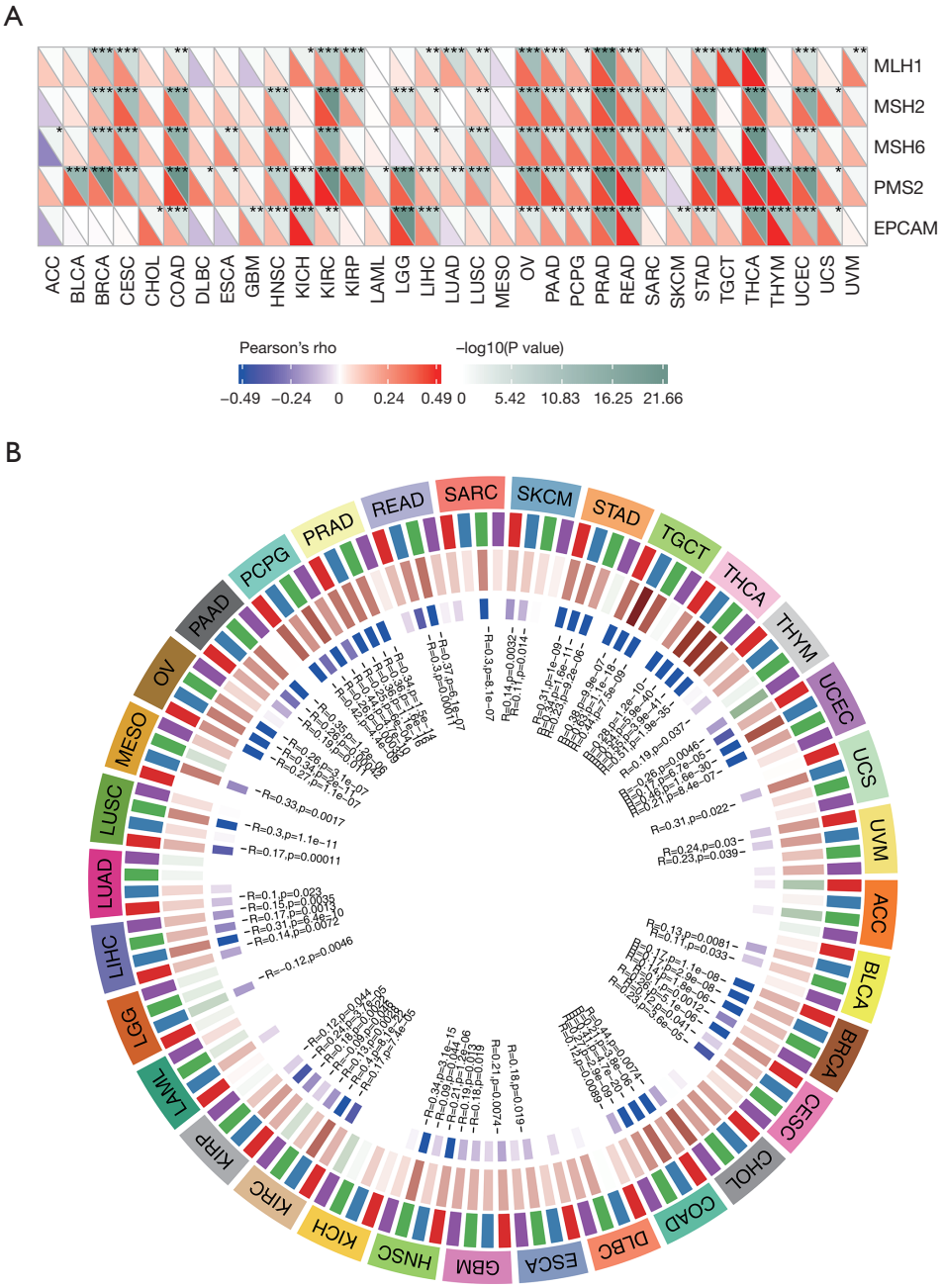


Figure 11 Analysis of the correlation between *ANK3* expression and the expression of DNA repair genes and methyltransferase in pan-cancer tissues. (A) Analysis of the correlation between the expression of five MMR genes and *ANK3* expression. (B) Analysis of the correlation between *ANK3* expression and four methyltransferases (red, blue, green, and purple denote *DNMT1*, *DNMT2*, *DNMT3a*, and *DNMT3b*, respectively). *, P<0.05; **, P<0.01; ***, P<0.001. *ANK3*, ankyrin-3; MMR, mismatch repair.

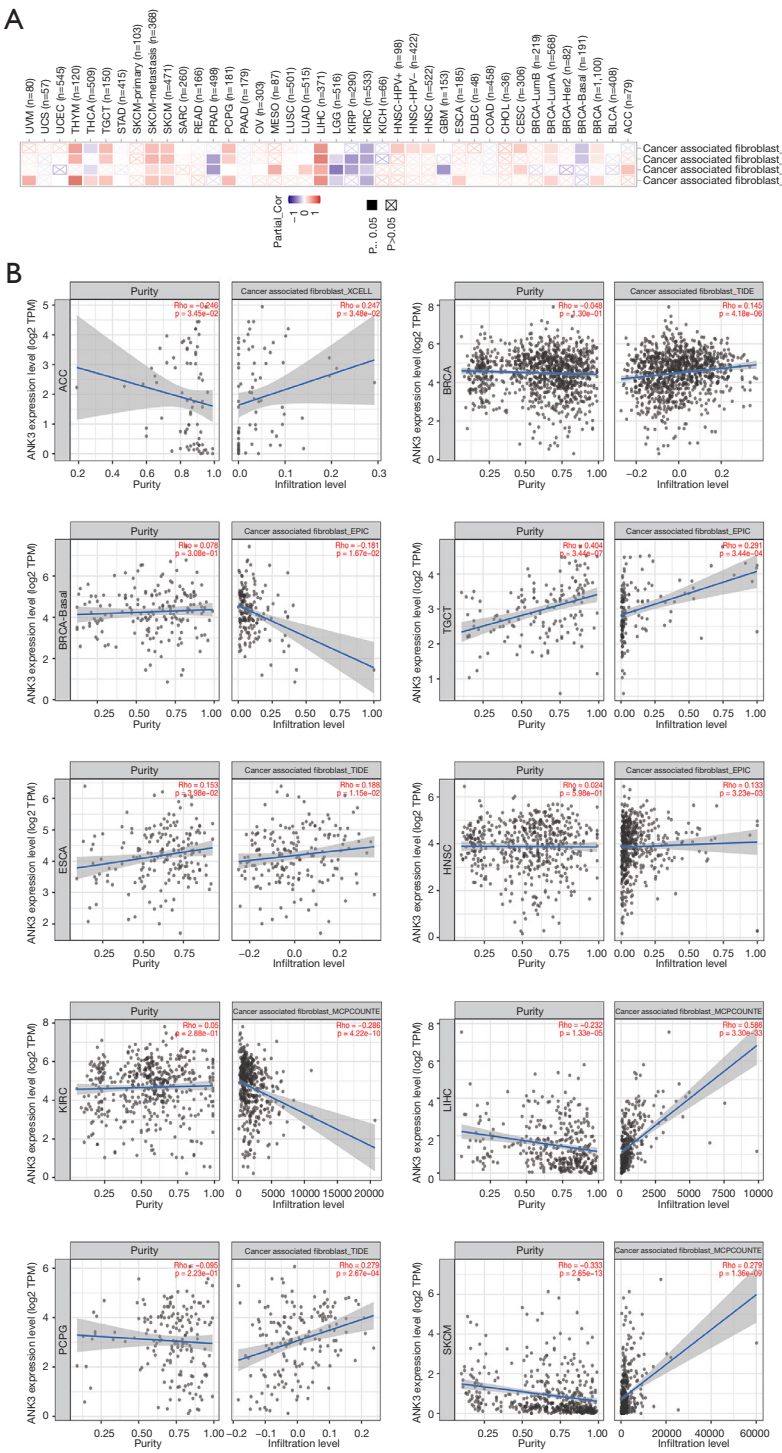


Figure 12 The relationship between *ANK3* expression and immune infiltration of TAFs. (A) Analysis of the correlation between *ANK3* expression and TAFs with immune infiltration. (B) Different algorithms were used to explore the correlation between *ANK3* gene expression and the TAFs infiltration level in TCGA. *ANK3*, ankyrin-3; TPM, transcripts per million; TAFs, tumor-associated fibroblasts; TCGA, The Cancer Genome Atlas.

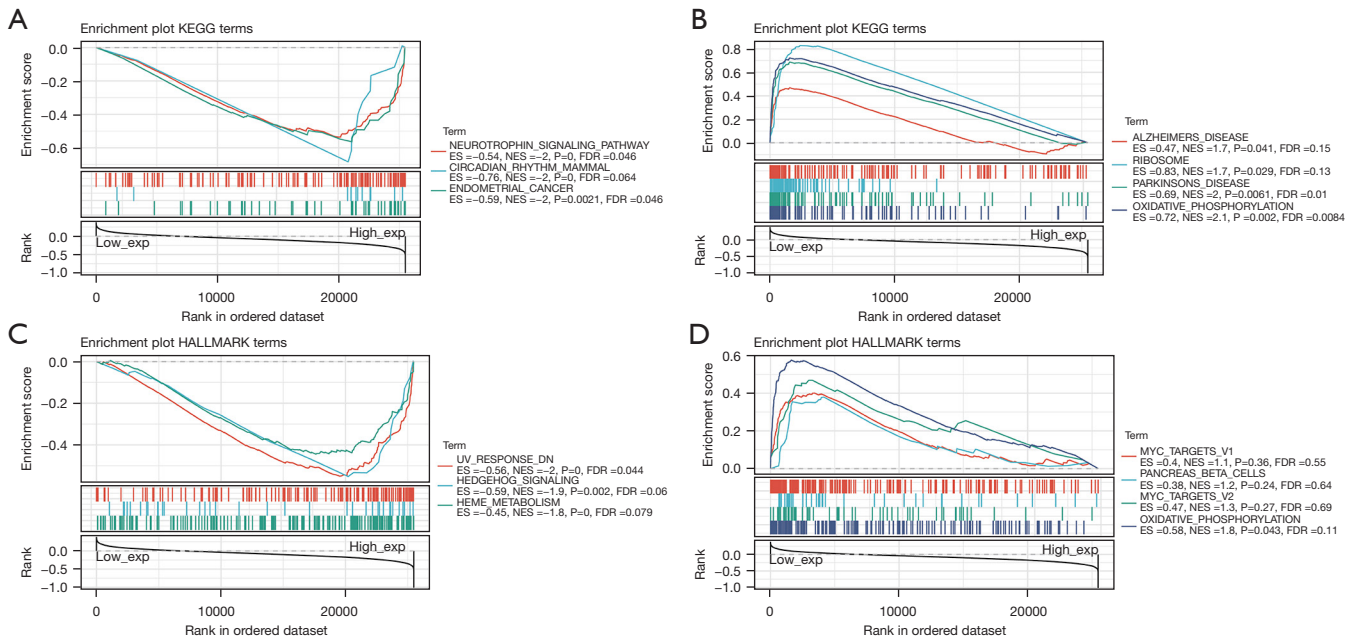


Figure 13 GSEA of patients with high and low expression levels of *ANK3*. (A) Abundant gene data sets from the KEGG collection of patients with high expression levels of *ANK3*. (B) The GSEA of KEGG sets of patients with low expression levels of *ANK3*. (C) The GSEA of HALLMARK sets of patients with high expression levels of *ANK3*. (D) The GSEA of HALLMARK sets of patients with low expression levels of *ANK3*. KEGG, Kyoto Encyclopedia of Genes and Genomes; ES, embryonic stem cell; NES, net enrichment score; FDR, false discovery rate; GSEA, gene set enrichment analysis; *ANK3*, ankyrin-3.

natural killers cells), adaptive immune cells (T and B cells), and cancer cells favor tumor growth and metastasis and decisively affect cancer survival and progression (28). Thus, our study also investigated the relationship between *ANK3* expression and immune cell abundance in pan-cancer, and found that the expression level of *ANK3* in LIHC, COAD, and KIRC was significantly positively correlated with macrophages, neutrophils, B cells, $CD4^+$ T cells, $CD8^+$ T cells, and dendritic cells, which indicates that the high expression of *ANK3* in pancarcinoma is more significantly correlated with immune invasion. It is roughly the same as high levels of *ANK3* expression being associated with higher immune scores in cancer. However, it should be noted that the expression level of *ANK3* in LGG was significantly negatively correlated with mesenchymal scores and immune scores, which has not been reported in any other studies. In conclusion, little is known about the biological function of *ANK3* in regulating the TME, and this needs to be explored further in future studies.

Tumor cells and their microenvironment typically produce a large number of immune regulators that upregulate immunosuppressive factors or downregulate immune activators to achieve immune escape induction. Positive regulators (*CD27*, *CD28*, *CD30*, and *ICOS*), and negative regulators (*CTLA4*, *PD-1*, *BTLA*, *TIM3*, and *LAG3*) affect the ability of the immune system to recognize tumor cells at immune loci (29-31). This study confirmed the positive association between *ANK3* and immunosuppressants, such as *CD274*, *KDR*, and *VTCN1*, which supports the findings of previous studies (20,32,33). *ANK3* may promote tumor metastasis and immunosuppression by expressing immunosuppressive genes (*CD274*, *KDR*, and *VTCN1*) in pan-cancer. Notably, in our study, *ANK3* was found to be positively correlated with the immune agonist *TNFSF15*, a cytokine that possesses anti-angiogenic activity and can promote the differentiation and polarization of macrophages to the M1 phenotype in KICH, PAAD, and UCS, which may in turn have a beneficial effect on the reconstitution of the immune microenvironment for tumor suppression (34). Thus, *ANK3* is co-expressed with various immunosuppressants, immunostimulatory factors, and MHC genes in pan-cancer, and can influence immunomodulatory gene expression or can act as the immunomodulatory gene itself.

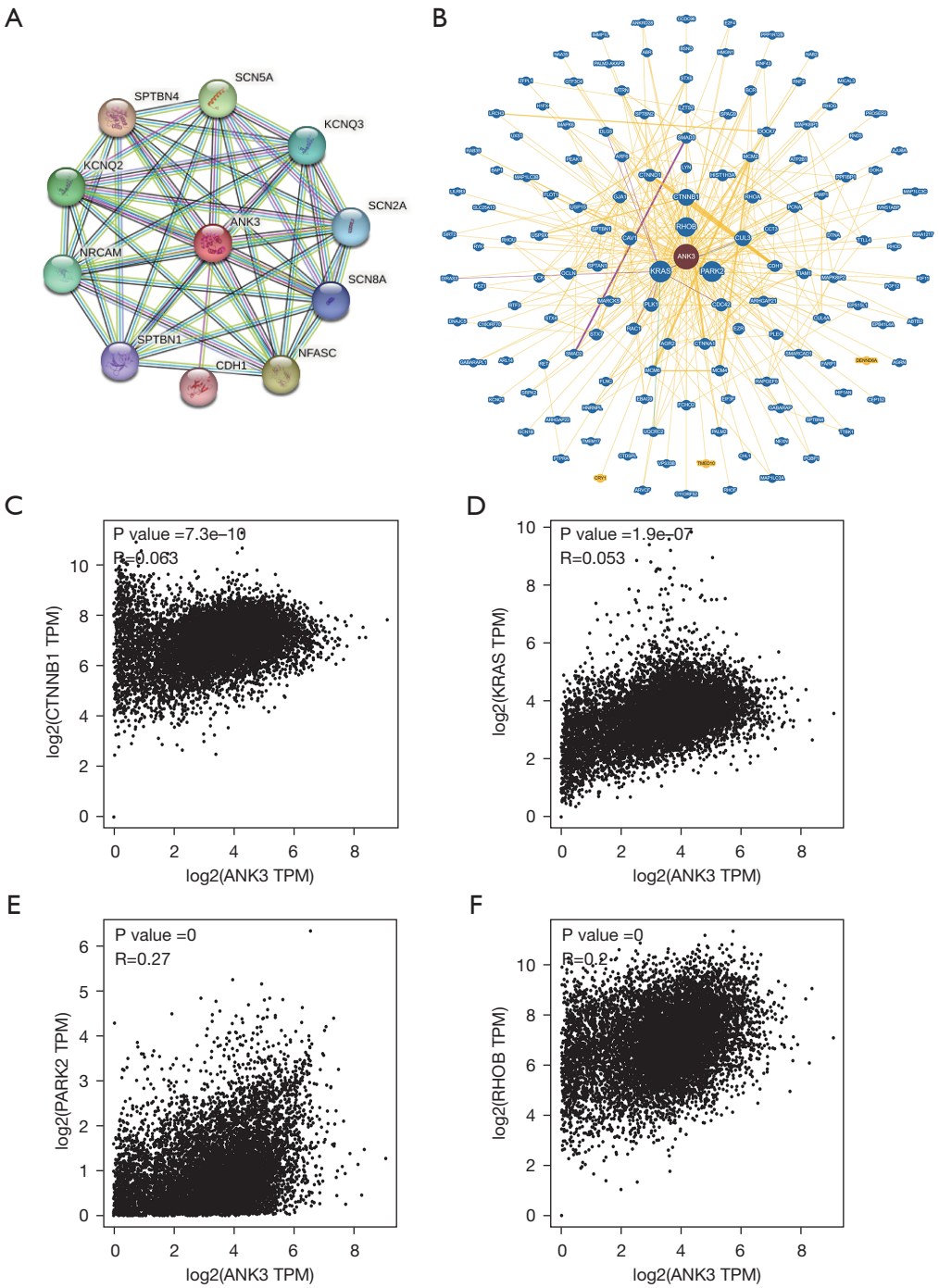


Figure 14 *ANK3*-related gene analysis. (A) The STRING tool was used to obtain *ANK3*-binding proteins. (B) BioGRID was used to screen the ANK3-targeting binding proteins. (C-F) Analysis of the correlation between ANK3 expression and the expression of selected target genes (*CINNB1*, *KRAS*, *PARK2*, and *RHOB*). *ANK3*, ankyrin-3; TPM, transcripts per million; STRING, Search Tool for the Retrieval of Interacting Genes.

The clinical stage, pathological grade, and immune and molecular subtype of a tumor may predict patient prognosis to some extent. According to the TISIDB analysis, *ANK3* expression indicates disease development in BLCA, BRCA, KIRC, LGG, LIHC, LUAD, and PRAD. ANK3 is also a gene that promotes LGG, LUSC, and OV, and UCEC. In the present study, we found that *ANK3* expression differed significantly in various immune and molecular subtypes of certain cancer types. Specifically, in ACC, *ANK3* was lowly expressed in the CIMP-high subtype, moderately expressed in the CIMP-medium subtype, and highly expressed in the CIMP-low subtype. Thus, *ANK3* may be an important biomarker associated with different tumor prognoses.

As oncogene sequencing and characterization methods continue to evolve, the selection of immunological neoantigens for tumors and ICGs has become increasingly important. For example, deficient MMR (dMMR) proteins or metastatic/unresectable cancers with high MSI exhibit a stronger susceptibility to the immune checkpoint inhibitors (ICIs) treatment with pembrolizumab blocking anti-PD-1. Thus, pembrolizumab is used to treat unresectable/metastatic cancers with a TMB ≥10 mutations/megabases (35). We explored this by examining the expression of over 40 ICGs and found that *ANK3* expression was negatively correlated with ICG expression in different tumor types (BLCA, LGG, and TGCT), which demonstrated that *ANK3* plays a regulatory role in the tumor immune pattern via the regulation of ICG expression. Notably, *ANK3* expression was only found to be negatively correlated with the neoantigen number in LUAD, which suggests that *ANK3* may serve as a new therapeutic marker in LUAD. TMB and MSI (microsatellite deletion), which are often present in tumor tissues, are closely associated with tumor prognosis after immunotherapy and thus can be used to predict cancer treatment outcomes. According to our findings, *ANK3* was positively correlated with the TMB in THYM, KIRP, and LAML, which suggests that a higher expression of *ANK3* is associated with a higher mutation degree in these tumors. *ANK3* expression was also found to be positively associated with MSI in COAD, KIRC, and LUSC and negatively associated with MSI in BRCA, HNSC, PRAD, SKCM, THCA, and UCS. This suggests that *ANK3* expression affects the TMB and MSI and patients' responses to ICIs, and thus may be used to predict the immunotherapy outcomes of many different tumors.

We also analyzed the effect of *ANK3* on pan-cancer DNA MMR gene expression and methyltransferases expression, and found a positive correlation between *ANK3* expression and MMR genes in most tumor tissues, such as OV, PAAD, PRAD, PEAD, and STAD. Accordingly, *ANK3* may promote DNA MMR genes by upregulating DNA methylation (36). According to our study, *ANK3* expression was positively correlated with methyltransferase expression in almost all tumors, which suggests that *ANK3* may regulate tumorigenesis and development through the regulation of pan-cancer epigenetic status. TAFs cannot exist alone in tumor tissues but need to interact with tumor cells to promote tumor proliferation and survival and maintain their malignant propensity. Our study is the first to reveal a link between *ANK3* expression and the TII of TAFs, which indicates that TAF activity can be influenced by affecting *ANK3* expression, thereby inhibiting tumor growth and activity.

Finally, we conducted a KEGG analysis and GSEA to analyze the molecular mechanism of action of *ANK3*. The KEGG-enriched data set showed an obvious association between the high expression of *ANK3* and the neurotrophic factor signaling pathway, the mammalian circadian pathway, and the endometrial cancer signaling pathway. According to the HALLMARK analysis, the high expression of *ANK3* is correlated with the UV_RESPONSE_DN, HEDGEHOG, and HEME_METABOLISM signaling pathways. According to previous studies, Krüppel-like factor 9 inhibits the expression of factors, such as *ANK3*, thereby affecting uterine epithelial gene expression and promoting endometrial cancer development (37). A further analysis revealed a positive correlation between *ANK3* expression and *CINNB1*, *KRAS*, and *PARK2* expression. Thus, we can conclude that the *ANK3* gene regulates immune cell infiltration and immunomodulatory factor-related signaling pathways, thus affecting tumorigenesis and progression.

Conclusions

In summary, this study analyzed the association between *ANK3* gene expression and patient prognosis. The results suggest that *ANK3* is related to tumor prognosis. Additionally, this study revealed a strong association between *ANK3* and tumor immunity. *ANK3* may serve as a biomarker and treatment target for tumor immunity. The results of this study are based on a bioinformatics analysis of open databases and have not been verified in experiments. However, these findings may contribute to future related studies to some extent. This study comprehensively explored the potential action mechanism of *ANK3* in human tumors, while also supporting the findings of other

experimental investigations.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2379/rc

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2379/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2379/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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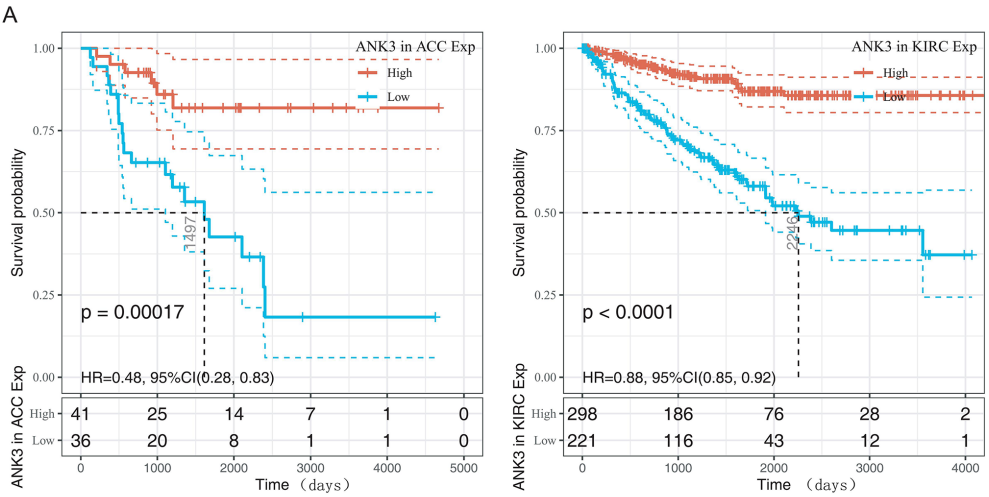

B

	HR	P Value
ACC	0.48(0.28 ~ 0.83)	8.5e−03
BLCA	1.03(0.98 ~ 1.08)	2.3e−01
BRCA	0.99(0.95 ~ 1.02)	4.4e−01
CESC	0.96(0.88 ~ 1.05)	4.1e−01
CHOL	0.98(0.89 ~ 1.09)	7.1e−01
COAD	0.91(0.76 ~ 1.09)	3.2e−01
DLBC	0.38(0.01 ~ 10.46)	5.7e−01
ESCA	0.98(0.88 ~ 1.08)	6.2e−01
GBM	1(0.93 ~ 1.07)	9.9e−01
HNSC	0.96(0.9 ~ 1.01)	1.3e−01
KICH	1.02(0.8 ~ 1.3)	8.8e−01
KIRC	0.88(0.85 ~ 0.92)	3.0e−09
KIRP	0.98(0.94 ~ 1.03)	4.7e−01
LAML	NA(NA ~ NA)	
LGG	0.97(0.94 ~ 1)	5.4e−02
LIHC	0.96(0.82 ~ 1.12)	6.0e−01
LUAD	0.99(0.96 ~ 1.03)	6.5e−01
LUSC	1.02(0.96 ~ 1.09)	4.8e−01
MESO	1.03(0.88 ~ 1.2)	7.3e−01
OV	1.02(0.95 ~ 1.08)	6.4e−01
PAAD	0.97(0.88 ~ 1.07)	5.6e−01
PCPG	1.12(0.92 ~ 1.37)	2.6e−01
PRAD	0.99(0.91 ~ 1.08)	8.8e−01
READ	0.93(0.6 ~ 1.46)	7.6e−01
SARC	0.99(0.9 ~ 1.08)	7.4e−01
SKCM	1(0.96 ~ 1.05)	9.1e−01
STAD	0.97(0.89 ~ 1.06)	5.2e−01
TGCT	0.9(0.43 ~ 1.9)	7.9e−01
THCA	0.96(0.81 ~ 1.15)	6.8e−01
THYM	0.87(0.59 ~ 1.28)	4.7e−01
UCEC	0.99(0.92 ~ 1.07)	8.3e−01
UCS	1.04(0.91 ~ 1.18)	5.7e−01
UVM	0.59(0.17 ~ 2.01)	4.0e−01

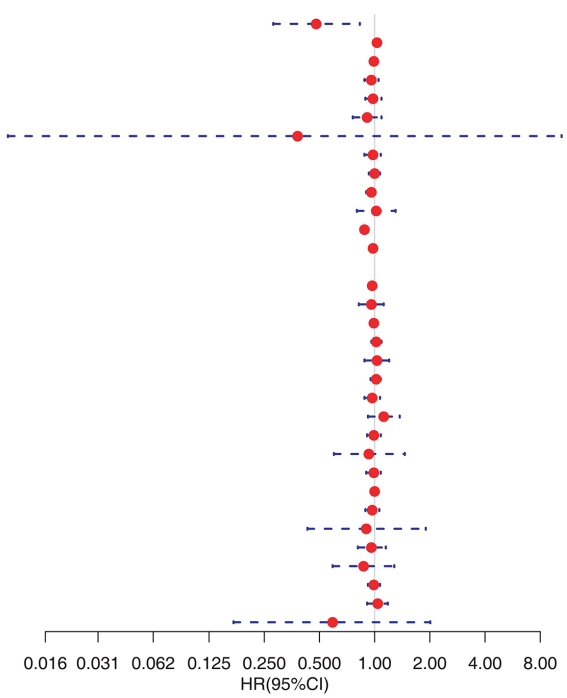

Figure S1 *ANK3* expression in pan-cancer and its relevance to DSS. *ANK3*, ankyrin-3; ACC, adrenocortical carcinoma; HR, hazard ratio; CI, confidence interval; KIRC, Kidney renal clear cell carcinoma; DSS, disease-specific survival.

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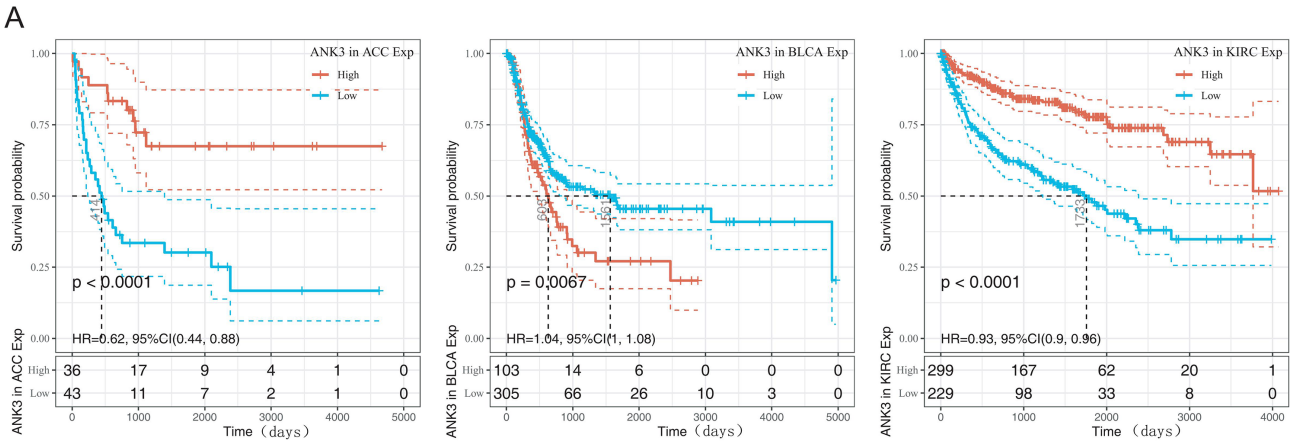

B

	HR	P Value
ACC	0.62(0.44 ~ 0.88)	6.4e−03
BLCA	1.04(1 ~ 1.08)	3.2e−02
BRCA	0.99(0.96 ~ 1.01)	3.0e−01
CESC	0.97(0.9 ~ 1.04)	4.1e−01
CHOL	0.97(0.87 ~ 1.07)	5.1e−01
COAD	0.92(0.8 ~ 1.05)	2.1e−01
DLBC	0.34(0.04 ~ 3.29)	3.5e−01
ESCA	1(0.93 ~ 1.07)	9.1e−01
GBM	0.99(0.91 ~ 1.07)	7.2e−01
HNSC	0.97(0.93 ~ 1.02)	1.9e−01
KICH	0.96(0.79 ~ 1.17)	7.0e−01
KIRC	0.93(0.9 ~ 0.96)	3.3e−07
KIRP	1(0.98 ~ 1.03)	8.0e−01
LAML	NA(NA ~ NA)	
LGG	0.98(0.96 ~ 1)	8.0e−02
LIHC	1.06(0.97 ~ 1.16)	2.1e−01
LUAD	1(0.97 ~ 1.02)	7.7e−01
LUSC	1.01(0.96 ~ 1.06)	7.0e−01
MESO	0.92(0.79 ~ 1.08)	3.1e−01
OV	0.98(0.93 ~ 1.04)	5.9e−01
PAAD	0.95(0.87 ~ 1.04)	2.3e−01
PCPG	1.05(0.96 ~ 1.15)	2.4e−01
PRAD	0.98(0.96 ~ 1.01)	1.9e−01
READ	0.84(0.64 ~ 1.11)	2.3e−01
SARC	0.99(0.93 ~ 1.05)	7.1e−01
SKCM	1.01(0.98 ~ 1.05)	4.4e−01
STAD	0.98(0.91 ~ 1.06)	6.3e−01
TGCT	1.01(0.84 ~ 1.22)	9.3e−01
THCA	0.94(0.88 ~ 1.01)	9.2e−02
THYM	0.95(0.85 ~ 1.07)	4.1e−01
UCEC	0.98(0.92 ~ 1.03)	4.3e−01
UCS	1.05(0.94 ~ 1.17)	3.9e−01
UVM	0.97(0.77 ~ 1.22)	8.1e−01

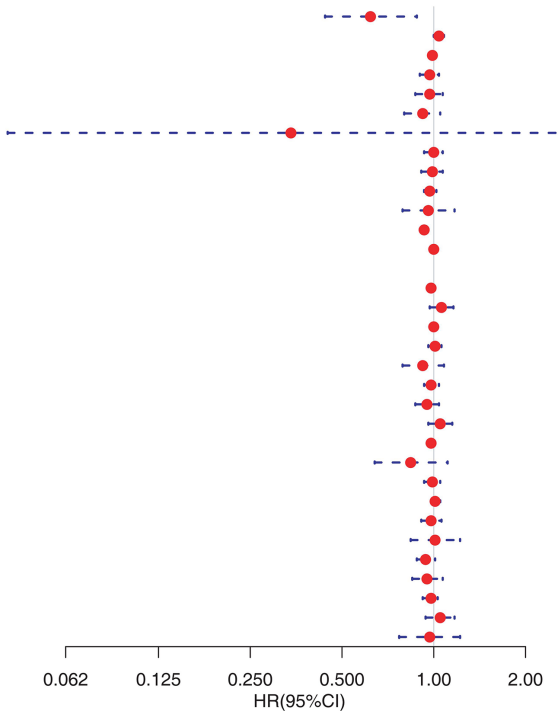

Figure S2 *ANK3* expression in pan-cancer and its relevance to PFI. *ANK3*, ankyrin-3; ACC, adrenocortical carcinoma; HR, hazard ratio; CI, confidence interval; BLCA, Bladder Cancer; KIRC, Kidney renal clear cell carcinoma; PFI, progression-free interval.

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