Original Article

Pan-cancer analyses demonstrate that ANKRD6 is associated with a poor prognosis and correlates with M2 macrophage infiltration in colon cancer

Rui Bai[1,2*], Dehao Wu[2*], Zhong Shi[3], Wangxiong Hu[2], Juan Li[4], Yuanyuan Chen[4], Weiting Ge[2], Ying Yuan[1,2], Shu Zheng[2]

[1]Department of Medical Oncology, Key Laboratory of Cancer Prevention and Intervention, Ministry of Education, the Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310009, China; [2]Cancer Institute, Key Laboratory of Cancer Prevention and Intervention, Ministry of Education, the Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310009, China; [3]Department of Medical Oncology, Institute of Cancer and Basic Medicine (ICBM) Chinese Academy of Sciences, Cancer Hospital of the University of Chinese Academy of Sciences, Hangzhou 310022, China; [4]Department of Radiation Oncology, Hangzhou Cancer Hospital, Hangzhou 310002, China

*These authors contributed equally to this work.

Correspondence to: Shu Zheng. Cancer Institute, Key Laboratory of Cancer Prevention and Intervention, Ministry of Education, the Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310009, China. Email: zhengshu@zju.edu.cn; Ying Yuan. Department of Medical Oncology, Key Laboratory of Cancer Prevention and Intervention, Ministry of Education, the Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310009, China. Email: yuanying1999@zju.edu.cn.

Abstract

Objective: Ankyrin repeat domain-containing protein 6 (ANKRD6) is an ankyrin repeat-containing protein which is structurally related to vertebrate inversin and Drosophila Diego. However, the correlations between ANKRD6 and tumor-infiltrating immune cells in cancers is not clear.

Methods: ANKRD6 expression was analyzed by Oncomine, Tumor Immune Estimation Resource (TIMER) and Gene Expression Profiling Interactive Analysis (GEPIA). PrognoScan and GEPIA were used to evaluate the influence of ANKRD6 on clinical prognosis. TIMER and CIBERSORT were used to analyze correlations between ANKRD6 expression levels and tumor immune cell infiltrates. Immunohistochemical analysis of the relationship between ANKRD6 expression and overall survival, as well as the relationship between ANKRD6 expression and M2 macrophage infiltration, was performed.

Results: High level of ANKRD6 expression was associated with poor prognosis of colon cancer. ANKRD6 expression level was positively correlated with infiltrating levels of CD8+ T cells, CD4+ T cells, macrophages, neutrophils and dendritic cells in colon cancer by using TIMER. Using CIBERSORT, we found that in plasma cells, CD8+ T cells, CD4+ memory resting T cells, follicular helper T cells and activated natural killer cells were significantly lower in the ANKRD6-high group than in the ANKRD6-low group. M0 and M2 macrophages were significantly higher in the ANKRD6-high group than in the ANKRD6-low group. Immunohistochemistry confirmed that M2 macrophage infiltration in the ANKRD6-high group significantly increased.

Conclusions: The high ANKRD6 expression is associated with poor prognosis of colon cancer. ANKRD6 expression is positively correlated with M2 macrophage infiltration in colon cancer.

Keywords: ANKRD6; colon cancer; pan-cancer; prognosis; tumor-infiltrating

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Introduction

According to the 2015 China Cancer Statistics, colorectal cancer has high morbidity and mortality rates and is the fifth most common malignant tumor (1). Immune checkpoint inhibitors, such as anti-programmed cell death 1 (PD-1)-inducing antibodies, anti-programmed cell death 1 ligand (PD-L1) inhibitory antibodies, and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibodies, alter cancer treatment. Efforts have been made to eliminate tumor immune interactions and identify prognostic predictors.

Ankyrin repeat domain-containing protein 6 (ANKRD6), also known as Diversin, is an ankyrin repeat protein that activates nonneural planar cell polarity (PCP) signals while inhibiting typical pathways. ANKRD6 is a modular protein located on chromosome 6 (q14.2−q16.1) and a member of the ankyrin repeat domain protein family. The ankyrin repeat domain protein family mediates physiologically important protein-protein interactions and acts as an adapter for signaling pathways (2). ANKRD6 is localized in the Xenopus ectoderm and mammalian cell centrosome (3). ANKRD6 is highly expressed in non-small cell lung cancer (NSCLC), breast cancer, and human gliomas (4-6). ANKRD6 overexpression is a predictor of poor prognosis of NSCLC and breast cancer (4,5). ANKRD6 promotes cell proliferation and invasion through the c-Jun N-terminal kinase (JNK) pathway (4,5). ANKRD6 has a stronger cytotoxic effect on 5,637 cells (bladder cancer cells) than human dermal fibroblast (HDF1) and human foreskin fibroblast (HFF3) cells (normal cells) (7).

WNT signals are transduced to the β-catenin pathway or PCP pathway. PCP is an important branch of WNT signaling (8) and is highly involved in embryogenesis and is essential for human disease (9,10). It activates the cytoskeletal pathway (11) to affect organ morphogenesis and cell polarization movement. The WNT-β-catenin pathway is related to tumorigenesis, and the WNT-PCP pathway is related to cell movement and metastasis and promotes tumor invasion, survival, and treatment resistance (12,13). The WNT-β-catenin pathway and the WNT-PCP pathway affect T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) and AP-1 (14) and regulate the immune response of tumors (15,16). The AP-1 components (c-Jun, JunB, c-Fos, Batf), by binding to the enhancer region (15), induce the expression of genes encoding cosuppressive immune checkpoints (PD-1, PD-L1). WNT inhibitors reduce PD-L1 expression, and WNT agonists enhance PD-L1 expression at the transcription and protein levels (17). TCF/LEF shows dynamic expression during T cell maturation, and the constitutive activation of the Wnt/β-catenin pathway reduces the expansion of mature cytotoxic T cells (18). So we speculate that ANKRD6 may affect the immune microenvironment through the WNT pathway.

The tumor immune microenvironment is mediated by immune cells infiltrating the tumor. Immunotherapy for tumors acts on the immune microenvironment, and the heterogeneity of the tumor immune microenvironment is bound to have a close relationship with the different response rates of immunotherapy. The immune microenvironment is expected to serve as a biomarker to guide clinical treatment and screen tumor patients who can benefit from immunotherapy. The correlations between ANKRD6 and tumor-infiltrating immune cells in cancers remain unclear. In this study, we evaluated the expression of ANKRD6 on clinical prognosis and analyzed correlations between ANKRD6 expression levels and tumor immune cell infiltrates.

Materials and methods

Analysis of differential expression in tumor and matched normal tissues

Oncomine database analysis

ANKRD6 expression in cancers was analyzed with the Oncomine database (https://www.oncomine.org/resource/login.html) (19). This database has identified the genes across 412 unique analyses of gene expression microarrays, spanning the majority of cancer types and subtypes. The threshold was determined as follows: P-value of 0.001, fold change of 1.5, and gene ranking of "ALL".

Tumor Immune Estimation Resource (TIMER) database analysis

TIMER is a comprehensive resource for systematic analysis of immune infiltrates (https://cistrome.shinyapps.io/timer/) (20). TIMER consists of six functional modules, including the association of tumor-infiltrating immune cell (TIIC) abundance with gene expression (Gene), overall survival (Survival), somatic mutations (Mutation), DNA somatic copy number alterations (SCNA), differential gene expression (DiffExp) and gene-gene correlations

(Correlation). The data comprise 10,897 samples across 32 cancer types from The Cancer Genome Atlas (TCGA). The gene expression levels are displayed using box plots, and the statistical significance of differential expression levels was evaluated using the Wilcoxon test. We can identify genes that are up- or down-regulated in the tumors compared to normal tissues for each cancer type, as displayed in gray columns, when normal data are available.

Relationship between ANKRD6 expression and prognosis

PrognoScan database analysis

A PrognoScan database analysis of the correlation between ANKRD6 expression and survival in cancers was performed (http://dna00.bio.kyutech.ac.jp/PrognoScan/) (21). Cancer microarray datasets with clinical annotations were intensively collected from the Gene Expression Omnibus (GEO), ArrayExpress and individual laboratory web sites with the following criteria: 1) patient information on survival events and duration; 2) large enough sample sizes to enable survival analysis; 3) derived from a "whole genome" platform and has no missing values so quantile normalization can function properly; and 4) derived from a platform for which probe annotations for a public identifier is available. The relationships between gene expression and patient overall survival (OS), disease-specific survival (DSS) and disease-free survival (DFS) were evaluated across publicly available cancer microarray datasets. The relationship between gene expression and prognosis was investigated using the minimum P-value approach. The threshold was adjusted to a Cox $P<0.05$.

Gene Expression Profiling Interactive Analysis (GEPIA) database analysis

GEPIA (http://gepia.cancer-pku.cn/index.html) (22) was used to obtain survival curves, including OS and DFS curves, based on gene expression with the log-rank test and the Mantel-Cox test in 33 types of cancer. The GEPIA web server features are divided into seven major tabs: General, Differential Genes, Expression DIY, Survival, Similar Genes, Correlation and PCA based on TCGA and GTEx data. GEPIA is a web-based tool that delivers fast and customizable functionalities based on TCGA and GTEx data. GEPIA uses the log-rank test, sometimes called the Mantel-Cox test, for hypothesis evaluation. The group cutoff "median" was determined. The Cox proportional hazard ratio (HR) and 95% confidence interval (95% CI) information can also be included in the survival plot.

Relationship between ANKRD6 expression and infiltrating immune cells

TIMER database analysis

TIMER applies a previously published statistical method to infer the abundance of six TIIC subsets [B cells, CD4 T cells, CD8 T cells, macrophages, neutrophils and dendritic cells (DCs)] from the gene expression profiles of six TIIC subsets that are precalculated for 10,897 tumors across 32 cancer types from the TCGA. Tumor purity is the key to selecting genes that contribute to the deconvolution of immune cells in tumor tissue and is inferred from DNA SNP array data using the R package CHAT (23). Cancer and blood cell lines diluted to known ratios (24) verified the tumor purity. The ANKRD6 expression was negatively correlated with tumor purity and TIIC subsets when Pearson's $r\leq-0.2$ and $P\leq0.05$, and positively correlated when Pearson's $r\geq0.2$ and $P\leq0.05$.

CIBERSORT analysis

The relative abundance of 22 tumor-infiltrating lymphocytes (TILs) in colon cancer with different ANKRD6 expression levels (TCGA) was estimated by the CIBERSORT algorithm, a computational approach for inferring leukocyte representation in bulk tumor transcriptomes (25). Using a group cutoff "median", low and high ANKRD6 expression groups were formed, and 135 patients were included. The TILs were compared between two groups by Student's *t* test using GraphPad Prism software (Version 8.0; GraphPad Software, Inc., LaJolla, USA).

Immunohistochemistry (IHC)

The experimental protocol was established according to the ethical guidelines of the Helsinki Declaration. This study was approved by the Institutional Review Board of Clinical Research, the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine. The ethics approval number is 2018-159. Written informed consent was obtained from each subject before collection of tissue samples and clinical information. Detailed clinical data for each individual human subject are shown in *Supplementary Table S1,2*. Survival curves of IHC were generated by GraphPad Prism software version 8.0.

IHC of samples from the Second Affiliated Hospital of Zhejiang University School of Medicine: IHC staining and semiquantitative analysis were performed as described in a previous work. The 4 μm sections were incubated with the

anti-ANKRD6 (1:200, Proteintech, 24333-1-AP), anti-CD68 (1:500, Huabio, EM1706-11) and CD163 (1:800, Huabio, ER1804-03) antibodies.

ANKRD6 expression scores were blindly evaluated by two pathologists using the immunoreactivity score, based on the percentage of positive cells and the intensity of staining. The percentage of positive cells was graded as follows: 0 (negative), 1 (<10%), 2 (10%−50%), 3 (51%−80%), 4 (>80%). The intensity of staining was graded as follows: 0 (no color reaction), 1 (mild reaction), 2 (moderate reaction), 3 (intense reaction). The scores of the first two parts are added together, and the final positive result is divided into 0−3 levels: 0 (0−1), 1 (2−3), 2 (4−5), 3 (6−7). ANKRD6 expression was defined as low (0, 1, 2) or high (3).

M2 macrophages: The whole section was observed under lowpower fields (magnification, ×100); hot spots with a large number of CD68+/CD163+ tumor-associated macrophages (TAMs) were selected, and the positive cells were counted in 5 fields of view in the hot spots under highpower fields (magnification, ×400). The average value was taken as the count value of CD68+/CD163+ TAMs.

Statistical analysis

The results of survival curves are displayed with HR (95% CI) and P or Cox P-values from a log-rank test. The results generated in Oncomine are displayed with P-values, fold changes and ranks. The gene expression correlation was evaluated by Spearman's correlation or Pearson's correlation and statistical significance, and the strength of the correlation was used the following guide: 0−0.19 "very weak", 0.20−0.39 "weak", 0.40−0.59 "moderate", 0.60−0.79 "strong", and 0.80−1.00 "very strong". Two-sided P<0.05 was considered statistically significant.

Results

mRNA expression of ANKRD6 in different types of human cancers

As shown in *Figure 1A*, the expression of ANKRD6 mRNA in different tumors and normal tissues was analyzed by Oncomine (gene expression microarray data). ANKRD6 expression is higher in brain and central nervous system (CNS) cancer, breast cancer, colorectal cancer, kidney cancer, lymphoma, myeloma, pancreatic cancer, sarcoma and other cancer tissues than in normal tissues. In some data sets, ANKRD6 expression is low in bladder cancer, brain and CNS cancer, breast cancer, colorectal cancer, head and neck squamous cell carcinoma, kidney cancer, leukemia, liver cancer, lung cancer, lymphoma, melanoma, prostate cancer, sarcoma and other cancers. The results of ANKRD6 expression are summarized in *Supplementary Table S3*.

As shown in *Figure 1B*, the differential expression of ANKRD6 mRNA between tumor and normal tissues was analyzed by TIMER (TCGA gene expression RNA-seq data). Compared with normal tissues, bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), colon adenocarcinoma (COAD), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal papillary cell carcinoma (KIRP), lung adenocarcinoma (LUAD), prostate adenocarcinoma (PRAD), lung squamous cell carcinoma (LUSC), rectum adenocarcinoma (READ), stomach adenocarcinoma (STAD), and uterine corpus endometrial carcinoma (UCEC) tissues had significantly lower ANKRD6 expression. However, the expression of ANKRD6 was significantly higher in cholangiocarcinoma (CHOL) and thyroid carcinoma (THCA) tissues than in normal tissues. We also used GEPIA to compare tumor and normal tissues in colon cancer. The expression of ANKRD6 was significantly lower in colon adenocarcinoma (COAD) than in normal tissues (P<0.05) (*Supplementary Figure S1*).

Prognostic potential of ANKRD6 in cancers

In Affymetrix microarrays (HG-U133A/HG-U133_Plus_2), ANKRD6 was present in 204671_s_at and 204672_s_at. Based on the Affymetrix microarray, we used PrognoScan technology to study the relationship between ANKRD6 expression and the prognosis of different cancers. The results are shown in *Supplementary Table S4* (Cox P<0.05). ANKRD6 expression significantly affects the prognosis of colorectal cancer (*Figure 2*). GSE17536 and GSE17537 showed that high ANKRD6 expression was associated with a poor prognosis. GSE14333 204672_s_at showed that high ANKRD6 expression was associated with poor DFS (HR=2.03, 95% CI, 1.33−3.09; Cox P<0.001). In other cancers, such as brain, breast and lung cancers, low ANKRD6 expression is associated with a poor prognosis, and high ANKRD6 expression is associated with a poor prognosis in skin cancer (*Supplementary Figure S2*).

TCGA-RNA sequencing data were also used to analyze the prognostic potential of ANKRD6 in different cancers by GEPIA (*Figure 3*, *Supplementary Figure S3*). In colorectal cancer, high levels of ANKRD6 expression are associated with poor OS (P=0.001) and DFS (P=0.001) (*Figure 3A*). In colon cancer, high levels of ANKRD6

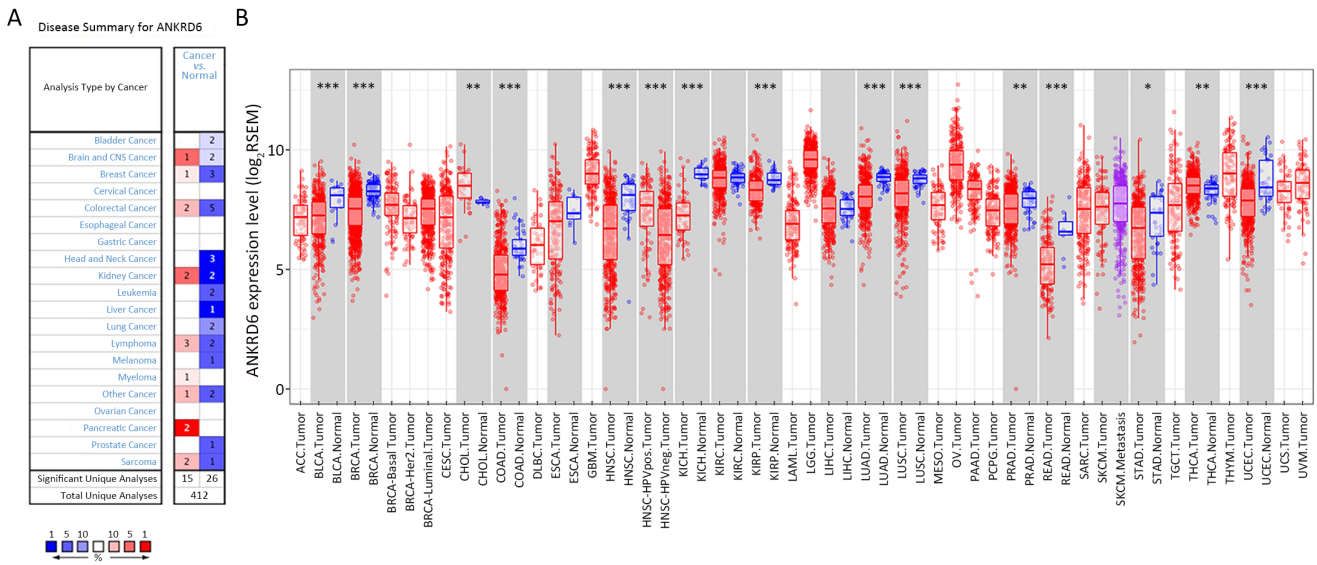

Figure 1 ANKRD6 expression levels in different types of human cancers. (A) Increased or decreased ANKRD6 in data sets of different cancer tissues compared with normal tissues in Oncomine; (B) ANKRD6 expression in different tumor types from TCGA database was determined by TIMER. *, P<0.05; **, P<0.01; ***, P<0.001. ANKRD6, ankyrin repeat domain-containing protein 6; TCGA, The Cancer Genome Atlas; CNS, central nervous system; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; HER2, human epidermal growth factor receptor 2; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; HPV, human papillomavirus; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian cancer; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

expression are associated with poor OS (P=0.002) and DFS (P=0.002) (*Figure 3B*). However, high levels of ANKRD6 expression are not associated with poor OS (P=0.400) and DFS (P=0.480) in rectal cancer (*Figure 3C*). We also analyzed the data of TCGA colon cancer by multivariate analysis. The results showed that ANKRD6 expression was an independent prognostic factor (P=0.008) (*Supplementary Table S5*). High ANKRD6 expression leads to a poor prognosis in patients with colon cancer.

ANKRD6 expression is correlated with immune infiltration levels in cancers

Using TIMER, we investigated the correlations between ANKRD6 expression and immune infiltration levels. The ANKRD6 expression level had significant positive correlations with CD8+ T cells (r=0.119, P=1.66E−02), CD4+ T cells (r=0.344, P=1.40E−12), macrophages (r=0.512, P=2.26E−28), neutrophils (r=0.241, P=1.04E−06) and DCs (r=0.305, P=4.50E−10) (*Figure 4A*). ANKRD6 may play a special role in immune invasion in colon cancer, especially in the infiltration of CD4+ T cells, macrophages and DCs.

In other 6 cancers, ANKRD6 expression has significantly negative correlations with tumor purity. ANKRD6 expression has significant positive correlations with B cell infiltration levels in 9 cancers, CD8+ T cells in 11 cancers, CD4+ T cells in 16 cancers, macrophages in 16 cancers, neutrophils in 12 cancers and DCs in 8 cancers (*Supplementary Table S6*).

Correlation analysis between different ANKRD6 expression levels and infiltrating immune cells in colon cancer

Using the CIBERSORT algorithm, we evaluated the

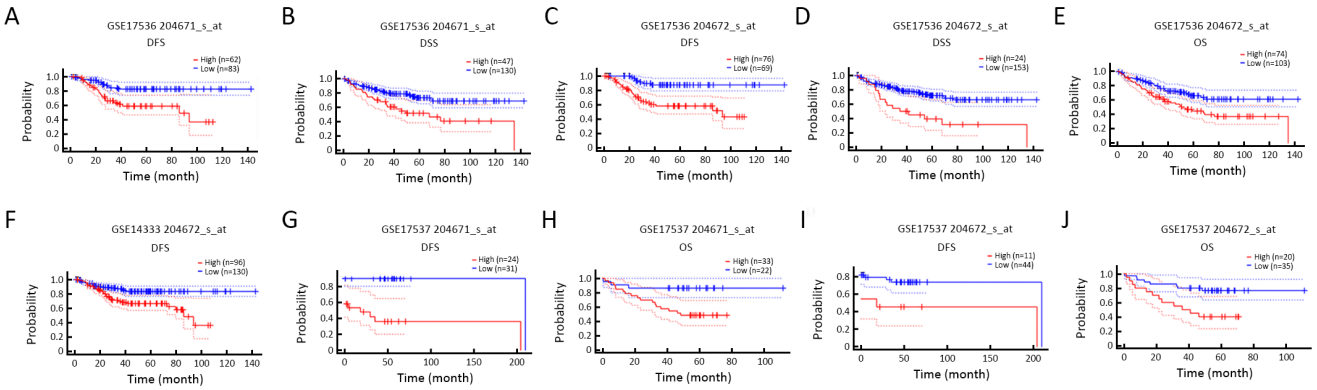

Figure 2 Kaplan-Meier survival curves comparing high and low expression of ANKRD6 in different types of colorectal cancer in PrognoScan. (A–E) Colorectal cancer GSE17536 showed that high ANKRD6 expression was associated with poor prognosis (204671_s_at DFS: HR=2.14, 95% CI=1.31–3.50, P=0.003; DSS HR=1.66, 95% CI=1.09–2.54, P=0.018; 204672_s_at DFS: HR=2.64, 95% CI=1.60–4.36, P<0.001; DSS: HR=1.89, 95% CI=1.25–2.85, P=0.002; OS: HR=1.58, 95% CI=1.07–2.33, P=0.021); (F) GSE14333 204672_s_at showed that high ANKRD6 expression was associated with poor DFS (HR=2.03, 95% CI=1.33–3.09, P<0.001); (G–J) Colorectal cancer GSE17537 showed that high ANKRD6 expression was associated with poor prognosis (204671_s_at DFS: HR=2.59, 95% CI=1.40–4.78, P=0.002; OS: HR=2.18, 95% CI=1.07–4.43, P=0.031; 204672_s_at DFS: HR=2.60, 95% CI=1.19–5.68, P=0.017; OS: HR=2.89, 95% CI=1.27–6.57, P=0.011). ANKRD6, ankyrin repeat domain-containing protein 6; DFS, disease-free survival; HR, hazard ratio; 95% CI, 95% confidence interval; DSS, disease-specific survival; OS, overall survival.

abundance of TILs in the colon microenvironment using the TCGA gene expression data. We found that the number of plasma cells (P=0.010), CD8+ T cells (P=0.011), CD4+ memory resting T cells (P=0.001), follicular helper T cells (P=0.001) and activated NK cells (P=0.047) were significantly lower in the ANKRD6-high group than in the ANKRD6-low group. The number of M0 macrophages (P<0.001) and M2 macrophages (P=0.020) was significantly higher in the ANKRD6-high group than in the ANKRD6-low group (*Figure 4B*). We used IHC to analysis the correlation between different ANKRD6 expression levels and OS and M2 macrophages in colon cancer. To probe the correlations among CD68, CD163 and ANKRD6, we use serial paraffin sections and the represent images of CD68, CD163 and ANKRD6 showed are serial paraffin sections from the same case (*Figure 5A*). Combining the results of TIMER and CIBERSORT, we speculated that ANKRD6 is related to M2 macrophage infiltration, which was verified by IHC. M2 macrophages were significantly higher in ANKRD6-high group than in ANKRD6-low group (P=0.014, *Figure 5B*). In colon cancer, high levels of ANKRD6 IHC expression are associated with poor OS (*Figure 5C*).

Discussion

ANKRD6 is a cytoplasmic protein with eight N-terminal ankyrin repeat domains, a conserved central domain and C-terminal domain. Noncanonical WNT signaling through Frizzled or ROR receptors activates DVL-dependent Rho-ROCK and Rac-JNK cascades (WNT/PCP signaling). The function of ANKRD6 as a PCP activator is mediated by its ankyrin repeat domain. ANKRD6 binds to CKIε through its central domain and binds to Conductin or Axin through its C-terminal domain. This leads to the recruitment of CKIε to the β-catenin degradation complex. Then, β-catenin is phosphorylated and degraded, and β-catenin/TCF signaling is subsequently inhibited (14,26).

The WNT signaling pathway is involved in embryonic development and normal adult homeostasis. Mutated or deregulated expression of the WNT signaling pathway components can induce disease, including tumors (27). Loss of E-cadherin is a major contributor to the epithelial-mesenchymal transition (EMT) process and is largely influenced by WNT/β-catenin signaling. An E-cadherin/β-catenin complex maintains epithelial integrity, and disturbances to this complex and the WNT/β-catenin pathway will ultimately lead to the nuclear translocation of β-catenin and transcription of EMT-promoting genes (28). Cancer cells undergoing EMT have been shown to promote immune suppression through several aspects, including the following: 1) PD-L1/PD-L2 expression; 2) increased autophagy; 3) reduced sensitivity to the cell death receptor pathway; 4) immunoproteasome deficiency and

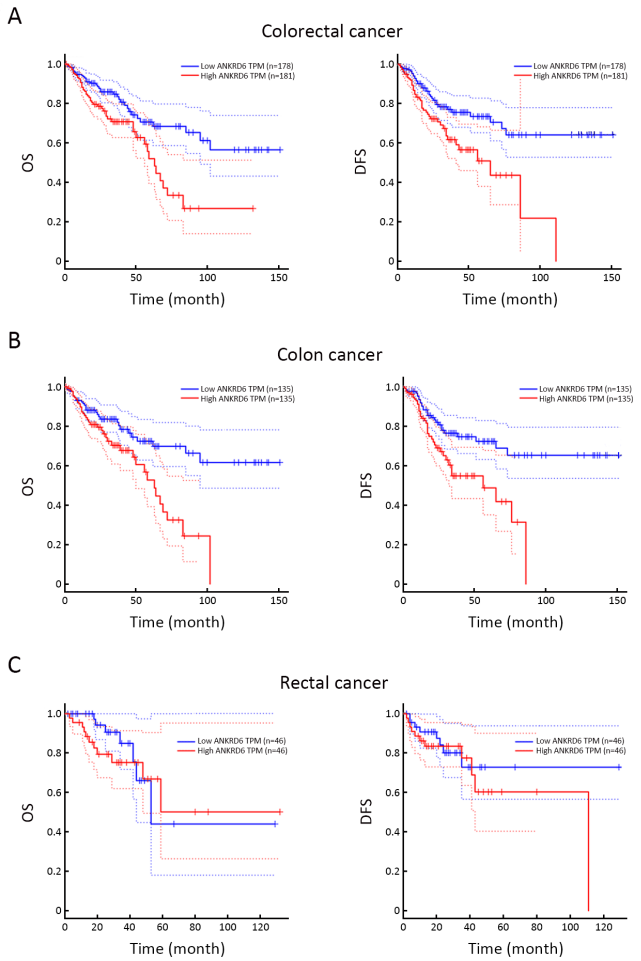

Figure 3 Kaplan-Meier survival curves comparing high and low expression of ANKRD6 in GEPIA databases. (A) Colorectal, high levels of ANKRD6 expression are associated with poor OS (P=0.001, HR=2.0) and DFS (P=0.001, HR=2.1); (B) Colon cancer, high levels of ANKRD6 expression are associated with poor OS (P=0.002, HR=2.1) and DFS (P=0.002, HR=2.1); (C) Rectal cancer, high levels of ANKRD6 expression are not associated with poor OS (P=0.400, HR=1.5) and DFS (P=0.480, HR=1.4).

pMHC reduction; 5) reduced E-cadherin expression; 6) immunological synapse dysfunction; and 7) other known or still unknown mechanisms (29). Here, we report that ANKRD6 expression correlates with the prognosis of different cancers. The high expression of ANKRD6 correlates with a poor prognosis in colon cancer. This may be because ANKRD6 can promote the EMT of tumors, which may lead to the invasion and metastasis of tumors. On the other hand, EMT may lead to the suppression of tumor immunity, which may lead to poor patient prognoses.

There are many methods to study the immune microenvironment of tumors. Sequencing and bioinformatics analyses are the current strategies. Through transcriptome sequencing, we can obtain the expression data of different genes in tumor samples. Through corresponding bioinformatics software, we can obtain the expression of various cells to classify tumor microenvironment (TME), identify infiltrating immune cell subsets, compare expression of different subsets, and further screen microenvironment by combining survival analysis subtypes or immune cell subsets as biomarkers. At present, there are three basic algorithms for immune analysis: 1) Marker genes (*TIminer*, *xCell* and *MCP-counter*); 2) Deconvolution algorithms (CIBERSORT, TIMER, EPIC and quanTIseq); and 3) Nonnegative matrix factorization (NMF, deconf, ssKL and ssFrobenius). TIMER can quickly determine the immune invasion of various tumors, but to ultimately focus on colon cancer to distinguish the types of immune infiltration in more detail, we used the same algorithm in CIBERSORT.

In recent years, breakthroughs have been made in tumor immunotherapy, and research on tumor immunotherapy has attracted much attention. The immune system recognizes and clears abnormal cells, but tumor cells can avoid recognition and cell death by the immune system through various immune escape mechanisms, leading to the occurrence and development of tumors. In recent years, PD-1-, PD-L1- and CTLA-4-targeted antibodies have significantly improved the response rate of tumors such as melanoma, lung cancer and colorectal cancer. TME consists of tumor cells, fibroblasts, stromal cells, blood vessels, immune cells and the extracellular matrix. Immune cell infiltration plays a variety of roles in promoting or delaying tumor progression (30). Which immune cells are involved in the expression of ANKRD6 in the TME? Using TIMER, we easily determined the relationship between ANKRD6 expression and infiltrating immune cells from a pan-cancer analysis.

ANKRD6 plays an important role in the recruitment and regulation of colon cancer infiltrating immune cells. Recent studies have provided possible mechanisms to explain why ANKRD6 expression is associated with immune infiltration and a poor prognosis. Many studies have reported that immune infiltration may affect patient survival. ANKRD6 may be related to M2 macrophage. Therefore, the high expression of ANKRD6 promotes M2 macrophages infiltration, leading to a poor prognosis. It is worth noting

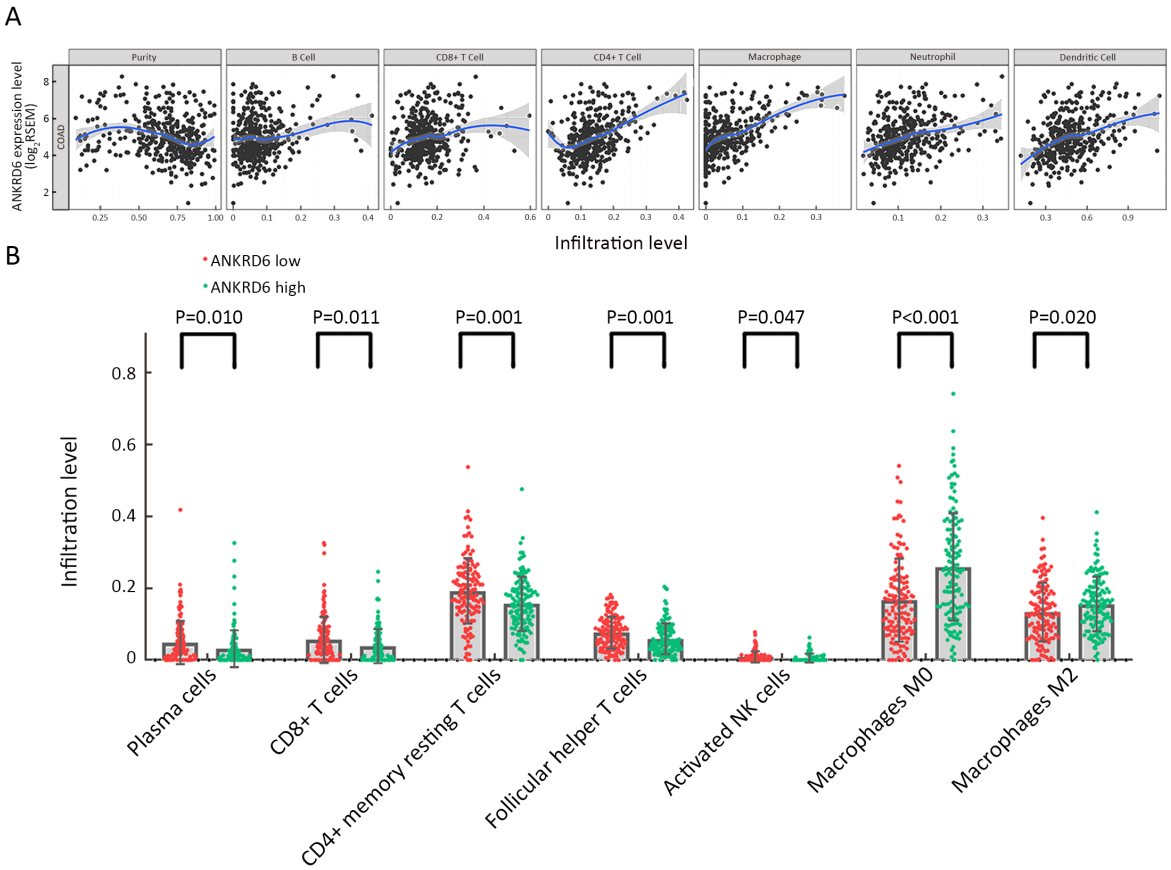

Figure 4 ANKRD6 expression and immune infiltration in colon cancer. (A) Correlation between ANKRD6 expression and immune infiltration level in colon cancer. ANKRD6 expression is significantly negatively related to tumor purity (r=−0.241, P=9.11E−07) and has significant positive correlations with infiltrating levels of CD8+ T cells (r=0.119, P=1.66E−02), CD4+ T cells (r=0.344, P=1.40E−12), macrophages (r=0.512, P=2.26E−28), neutrophils (r=0.241, P=1.04E−06) and DCs (r=0.305, P=4.50E−10) in colon cancer, other than B cells (r=0.027, P=5.94E−01) (n=457); (B) Relative abundance of tumor infiltrating immune cells in ANKRD6-low expression group *vs.* ANKRD6-high expression group. Plasma cells (P=0.010), CD8+ T cells (P=0.011), CD4+ memory resting T cells (P=0.001), follicular helper T cells (P=0.001) and activated NK cells (P=0.047) are significantly lower in ANKRD6-high group than in ANKRD6-low group. Macrophages M0 (P<0.001) and Macrophages M2 (P=0.020) are significantly higher in ANKRD6-high group compared to ANKRD6-low group. ANKRD6, ankyrin repeat domain-containing protein 6; DC, dendritic cell.

that macrophages in TME exhibit an M2-like phenotype and help tumor growth and development through cancer cell proliferation, angiogenesis and metastasis (31).

Conclusions

In colon cancer patients, the infiltration of M2 macrophages can lead to immunosuppression, which can result in tumor recurrence and metastasis, affecting patient survival. High ANKRD6 expression is associated with poor prognosis of colon cancer, and ANKRD6 expression is positively correlated with M2 macrophage infiltration in colon cancer. Therefore, ANKRD6 will be a very simple and widely used marker for colon cancer prediction and individualized immunotherapy.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

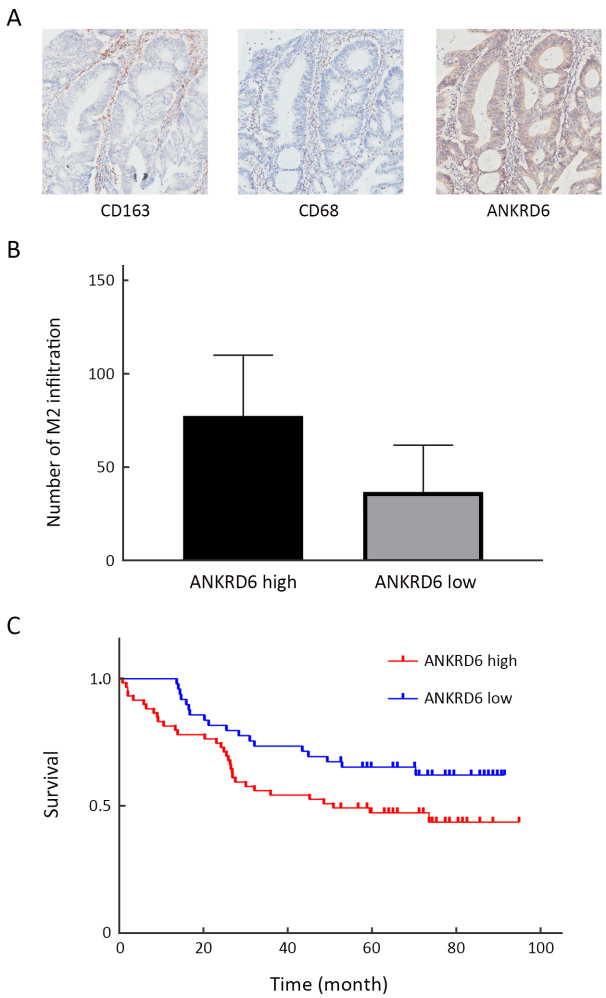

Figure 5 M2 infiltrating macrophages immune cells in ANKRD-low group and ANKRD6-high group. (A) Represent images of CD163, CD68 and ANKRD6 showed are serial paraffin sections from the same case (×100); (B) M2 macrophages are significantly higher in ANKRD6-high group compared to ANKRD6-low group (P=0.014); (C) High levels of ANKRD6 IHC expression are associated with poor OS. ANKRD6, ankyrin repeat domain-containing protein 6; IHC, immunohistochemistry; OS, overall survival.

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Table S1 Patient and tumor characteristics for ANKRD6 IHC survival analysis

Characteristics	n (%)			P
	No. of cases	ANKRD6-high group	ANKRD6-low group	
Sex				0.622
Female	49 (45.37)	25 (42.37)	24 (48.98)	
Male	59 (54.63)	34 (57.63)	25 (51.02)	
Age (year)				1.000
<65	54 (50.00)	30 (50.85)	24 (48.98)	
≥65	54 (50.00)	29 (49.15)	25 (51.02)	
Stage				0.310
II	53 (49.07)	27 (45.76)	26 (53.06)	
III	35 (32.41)	18 (30.51)	17 (34.69)	
IV	20 (18.52)	14 (23.73)	6 (12.24)	
Position				0.198
Left	15 (13.89)	11 (18.64)	4 (8.16)	
Right	93 (86.11)	48 (81.36)	45 (91.84)	

IHC, immunohistochemistry; ANKRD6, ankyrin repeat domain-containing protein 6.

Table S2 Patient information for ANKRD6 and M2 macrophages IHC

Patients	M2 macrophages	ANKRD6	Sex	Age (year)	Stage	Position
P1	43.2	1	Female	69	II	Descending colon
P2	63.0	2	Male	62	II	Transverse colon
P3	50.6	2	Female	75	II	Cecum
P4	15.8	2	Female	75	II	Ascending colon
P5	58.0	2	Female	78	II	Descending colon
P6	15.0	2	Male	70	III	Ascending colon
P7	70.0	2	Female	46	II	Sigmoid colon
P8	6.6	2	Male	48	II	Ascending colon
P9	9.6	0	Male	67	II	Ascending colon
P10	57.8	3	Female	44	III	Descending colon
P11	150.0	3	Male	74	III	Descending colon
P12	88.6	3	Male	34	III	Ascending colon
P13	67.0	3	Male	43	II	Splenic flexure
P14	80.0	3	Male	59	II	Ascending colon
P15	76.8	3	Male	59	I	Splenic flexure
P16	56.0	3	Female	71	II	Transverse colon
P17	43.8	3	Male	48	II	Sigmoid colon

IHC, immunohistochemistry; ANKRD6, ankyrin repeat domain-containing protein 6.

Table S3 ANKRD6 expression in cancers *vs.* normal tissues in Oncomine database

Cancer type	P	Fold change	Rank (%)	Sample
Bladder cancer				
Superficial bladder cancer	4.52E−06	−2.125	12	157
Infiltrating bladder urothelial carcinoma	7.35E−06	−1.565	14	157
Brain cancer				
Anaplastic oligodendroglioma	7.32E−07	3.337	3	33
Brain glioblastoma	2.60E−04	−1.658	/	557
Glioblastoma	1.97E−05	−1.529	/	180
Breast cancer				
Invasive breast carcinoma	5.61E−08	3.838	/	59
Invasive ductal breast carcinoma	2.58E−31	−2.019	4	593
Invasive breast carcinoma	3.82E−10	−1.630	12	593
Invasive lobular breast carcinoma	3.49E−07	−1.527	13	593
Colorectal cancer				
Colon carcinoma Epithelia	5.45E−07	2.520	6	40
Colon carcinoma	9.77E−06	1.951	13	40
Colon adenoma	5.86E−06	−1.768	/	40
Cecum adenocarcinoma	1.39E−06	−2.251	/	237
Colon adenocarcinoma	7.55E−08	−2.153	/	237
Colon mucinous adenocarcinoma	5.77E−05	−1.959	/	237
Rectal adenocarcinoma	1.25E−06	−2.070	/	237
Head-neck cancer				
Oral cavity squamous cell carcinoma	5.49E−25	−2.642	1	79
Head and neck squamous cell carcinoma	1.82E−13	−2.560	/	54
Tongue squamous cell carcinoma	2.80E−06	−2.603	4	58
Kidney cancer				
Renal wilms tumor	4.16E−04	1.745	/	35
Hereditary clear cell renal cell carcinoma	6.32E−04	1.569	22	70
Chromophobe renal cell carcinoma	5.42E−05	−2.457	1	67
Clear cell sarcoma of the kidney	3.65E−05	−1.981	/	35
Leukemia				
Chronic adult T-cell leukemia/lymphoma	6.38E−05	−3.020	2	47
Acute adult T-cell leukemia/lymphoma	9.52E−05	−2.543	2	47
Liver cancer				
Hepatocellular adenoma	1.01E−05	−2.213	/	64
Lung cancer				
Lung adenocarcinoma	8.63E−10	−1.717	/	156
Squamous cell lung carcinoma	3.50E−04	−1.586	/	156
Lymphoma				
Follicular lymphoma	1.34E−11	1.818	/	136
Unspecified peripheral T-cell lymphoma	1.66E−06	1.514	/	60
Nodular lymphocyte predominant hodgkin's lymphoma	2.42E−04	1.560	6	67

Table S3 (*continued*)

Table S3 (*continued*)

Cancer type	P	Fold change	Rank (%)	Sample
Chronic adult T-cell leukemia/lymphoma	6.38E−05	−3.020	2	47
Acute adult T-cell leukemia/lymphoma	9.52E−05	−2.543	2	47
Melanoma				
Cutaneous melanoma	4.96E−07	−4.460	/	70
Myeloma				
Monoclonal gammopathy of undetermined significance	6.57E−05	1.530	12	78
Other cancers				
Seminoma	3.37E−05	4.354	/	107
Mesothelima	4.21E−04	−1.512	/	54
Vulva	9.43E−04	−2.865	/	19
Pancreas cancer				
Pancreatic carcinoma	2.16E−05	1.745	1	17
Pancreatic ductal adenocarcinoma	2.92E−11	2.279	/	78
Prostate cancer				
Prostate carcinoma	5.10E−04	−2.519	/	35
Sarcoma				
Myxoid/round cell liposarcoma	2.73E−07	1.790	9	158
Leiomyosarcoma	1.73E−06	1.516	10	158
Clear cell sarcoma of the kidney	3.65E−05	−1.981	/	35

ANKRD6, ankyrin repeat domain-containing protein 6; TCGA, The Cancer Genome Atlas.

Table S4 Relationship between ANKRD6 expression and patient prognosis Cox P<0.05 in PrognoScan database

Cancers	Dataset	Probe ID	Endpoint	N	HR (95% CI)	Cox P
Brain cancer	GSE4412-GPL96	204671_s_at	OS	74	0.48 (0.25–0.94)	0.032
Breast cancer	GSE1456-GPL96	204671_s_at	OS	159	0.48 (0.27–0.85)	0.012
	GSE3494-GPL96	204672_s_at	DSS	236	0.46 (0.24–0.88)	0.020
	GSE4922-GPL96	204671_s_at	DFS	249	0.62 (0.42–0.90)	0.012
	GSE4922-GPL96	204672_s_at	DFS	249	0.56 (0.33–0.95)	0.033
Colorectal cancer	GSE17536	204672_s_at	DFS	145	2.64 (1.60–4.36)	<0.001
	GSE17536	204671_s_at	DFS	145	2.14 (1.31–3.50)	0.002
	GSE17536	204672_s_at	DSS	177	1.89 (1.25–2.85)	0.002
	GSE17536	204671_s_at	DSS	177	1.66 (1.09–2.54)	0.018
	GSE17536	204672_s_at	OS	177	1.58 (1.07–2.33)	0.021
	GSE14333	204672_s_at	DFS	226	2.03 (1.33–3.09)	0.001
	GSE17537	204671_s_at	DFS	55	2.59 (1.40–4.78)	0.002
	GSE17537	204672_s_at	OS	55	2.89 (1.27–6.57)	0.011
	GSE17537	204671_s_at	OS	55	2.18 (1.07–4.43)	0.031
	GSE17537	204672_s_at	DFS	55	2.60 (1.19–5.68)	0.017
Lung cancer	GSE31210	204672_s_at	RFS	204	0.30 (0.15–0.61)	0.001
	GSE31210	204671_s_at	RFS	204	0.40 (0.22–0.72)	0.002
	GSE4573	204672_s_at	OS	129	0.59 (0.36–0.96)	0.032
Skin cancer	GSE19234	204671_s_at	OS	38	2.51 (1.17–5.36)	0.018

ANKRD6, ankyrin repeat domain-containing protein 6; OS, overall survival; DSS, disease-specific survival; DFS, disease-free survival; RFS, relapse-free survival; HR, hazard ratio; 95% CI, 95% confidence interval.

Table S5 Data of TCGA colon cancer by multivariate analysis

Characteristics	HR	95% CI	P
Sex			
Female	1		
Male	1.75	0.89–3.41	0.103
Age (year)			
<65	1		
≥65	1.96	0.91–4.23	0.085
Stage			
I	1		
II	1.11	0.32–3.90	0.868
III	1.57	0.44–5.60	0.487
IV	4.68	1.24–17.58	0.022
Position			
Left	1		
Right	0.55	0.29–1.07	0.078
ANKRD6			
High	1		
Low	0.41	0.22–0.79	0.008
Race			
Asian	1		
Black	0.73	0.06–8.71	0.807
White	0.93	0.12–7.28	0.945
Others	0.88	0.10–8.00	0.907

TCGA, The Cancer Genome Atlas; ANKRD6, ankyrin repeat domain-containing protein 6; HR, hazard ratio; 95% CI, 95% confidence interval.

Table S6 Correlation of ANKRD6 expression with immune infiltration in other cancers (without COAD)

Cancers	Purity		B cell		CD8+ T cell		CD4+ T cell		Macrophage		Neutrophil		DC	
	Cor	P	Cor	P	Cor	P	Cor	P	Cor	P	Cor	P	Cor	P
ACC	−0.081	<0.01	0.035	<0.01	0.301	<0.01	0.199	<0.01	0.132	<0.01	0.116	<0.01	0.018	<0.01
BLCA	−0.015	<0.01	0.191	<0.01	−0.013	<0.01	0.058	<0.01	0.173	<0.01	0.047	<0.01	−0.060	<0.01
BRCA	−0.259	<0.01	0.003	<0.01	0.228	<0.01	0.223	<0.01	0.318	<0.01	0.220	<0.01	0.197	<0.01
BRCA-Basal	0.207	<0.01	0.048	<0.01	0.165	<0.01	0.205	<0.01	0.200	<0.01	0.080	<0.01	0.133	<0.01
BRCA-HER2	−0.123	<0.01	−0.027	<0.01	−0.051	<0.01	0.089	<0.01	0.412	<0.01	0.073	<0.01	0.131	<0.01
BRCA-Luminal	−0.358	<0.01	0.092	<0.01	0.272	<0.01	0.277	<0.01	0.386	<0.01	0.293	<0.01	0.256	<0.01
CESC	0.008	<0.01	0.104	<0.01	0.020	<0.01	0.129	<0.01	0.050	<0.01	−0.071	<0.01	−0.096	<0.01
CHOL	0.019	<0.01	0.177	<0.01	0.209	<0.01	0.269	<0.01	0.164	<0.01	0.244	<0.01	0.135	<0.01
DLBC	−0.315	<0.01	−0.150	<0.01	−0.356	<0.01	−0.100	<0.01	0.466	<0.01	−0.269	<0.01	−0.420	<0.01
ESCA	−0.112	<0.01	0.069	<0.01	0.067	<0.01	−0.051	<0.01	0.244	<0.01	0.077	<0.01	0.085	<0.01
GBM	0.198	<0.01	0.099	<0.01	0.062	<0.01	0.168	<0.01	0.087	<0.01	0.314	<0.01	−0.100	<0.01
HNSC	0.092	<0.01	0.371	<0.01	0.240	<0.01	0.239	<0.01	0.192	<0.01	0.023	<0.01	0.181	<0.01
HNSC-HPVpos	0.134	<0.01	0.385	<0.01	0.406	<0.01	0.253	<0.01	0.051	<0.01	0.114	<0.01	0.273	<0.01
HNSC-HPVneg	0.027	<0.01	0.316	<0.01	0.147	<0.01	0.207	<0.01	0.189	<0.01	−0.035	<0.01	0.123	<0.01
KICH	−0.210	<0.01	0.583	<0.01	0.303	<0.01	0.057	<0.01	0.214	<0.01	0.148	<0.01	0.393	<0.01
KIRC	−0.106	<0.01	0.185	<0.01	0.126	<0.01	0.233	<0.01	0.236	<0.01	0.293	<0.01	0.165	<0.01
KIRP	−0.056	<0.01	0.243	<0.01	0.311	<0.01	−0.049	<0.01	0.165	<0.01	0.054	<0.01	0.176	<0.01
LGG	0.150	<0.01	−0.019	<0.01	−0.074	<0.01	−0.062	<0.01	−0.047	<0.01	−0.087	<0.01	−0.091	<0.01
LIHC	−0.118	<0.01	0.151	<0.01	0.208	<0.01	0.294	<0.01	0.340	<0.01	0.298	<0.01	0.261	<0.01
LUAD	0.014	<0.01	0.264	<0.01	0.101	<0.01	0.216	<0.01	0.180	<0.01	0.111	<0.01	0.211	<0.01
LUSC	0.042	<0.01	0.170	<0.01	0.035	<0.01	0.163	<0.01	0.133	<0.01	0.141	<0.01	0.054	<0.01
MESO	−0.091	<0.01	0.123	<0.01	−0.025	<0.01	−0.025	<0.01	0.284	<0.01	−0.136	<0.01	−0.119	<0.01
OV	0.064	<0.01	0.229	<0.01	0.096	<0.01	0.004	<0.01	−0.06	<0.01	0.079	<0.01	0.099	<0.01
PAAD	−0.314	<0.01	0.196	<0.01	0.337	<0.01	0.237	<0.01	0.470	<0.01	0.434	<0.01	0.315	<0.01
PCPG	−0.104	<0.01	−0.032	<0.01	0.069	<0.01	0.184	<0.01	0.081	<0.01	0.104	<0.01	0.036	<0.01
PRAD	−0.108	<0.01	0.181	<0.01	0.096	<0.01	0.069	<0.01	0.151	<0.01	0.231	<0.01	0.077	<0.01
READ	−0.259	<0.01	0.143	<0.01	−0.036	<0.01	0.323	<0.01	0.273	<0.01	0.092	<0.01	0.279	<0.01
SARC	0.314	<0.01	−0.009	<0.01	−0.012	<0.01	−0.176	<0.01	−0.279	<0.01	−0.162	<0.01	−0.255	<0.01
SKCM	−0.013	<0.01	−0.005	<0.01	0.181	<0.01	0.049	<0.01	0.110	<0.01	0.231	<0.01	0.084	<0.01
SKCM-primary	0.108	<0.01	−0.123	<0.01	0.226	<0.01	−0.008	<0.01	0.163	<0.01	0.355	<0.01	0.168	<0.01
SKCM-metastasis	0.050	<0.01	−0.018	<0.01	0.145	<0.01	0.034	<0.01	0.076	<0.01	0.168	<0.01	0.028	<0.01
STAD	−0.097	<0.01	0.190	<0.01	0.066	<0.01	0.389	<0.01	0.454	<0.01	0.024	<0.01	0.219	<0.01
TGCT	0.263	<0.01	−0.126	<0.01	−0.143	<0.01	0.295	<0.01	0.240	<0.01	0.143	<0.01	0.162	<0.01
THCA	−0.062	<0.01	0.347	<0.01	−0.343	<0.01	0.249	<0.01	0.307	<0.01	0.083	<0.01	0.089	<0.01
THYM	0.113	<0.01	−0.387	<0.01	−0.199	<0.01	−0.497	<0.01	−0.066	<0.01	0.204	<0.01	−0.494	<0.01
UCEC	−0.012	<0.01	0.031	<0.01	−0.073	<0.01	0.076	<0.01	0.268	<0.01	−0.004	<0.01	−0.049	<0.01
UCS	0.241	<0.01	0.032	<0.01	−0.124	<0.01	0.204	<0.01	0.160	<0.01	0.074	<0.01	0.113	<0.01
UYM	−0.146	<0.01	0.564	<0.01	−0.018	<0.01	−0.075	<0.01	−0.150	<0.01	0.243	<0.01	−0.218	<0.01

ANKRD6, ankyrin repeat domain-containing protein 6; COAD, colon adenocarcinoma; DC, dendritic cell; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; HER2, Human epidermal growth factor receptor 2; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; HPV, human papillomavirus; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian cancer; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

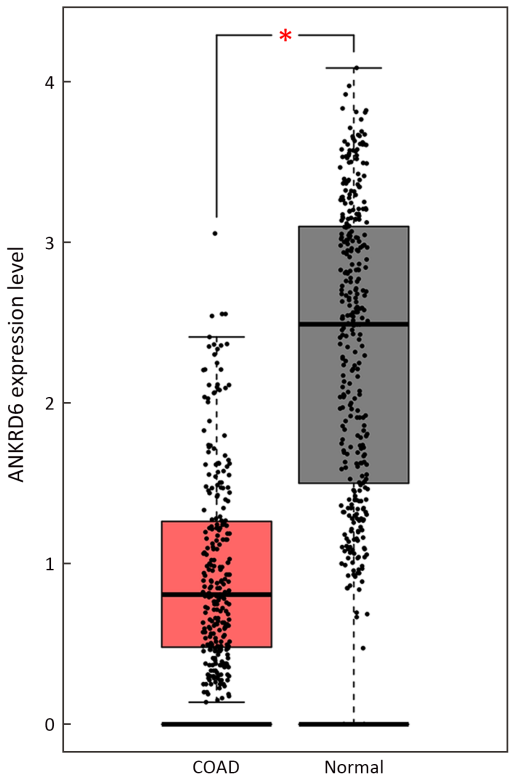

Figure S1 Expression of ANKRD6 was significantly lower in COAD than in normal tissues (P<0.05). ANKRD6, ankyrin repeat domain-containing protein 6; COAD, colon adenocarcinoma.

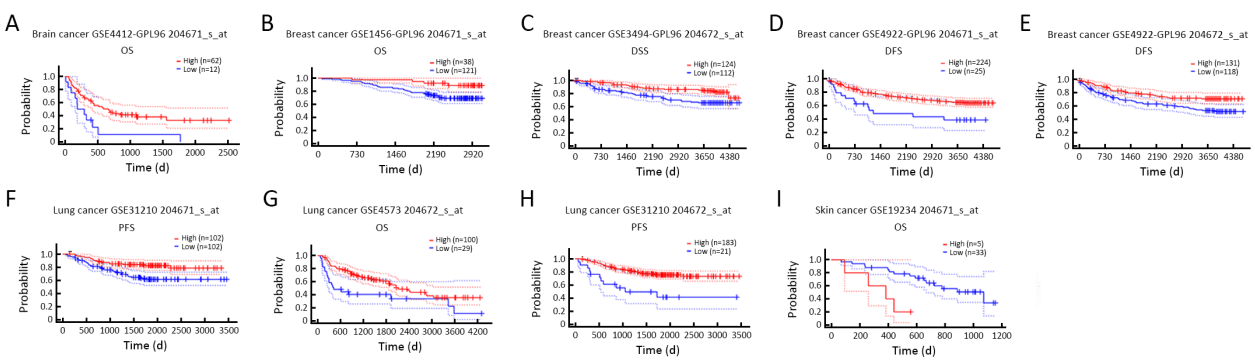

Figure S2 Kaplan-Meier survival curves comparing high and low expression of ANKRD6 in brain, breast, lung and skin cancer in PrognoScan. (A) Brain cancer GSE4412-GPL96 showed that low ANKRD6 expression was associated with poor prognosis (204671_s_at OS: HR=0.48, 95% CI=0.25−0.94, P=0.032); (B−E) Breast cancer GSE1456-GPL96 showed that low ANKRD6 expression was associated with poor prognosis (204671_s_at OS: HR=0.48, 95% CI=0.27−0.85, P=0.012; 204672_s_at DSS: HR=0.46, 95% CI=0.24−0.88, P=0.020; 204671_s_at DFS: HR=0.62, 95% CI=0.42−0.90, P=0.012; 204672_s_at DFS: HR=0.56, 95% CI=0.33−0.95, P=0.033); (F−H) Lung cancer GSE31210 and GSE4573 showed that low ANKRD6 expression was associated with poor prognosis (GSE31210 204671_s_at PFS: HR=0.40, 95% CI=0.22−0.72 P=0.002; GSE4573 204672_s_at OS: HR=0.59, 95% CI=0.36−0.96 P=0.032; GSE31210 204672_s_at PFS: HR=0.40, 95% CI=0.22−0.72, P<0.001); (I) Skin cancer GSE19234 high ANKRD6 expression was associated with poor prognosis. (204671_s_at OS: HR=2.51, 95% CI=1.17−5.36, P=0.018).

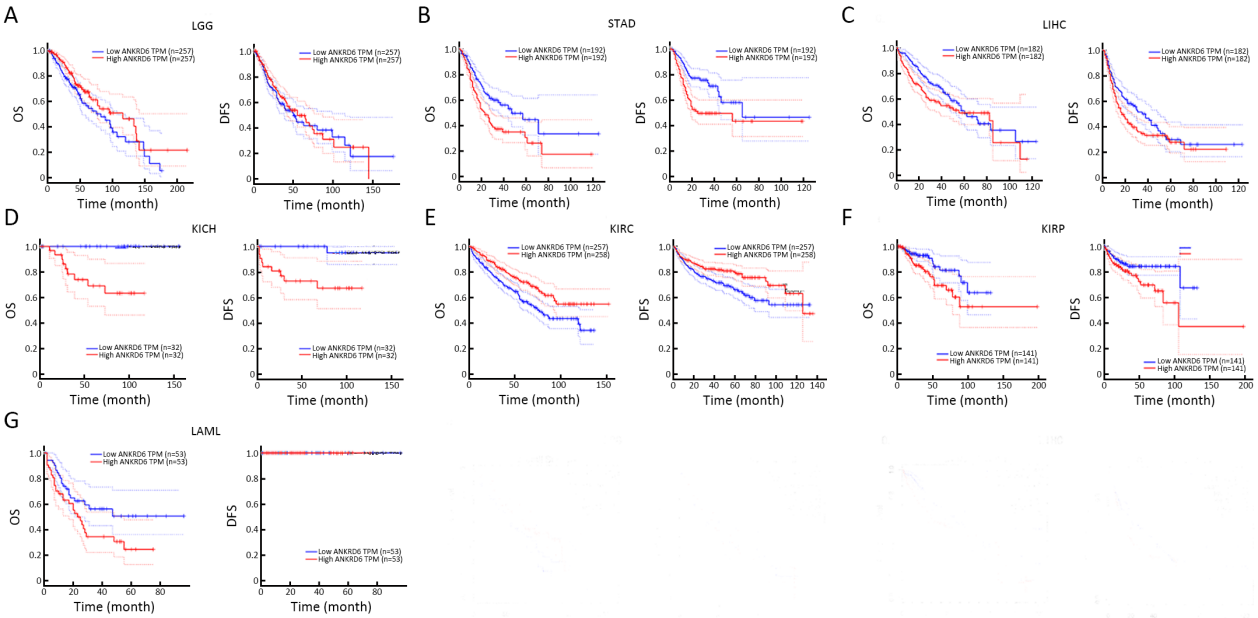

Figure S3 Kaplan-Meier survival curves comparing high and low expression of ANKRD6 in LGG, STAD, LIHC, KICH, KIRC, KIRP, LAML in GEPIA databases. (A) LGG, low levels of ANKRD6 expression is associated with poor OS (P=0.031); (B) STAD, high levels of ANKRD6 expression are associated with poor OS (P<0.001) and DFS (P<0.001); (C) LIHC, high levels of ANKRD6 expression are associated with poor OS (P=0.049) and DFS (P=0.035); (D) KICH, high levels of ANKRD6 expression are associated with poor OS (P<0.001) and DFS (P=0.003); (E) KIRC, low levels of ANKRD6 expression are associated with poor OS (P=0.001) and DFS (P=0.013); (F) KIRP, high levels of ANKRD6 expression are associated with poor OS (P=0.030) and DFS (P=0.029); G) LAML, high levels of ANKRD6 expression is associated with poor OS (P=0.042). LGG, brain lower grade glioma; STAD, stomach adenocarcinoma; LIHC, liver hepatocellular carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia.