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*Research Article*

# Comprehensive Pan-Cancer Analysis Reveals the Potential Biological, Immunological, and Prognostic Value of NKG2A

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*Background*. NKG2A (KLRC1) belongs to the NKG2 family, which has been shown to affect the activity of natural killer (NK) cells and CD8T cells. However, a comprehensive biological analysis and exploration of NKG2A in different cancers is lacking and this needs to be further investigated. *Methods*. A comprehensive pan-cancer analysis of NKG2A was performed based on multiple databases. The Cancer Genome Atlas (TCGA) and Genotype–Tissue Expression (GTEx) databases were used to analyze the expression profile of NKG2A in pan-cancer. The relevance of NKG2A to the prognosis of different cancers was assessed using Kaplan–Meier survival analysis. In addition, we explored the correlation between NKG2A expression and gene mutations, pathological staging, tumor-infiltrating immune cells (TIICs), DNA methyltransferase (DNMT) genes, tumor mutation burden (TMB), microsatellite instability (MSI), mismatch repair (MMR), and immune checkpoints (ICPs). Finally, the expression levels of NKG2A in several cancer cell lines were verified by qRT-PCR. *Results*. Pan-cancer comprehensive analysis showed that NKG2A expression levels were significantly different between multiple cancers and corresponding normal tissues. The differential expression of NKG2A was related to the prognosis and pathological staging of patients with multiple cancers, and was closely related to the excessive infiltration of immune cells and the regulation of ICP genes in the tumor microenvironment (TME). In addition, TMB, MSI, MMR, and DNMT genes in many cancer types are also affected by NKG2A expression. Gene set enrichment analysis (GSEA) showed that NKG2A was associated with multiple immune-related functions and pathways in malignant tumors. qRT-PCR results showed that NKG2A was underexpressed in liver, gastric, and colon cancer cell lines compared to normal cells, which was consistent with bioinformatics analysis. *Conclusion*. The present study suggests that NKG2A may be a potential predictive biomarker for cancer immune response and prognosis.

## 1. Introduction

Cancer is a common and highly lethal disease, and its prevalence and death rates are increasing every year [1]. In current clinical practice, it is imperative to explore new and more effective therapeutic approaches compared to traditional cancer treatments. In recent years, a growing number of immunomodulatory strategies have been developed for cancer treatment, including immune checkpoint inhibitors (ICIs), pericyte therapies, and cancer vaccines [2]. Of these, ICIs have made the most significant breakthroughs and have had the greatest impact on clinical trials [3]. Currently, PD-1 and CTLA-4 are the classical immune

checkpoints and have long been used in clinical treatment [4], and PD-1 and its ligands PD-L1 or PD-L2 have been shown to play an important role in suppressing the tumor microenvironment [5, 6]. High expression of PD-L1 or CTLA-4 is an important cause of uncontrolled T-lymphocyte-mediated adaptive immune responses or immune escape [7].

However, not all cancer patients have a significant response to first-generation ICIs or are already resistant to these ICIs. NKG2A (Also known as KLRC1 or CD159) has been dubbed the natural killer (NK) cell immunotherapeutic target due to its ability to inhibit NK cells and its value as a potential target [8]. This is similar to the T-cell-associated immune checkpoints PD-LI and CLTA-4, but has the added advantage over T-cell therapies that NK cells are not restricted by histocompatibility complex (MHC) molecules and can also fill the gap of tumor cells that lack a self-activation program to escape T-cell regulation [9, 10]. NKG2A is a protein-coding gene preferentially expressed in T and NK cells and is also a cell surface receptor containing an intracytoplasmic tyrosine inhibitory motif (ITIM) [11]. ITIM is phosphorylated to recruit the phosphatase SHP-1/2, which transduces inhibitory signals to immune cells [12]. In human peripheral blood, NKG2A is expressed on approximately one-half of NK cells and expressed on approximately 5% of $CD8^+$ T cells [13]. This expression pattern can be enhanced by a number of cytokines such as IL-15 and chronic antigenic stimulation [14]. NKG2A binds to the ligand HLA-E or mouse Qa-1 and inhibits cellular immune regulation by blocking the activation of NK cells [15], a nonclassical HLA1-like molecule that is often highly expressed in tumors and has a role in inducing lymphocyte activation [16, 17]. The CD94/NKG2A axis mediates the overexpression of HLA-E to enhance resistance to NK cells and detect antigenic abduction [18].

In the tumor immune microenvironment (TME), NKG2A plays an important role in the anticancer immune response. NKG2A is considered an NK cell-associated immunotherapeutic target due to its specific function of inhibiting NK cell expression. Its inhibitor, the anti-NKG2A monoclonal antibody (monalizumab), is now available for clinical trials. Monalizumab reverses the suppression or failure of NK cells and $CD8^+$ T cells by blocking the specific binding of the NKG2A receptor to HLA-E [19, 20]. Monalizumab alone stimulates NK cell toxicity, whereas in combination with other immunosuppressive agents it promotes NK and T-cell function more effectively and is more clinically beneficial [21]. Studies have shown that in HCC, NKG2A is highly expressed in tumor-infiltrating NK cells and high levels of HLA-E are found in HCC tissue [22]. Abnormal levels of NKG2A/HLA-E expression can affect many types of cancer, such as gastric, colorectal, ovarian, and lung cancers [23, 24]. However, current studies have focused more on the effect of NKG2A as an immune target and the study of its role in being blocked, lacking a cancer analysis perspective to explore the relevance of NKG2A to the prognosis of different types of cancer, the immune landscape, and its potential as a cancer marker. Therefore, we evaluated the variations in NKG2A expression in 33 different cancer tissues and healthy paracancerous tissues in the TCGA database, along with the relationship between NKG2A and prognosis in various cancer types and the TME, microsatellite instability (MSI), DNA mismatch repair system (MMR), DNA methyltransferases, and GSEA enrichment analysis and other aspects to observe the relevance analysis of immune regulation and survival of NKG2A impact on pan-cancer.

Table 1: Full names and abbreviations of the 33 cancers in the TCGA database.

| Abbreviation | Full name |
|---|---|
| ACC | Adrenocortical carcinoma |
| BLCA | Bladder urothelial carcinoma |
| BRCA | Breast invasive carcinoma |
| 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 |
| 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 serous cystadenocarcinoma |
| 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 |

## 2. Materials and Methods

*2.1. Acquisition of Sample Data.* The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/) contains clinical data and transcriptomic RNA sequence data for 33 malignancies, and researchers can collect and download these publicly available data for data analysis (Table 1). Information on the differential expression of NKG2A in cancer and normal tissues was obtained and analyzed using the TCGA and GTEx databases. Genotype–Tissue Expression (GTEx) (https://gtexportal.org/), a tissue repository and data resource established by the

National Institutes of Health (NIH) Common Fund, examined over 7,000 autopsies from 449 healthy human donors at the time of survival and included 44 tissue types. To clarify the correlation between NKG2A expression and the abundance of immune cell infiltration in pan-cancer, we searched and downloaded data resources for 33 cancer-associated immune cells through the TIMER database, focusing on the correlation between NKG2A and the immune microenvironment of pan-cancer.

*2.2. Gene Expression and Genetic Mutation Analysis.* The significance of the difference in expression of NKG2A in cancer and normal tissues was tested by Wilcoxon, with "*", "**," and "****," indicating $P<0.05$, $P<0.01$, and $P<0.001$, respectively. Based on sample analysis from the TCGA and GTEX databases, NKG2A expression differences between 33 cancers and corresponding paracancerous normal tissues were evaluated, and the results were presented as box plots. Through the cBioPortal (https://www.cbioportal.org/) database, we were able to assess genetic alterations in the NKG2A gene in different cancers, including missense mutations, deletions, splicing, and other alterations.

*2.3. Prognosis and Assessment of ROC.* Survival information data were retrieved and downloaded for each sample through the TCGA database, with the main aspects analyzed, including overall survival (OS), disease-specific survival (DSS), disease-free interval (DFI), and progression-free interval (PFI). The expression levels and survival prognosis of NKG2A in pan-cancer were assessed and analyzed using a univariate approach. The median NKG2A expression level was used as the cutoff point for cancer dichotomization, thereby dividing the patient population into high and low expression groups, and the Kaplan–Meier (K–M) method was then used to analyze and compare the survival rates of the two groups. K–M curves were plotted using the R packages "survminer" and "survivor", and forest plots were drawn using the R packages "survivor" and "forestplot." The K–M plotter (https://kmplot.com) was then used to assess the impact of NKG2A on survival in patients with different cancers based on data builds derived from databases such as GEO, EGA, and TCGA to analyze the relationship of NKG2A expression with OS and RFS. The R packages "pROC" and "ggplot2" were used to evaluate the RNAseq data of TCGA and GTEx and to visualize the results. The area under the ROC curve (AUC) was calculated to confirm the diagnosis and prognosis of cancer. In addition, to visualize the association between NKG2A gene expression levels and clinical outcome in pan-cancer, the R packages "limma" and "ggpubr" were used to show the association of NKG2A genes with clinicopathology.

*2.4. Analysis of Tumor Immune Microenvironment and Immune/Molecular Subtype.* To reliably assess the immune correlation of NKG2A gene expression levels in pan-cancer, we used immunedeconv, an R package that integrates two state of the art algorithms such as TIMER and CIBERSORT, allowing us to quantify the relationship between NKG2A and immune cell infiltration abundance and immune-associated gene coexpression analysis. We next performed coexpression analysis of NKG2A expression levels and expression levels of recognized immune checkpoints in pan-cancer using Spearman statistical methods. We were also able to calculate stroma/immune scores for each tumor sample by running the R packages "ESTIMATE" and "LIMMA." The TISIDB website (http://cis.hku.hk/TISIDB/index.php) was used to analyze the association between NKG2A expression and the immune and molecular subtypes of different cancers.

*2.5. Correlation Analysis of NKG2A Expression with TMB, MSI, MMR, and DMNT.* The correlation between tumor mutational burden (TMB) and microsatellite instability (MSI) and NKG2A gene expression can be calculated separately by Spearman's statistical method and the results can be visualized by creating radar plots using the R package "fmsb" ($^{***}P<0:001$; $^{**}P<0:01$; $^{*}P<0:05$). Abnormalities in the DNA mismatch repair system (MMR) and DNA methylation are also important factors contributing to tumor development. We downloaded the mutation levels of five MMR genes, namely MutL homolog 1 (MLH1), MutS homolog 2 (MSH2), MutS homolog 6 (MSH6), PMS1 homolog 2 (PMS2), and epithelial cell adhesion molecule (EPCAM) from the TCGA database and used Pearson statistical analysis to assess NKG2A expression levels in different cancers in relation to MMR mutations. The correlation between NKG2A and the levels of four methyltransferases, deoxyribonucleic acid methyltransferase 1 (DNMT1), TRNA aspartate methyltransferase 1 (TRDMT1), DNA methyltransferase3$\alpha$ (DNMT3A), and DNA methyltransferase 3$\beta$ (DNMT3B), was also analyzed by Pearson statistical analysis. The final results were presented as a heat map using the R package "pheatmap."

*2.6. GSEA Enrichment Analysis.* Gene set enrichment analysis (GSEA) website (https://www.gsea-msigdb.org/gsea/downloads.jsp) was obtained for the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets. We used the R packages "limma," "org.Hs.eg.db," "clusterProfiler," and "enrichplot" for functional annotation and pathway enrichment analysis of GO and KEGG. The data were also organized and analyzed using R software (version 4.13) and Perl scripts (version 5.32.1.1).

*2.7. Cell Culture.* Human normal gastric epithelial cell line GES-1 and human gastric cancer cell lines HGC-27, MKN-45, MGC-803, human normal hepatocyte line L-O2 and human liver cancer cell lines SMMC-7721, HUh7, H-97, human normal colonic epithelial cell line NCM460, and human colon cancer cell lines SW620, SW480, HCT116 were obtained from this subject group and stored in the central laboratory of Gansu Provincial People's Hospital. The cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS), 1% double antibodies (streptomycin and penicillin) and placed in a humidified incubator containing 5% concentration of $CO_2$ at 37°C.

*2.8. RNA Isolation and qRT-PCR.* RNA was extracted according to the instructions of the M5 Universal RNA Mini Kit. To determine the RNA concentration and purity in accordance with the experimental requirements,

absorbance values were measured at 260 and 280 nm. RNA was reverse transcribed to cDNA according to the instructions of M5 Sprint qPCR RT Kit with gDNA Remover Reverse Transcription Kit. 2×M5 HiPer SYBR Premix EsTaq (with Tli RnaseH) was used as a fluorescent dye for RT-qPCR assay. NKG2A and GAPDH primers were designed and synthesized by Bioengineering (Shanghai) Co. NKG2A forward primer: 5′-TCACTGCAAAGATTTACCATCAGC-3′, reverse primer: 5′-TTCAGGGAAGAATTGTTGTGCC-3′. GAPDH forward primer: 5′-GGAAGCTTGTCATCA ATGGAAATC-3′, reverse primer 5′-TGATGACCCTTTTG GCTCCC-3′. mRNA expression levels of NKG2A were calculated and analyzed by the $2^{-\Delta\Delta Ct}$ formula.

*2.9. Statistical Analysis.* All gene expression data were normalized by log2. Differential expression of target genes in pan-cancers was detected by Wilcoxon test. Survival prognosis analysis of NKG2A genes and pan-cancers was performed by K–M curve method. The relationship between NKG2A gene expression and immune cell infiltration abundance and gene coexpression analysis was assessed by Spearman method. Correlation of NKG2A expression levels with MMR and DNA methyltransferase mutations in pan-cancer was assessed using Pearson statistical analysis. All data analyses were performed using R software (version 4.2.0, https://www.R-project.org) and the R package was run to complete multiple visualization of the results. All findings were significantly correlated at $P < 0.05$.

## 3. Results

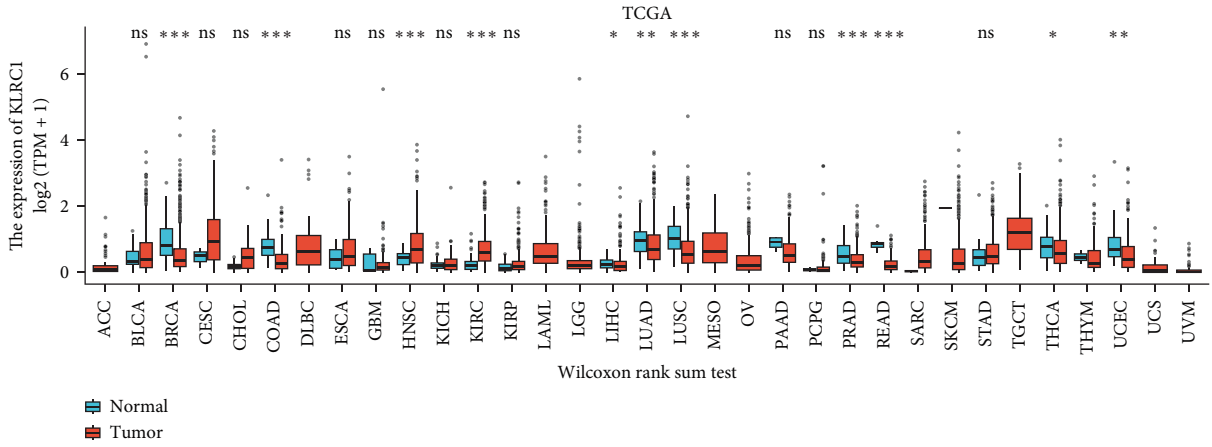
*3.1. Differential Expression of NKG2A in Pan-Cancers.* By analysis of NKG2A expression in tumor and normal tissues based on cancer data in TCGA, the results showed that the expression levels of NKG2A were lower in BRCA, COAD, LIHC, LUSC, PRAD, READ, and UCEC than in normal tissues, and significantly higher only in HNSC and KIRC (Figure 1(a)). To further explore the differences in NKG2A expression in different cancer types, we combined TCGA and GTEx databases to assess the differences in NKG2A expression profiles between 33 cancers and paracancerous normal tissues using an expanded sample size. New analysis reveals the addition of four cancer types with lower than normal tissue levels of NKG2A expression, namely LUAD, STAD, THCA, and UCS, and nine new cancer types with higher than normal tissue levels of NKG2A expression, namely CESC, ESCA, KIRP, LAML, LGG, OV, PAAD, SKCM, and TGCT (Figure 1(b)). cBioportal database was used to analyze the genetic variation of NKG2A in pan-cancer. The results showed that the main genetic variant type of NKG2A was amplification, and the three cancer types with the highest variant frequency were testicular germ cell tumors (6.04%), ovarian serous cystadenocarcinoma (3.91%), and uterine carcinosarcoma (3.51%) (Figure 1(c)).

*3.2. Prognostic Value of NKG2A in Pan-Cancer.* We investigated the association between NKG2A expression and survival prognosis in multiple cancers. Using forest plots to observe the correlation between NKG2A expression levels and OS, DSS, DFI, and PFI, it was found that the expression of NKG2A was significantly positively correlated with OS, DSS, and DFI of BLCA, OS and PFI of BRCA, OS and DSS of SKCM, and DFI and PFI of UCEC. Conversely, NKG2A expression was negatively correlated with the DFI of KIRP, DFI and PFI of THCA, and PFI of THYM (Figure 2). In addition, observing the K–M curve of TCGA showed that the expression of NKG2A was significantly correlated with the prognosis of a variety of cancers. High expression of NKG2A played a protective role in the prognosis of patients with eight types of cancer, including LGG, PCPG, SKCM, UCEC, CESC, ACC, COAD, and KIRC, and only had adverse outcomes in patients with THYM, HNSC, and THCA (Figure 3). In addition to these results, the K–M plotter analysis showed that patients with BLCA, BRCA, LIHC, OV, READ, LUSC, and PCPG with high levels of NKG2A showed good OS or RFS, while patients with PAAD with high levels of NKG2A showed poor OS (Figure 4).

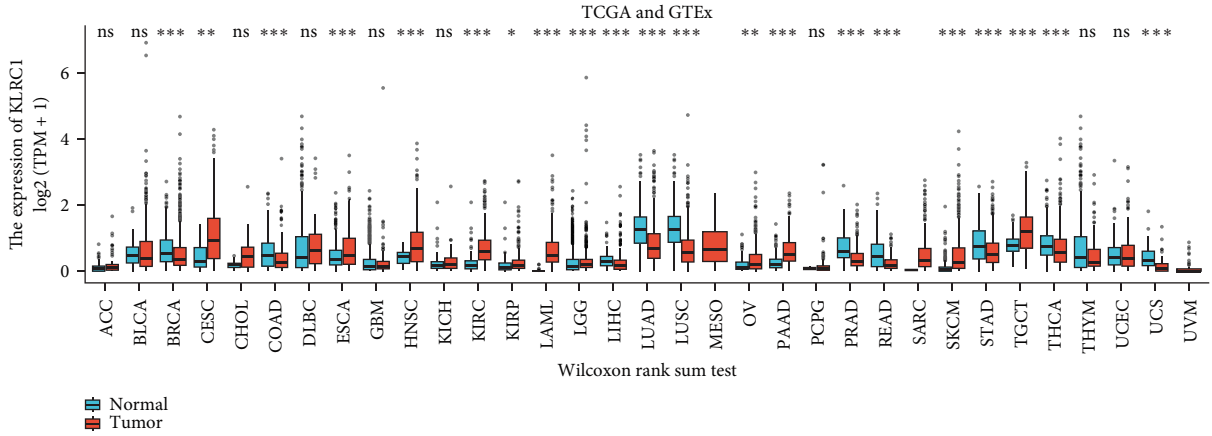
*3.3. Analysis of the Clinicopathology Associated with NKG2A Affecting Pan-Cancer.* Observing the results of clinicopathological analysis related to NKG2A, we were able to find a significant correlation between the expression of NKG2A and the age and tumor stage of some tumor patients. In BLCA, BRCA, KIRP, OV, and SKCM, NKG2A expression levels were significantly higher in patients aged ≤65 years. In contrast, in LAML and SARC, NKG2A expression levels were higher in patients aged >65 years (Figure 5(a)–5(g)). In addition, there was a positive correlation between the high expression of NKG2A and the tumor stage of pan-cancer. Specifically, NKG2A expression was higher in stage I–II and lower in stage III–IV in BLCA, COAD, and TGCT. In contrast, NKG2A expression was lower in stages I–II and higher in stages III–IV in THCA (Figure 5(h)–5(k)).

In addition, to further explore the potential value of NKG2A in pan-cancer, we plotted the ROC curves associated with NKG2A. The results showed that NKG2A had moderate diagnostic accuracy (AUC between 0.7 and 0.9) for CESC, KIRC, LUAD, LUSC, PAAD, PRAD, SKCM, and UCS in predicting tumor or nontumor prognosis while it was more accurate for LAML (AUC > 0.9) (Figure 5(l)). This suggests that NKG2A has a strong tumor predictive ability.

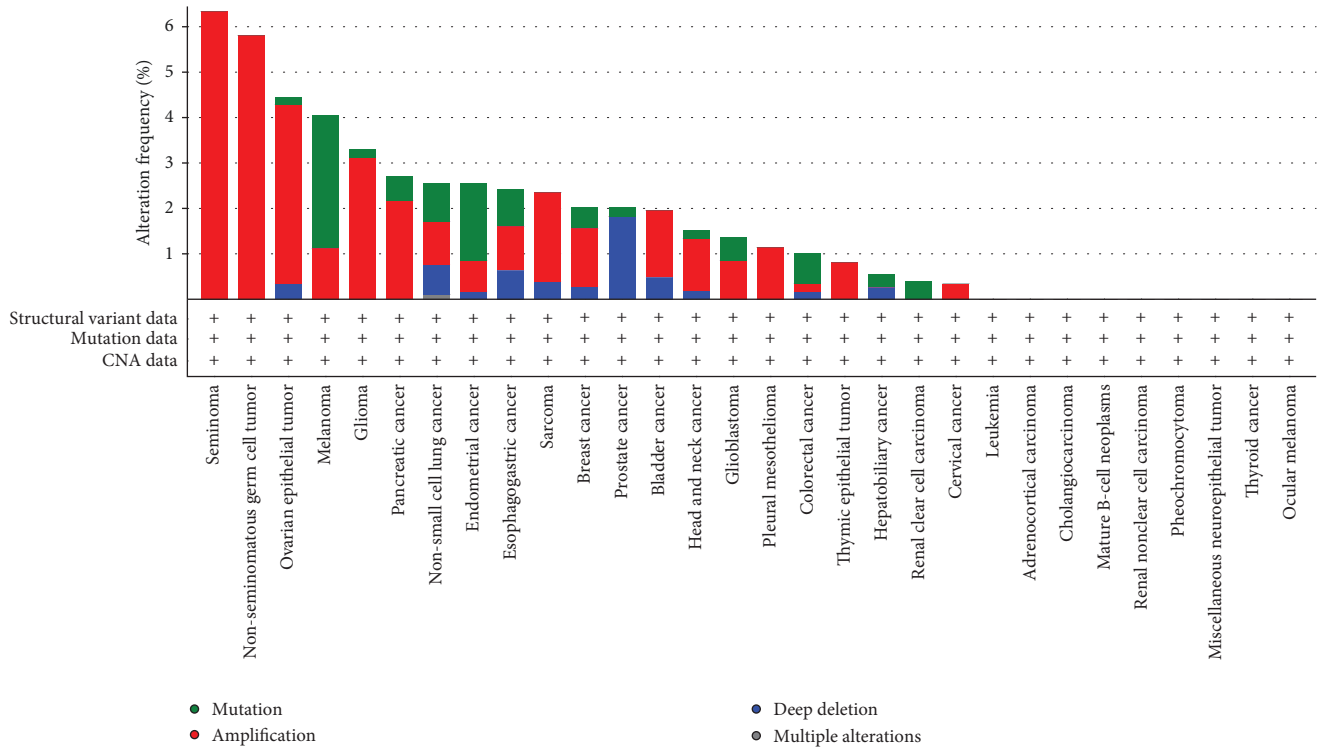
*3.4. NKG2A Is Associated with the Tumor Immune Microenvironment in Pan-Cancer.* We analyzed the correlation between NKG2A expression and the abundance of different immune cell infiltrates in pan-cancer based on TIMER and CIBERSORT methods. The results revealed that NKG2A expression was significantly correlated with the degree of infiltration of six major immune cells in all cancers except DLBC, GBM, LGG, and THYM. In these cancers, NKG2A expression was significantly positively correlated with the degree of infiltration of $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, bone marrow dendritic cells, macrophages, and B cells. In contrast, NKG2A expression in LGG was negatively correlated with the level of infiltration of $CD8^+$ T cells, and in THYM, NKG2A showed a significant negative correlation with the abundance of infiltration of $CD8^+$ T cells, $CD4^+$ T cells, and bone marrow-like dendritic cells (Figure 6(a)). Using the CIBEROR technique, we investigated the potential




(a)




(b)




(c)

Figure 1: Expression levels of NKG2A in different types of cancer. (a) Differential expression of NKG2A in tumor and normal tissues based on TCGA database ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). (b) Differential expression of NKG2A in tumor and normal tissues based on TCGA and GTEx database ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). (c) Type and frequency of genetic alterations in the NKG2A gene in pan-cancer.

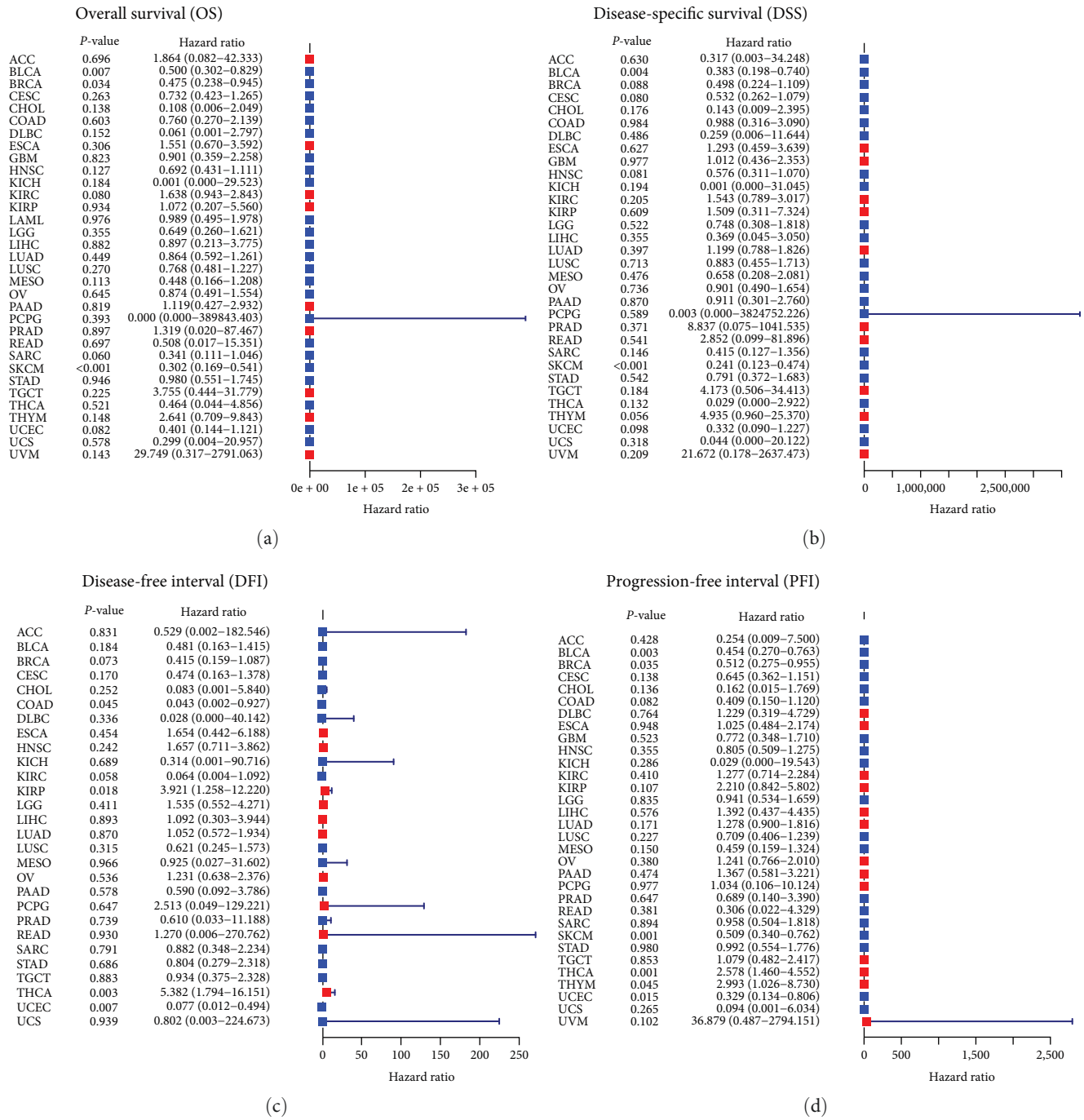
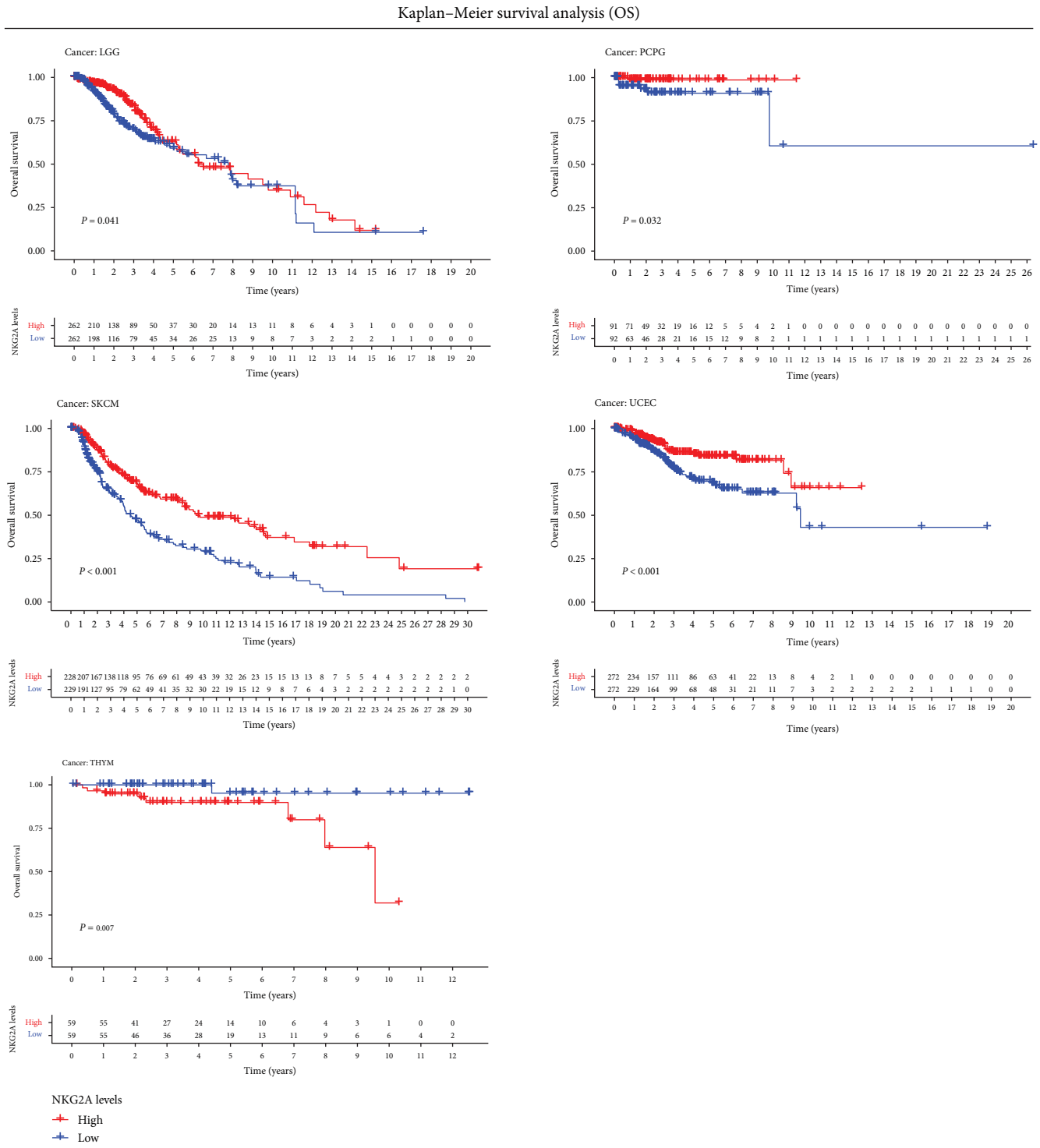



Figure 2: Correlation between NKG2A expression and survival using the COX method for different types of cancer in TCGA. (a) OS. (b) DSS. (c) DFI. (d) PFI. OS, overall survival; DSS, disease-specific survival; DFI, disease-free interval; PFI, progression-free interval.

relationship between NKG2A expression and infiltration of 22 immune cells and subtypes. Coexpression analysis of NKG2A with immune cells suggested that this gene expression was mainly associated with immune cell infiltration and infiltration. The cancer types enriched with the most NKG2A positively associated immune cells were SARC, COAD, and BRCA ($n = 8$), whereas the cancer types enriched with the most NKG2A negatively associated immune cells were KIRC, HNSC, and BLCA ($n = 8$) (Figure 6(b)).

Immunization checkpoint (ICP) genes have been shown to influence immune cell infiltration and immunotherapy. To further explore the immune relevance of NKG2A to cancer immunotherapy, we performed coexpression analysis of NKG2A and ICP genes. The results showed that NKG2A expression was significantly associated with the expression of multiple ICP genes in multiple cancers, such as PDCD1 (PD-L1), CTLA4, CD274, TIGIT, TMIGD2, LAG3, CD244, IDO2, and BTLA. Thus, NKG2A may be involved in the




(a)

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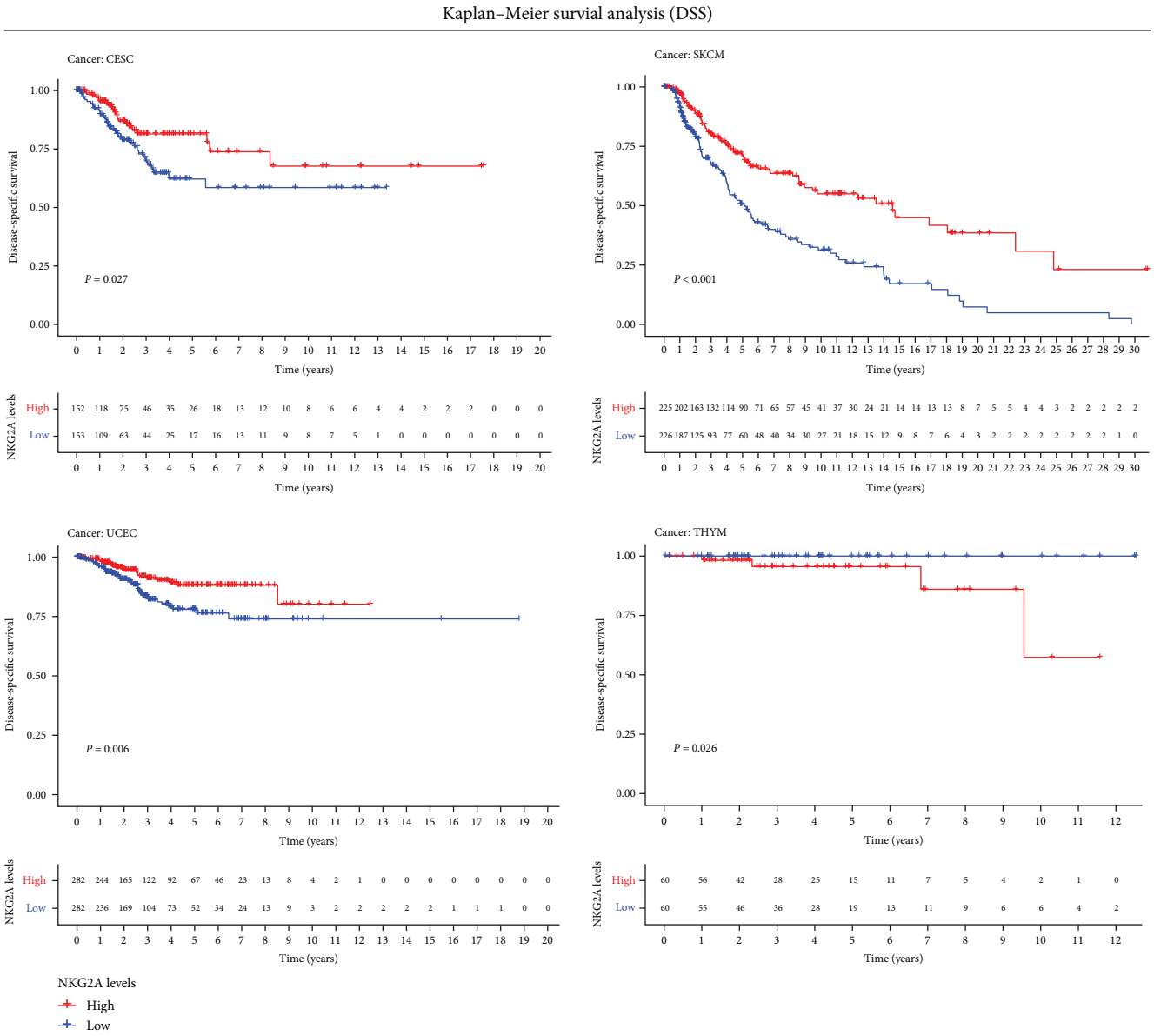


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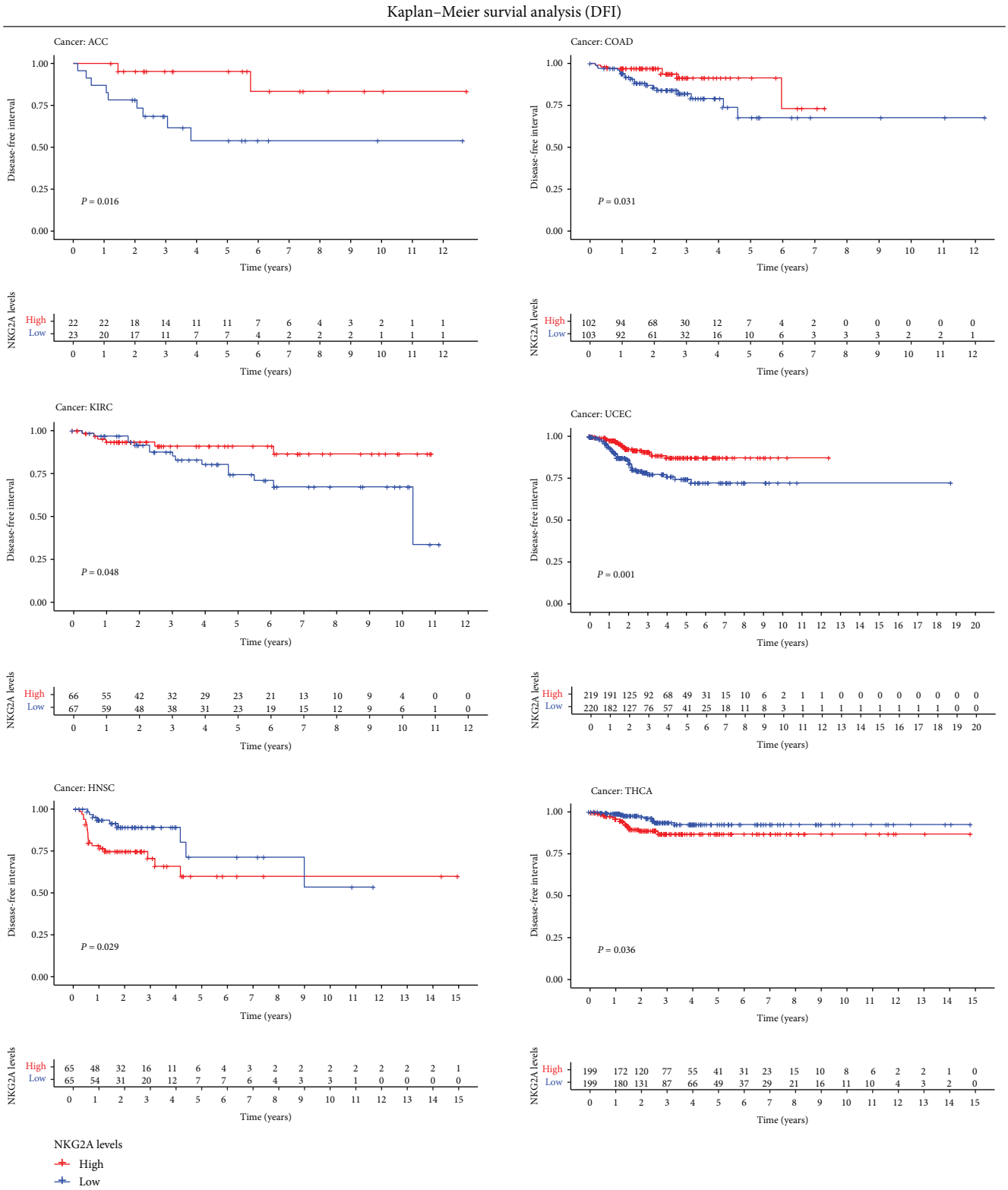


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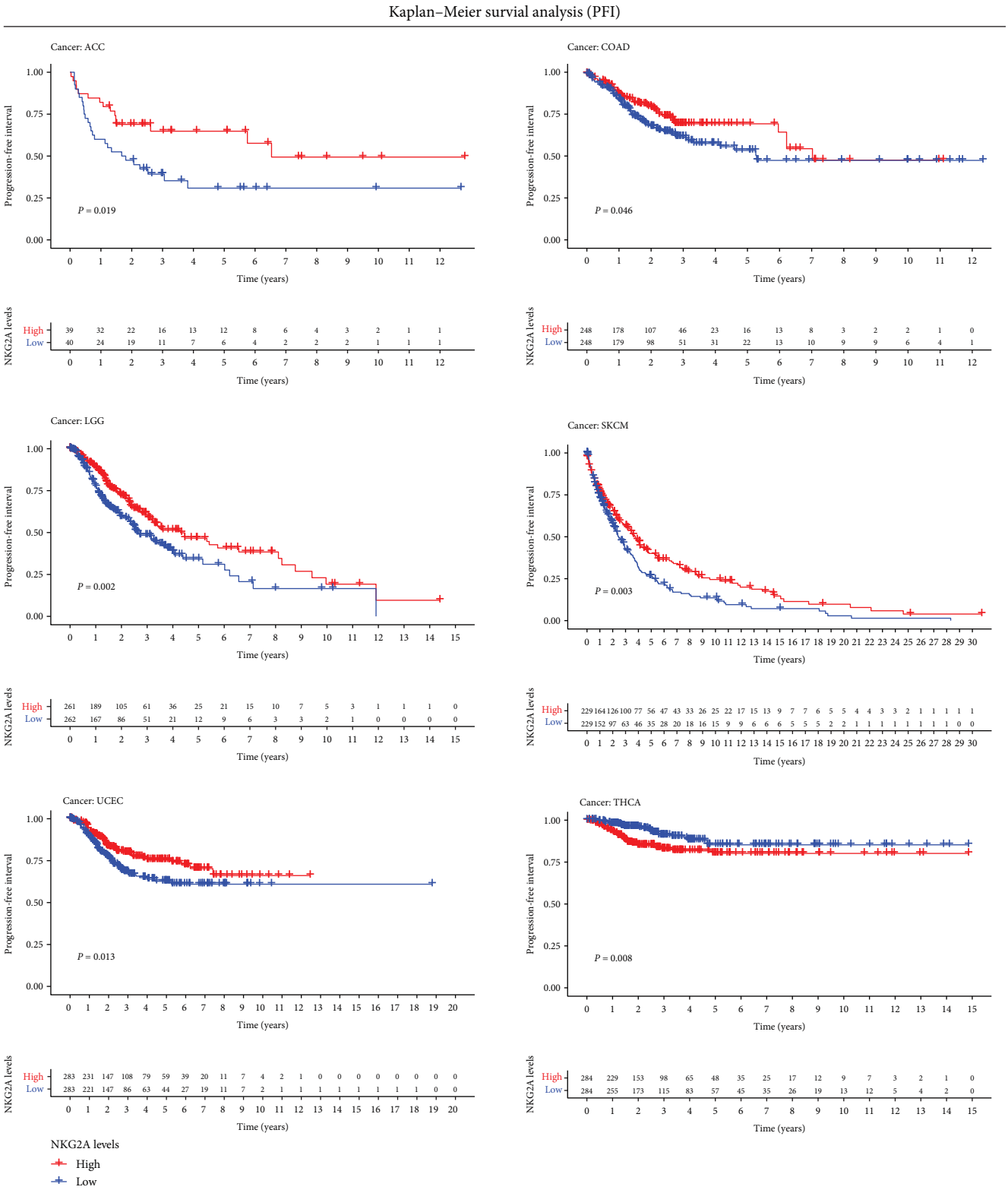
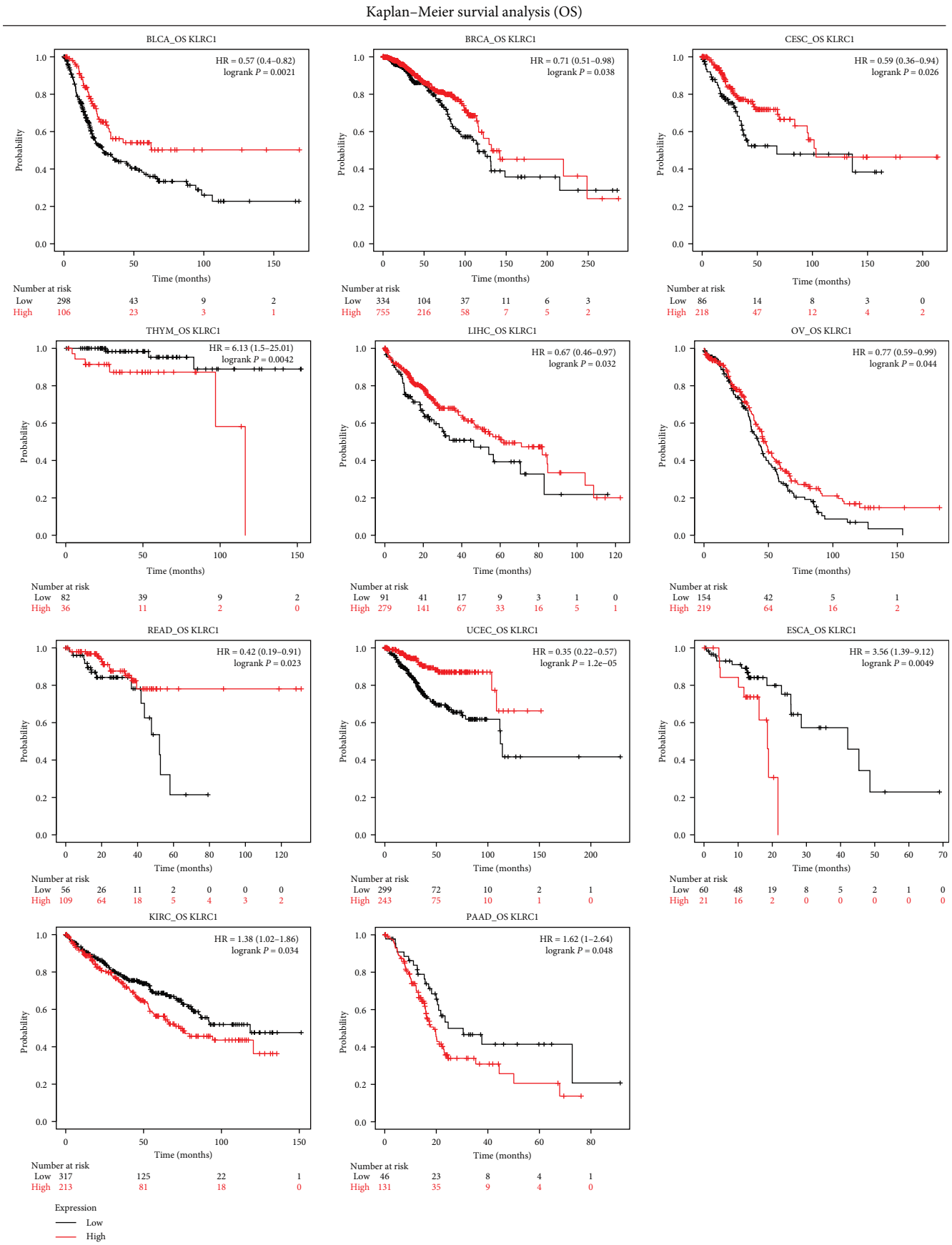



(d)

Figure 3: Comparison of Kaplan–Meier survival curves for differential expression of the NKG2A gene in different cancer types. (a) OS: KM curves of high and low NKGA2 expression in LGG, PCPG, SKCM, UCEC, and THYM patients. (b) DSS: KM curves of high and low NKGA2 expression in CESC, SKCM, UCEC, and THYM patients. (c) DFI: KM curves of high and low NKGA2 expression in ACC, COAD, KIRC, UCEC, HNSC, and THCA patients. (d) PFI: KM curves of high and low NKGA2 expression in ACC, COAD, LGG, SKCM, UCEC, and THCA patients.




(a)

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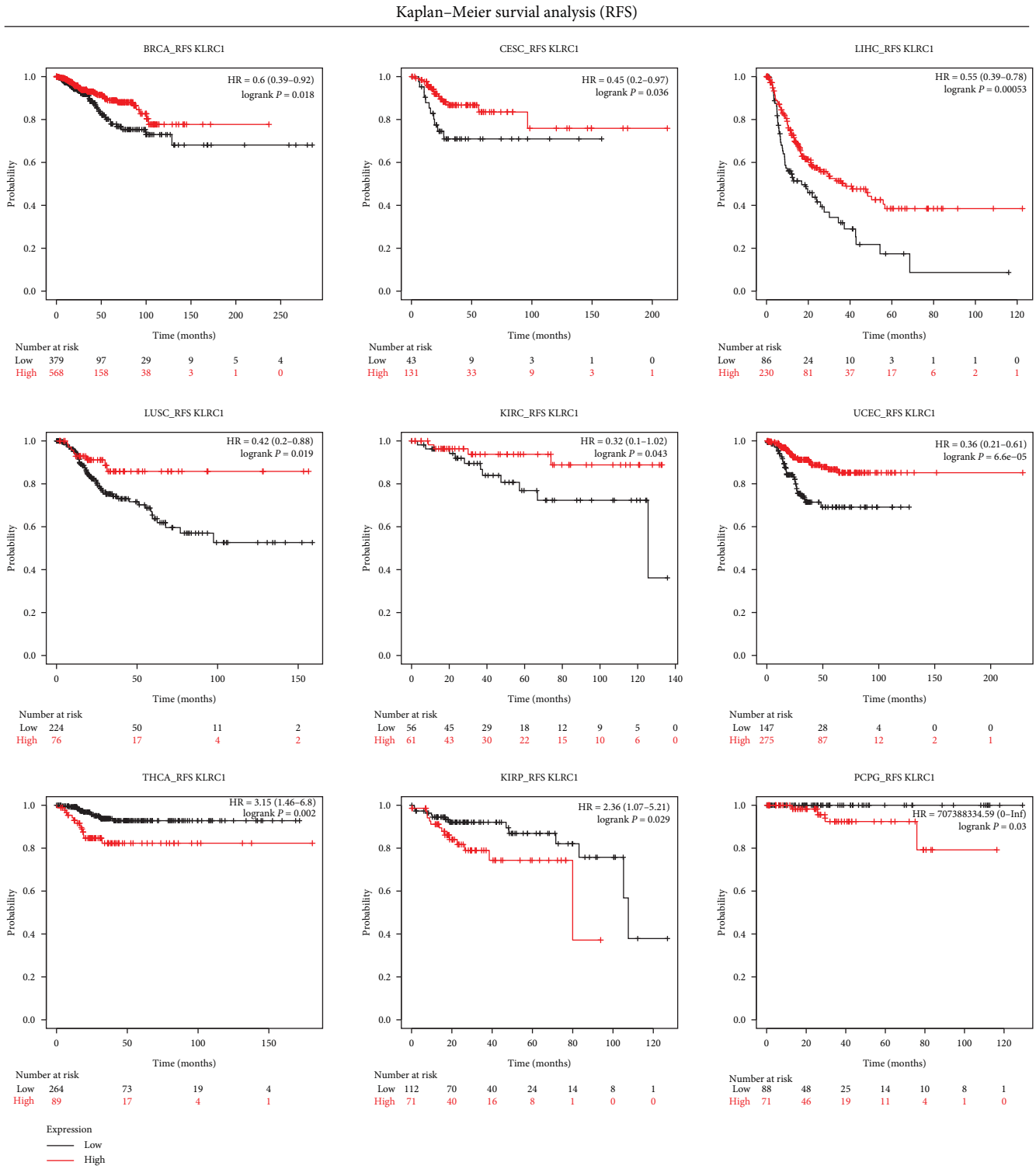
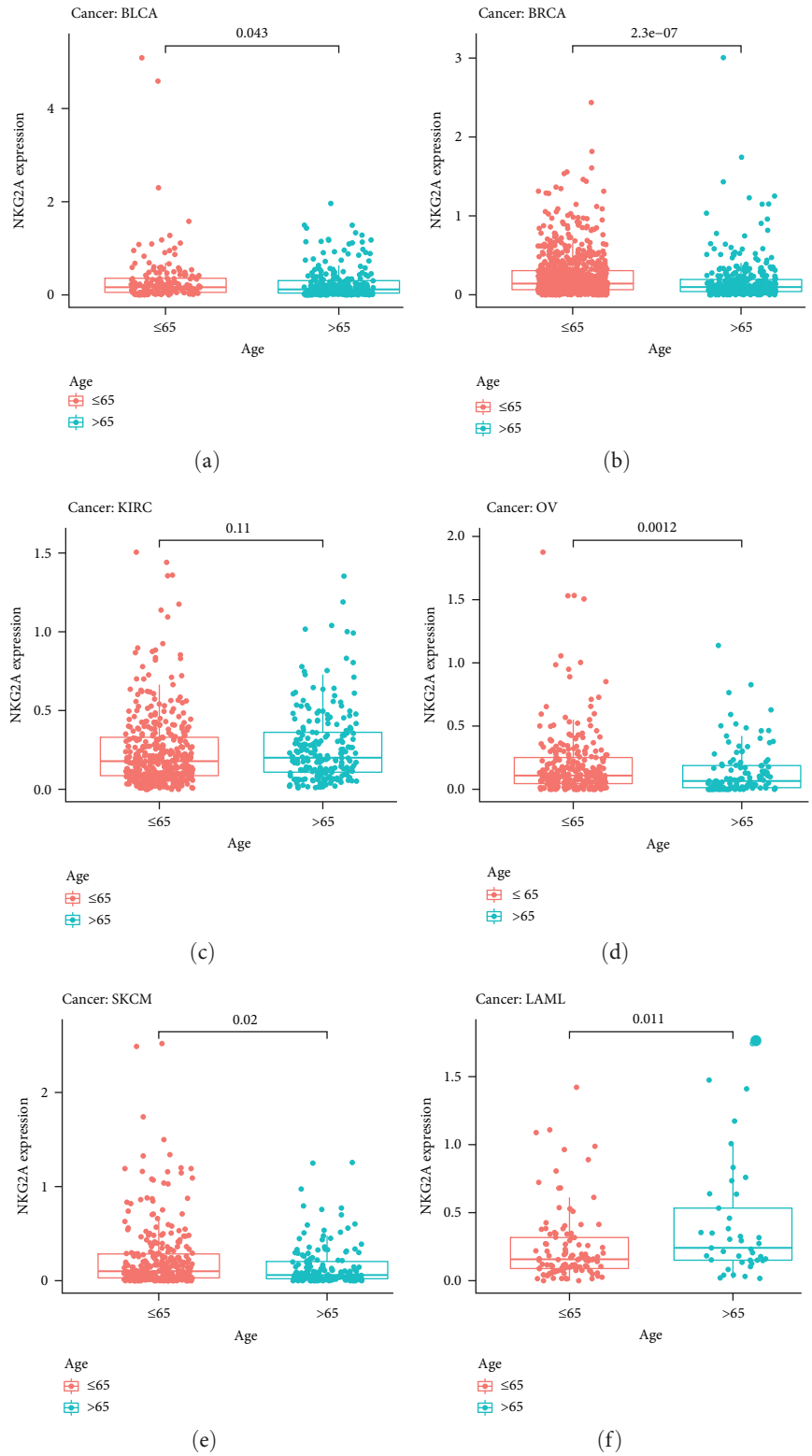



(b)

Figure 4: Kaplan–Meier survival curves assess high/low expression of the NKG2A gene in different cancers in the Kaplan–Meier plotter database. (a) Survival curves linking NKG2A gene expression to OS. (b) Survival curves linking NKG2A gene expression to RFS.

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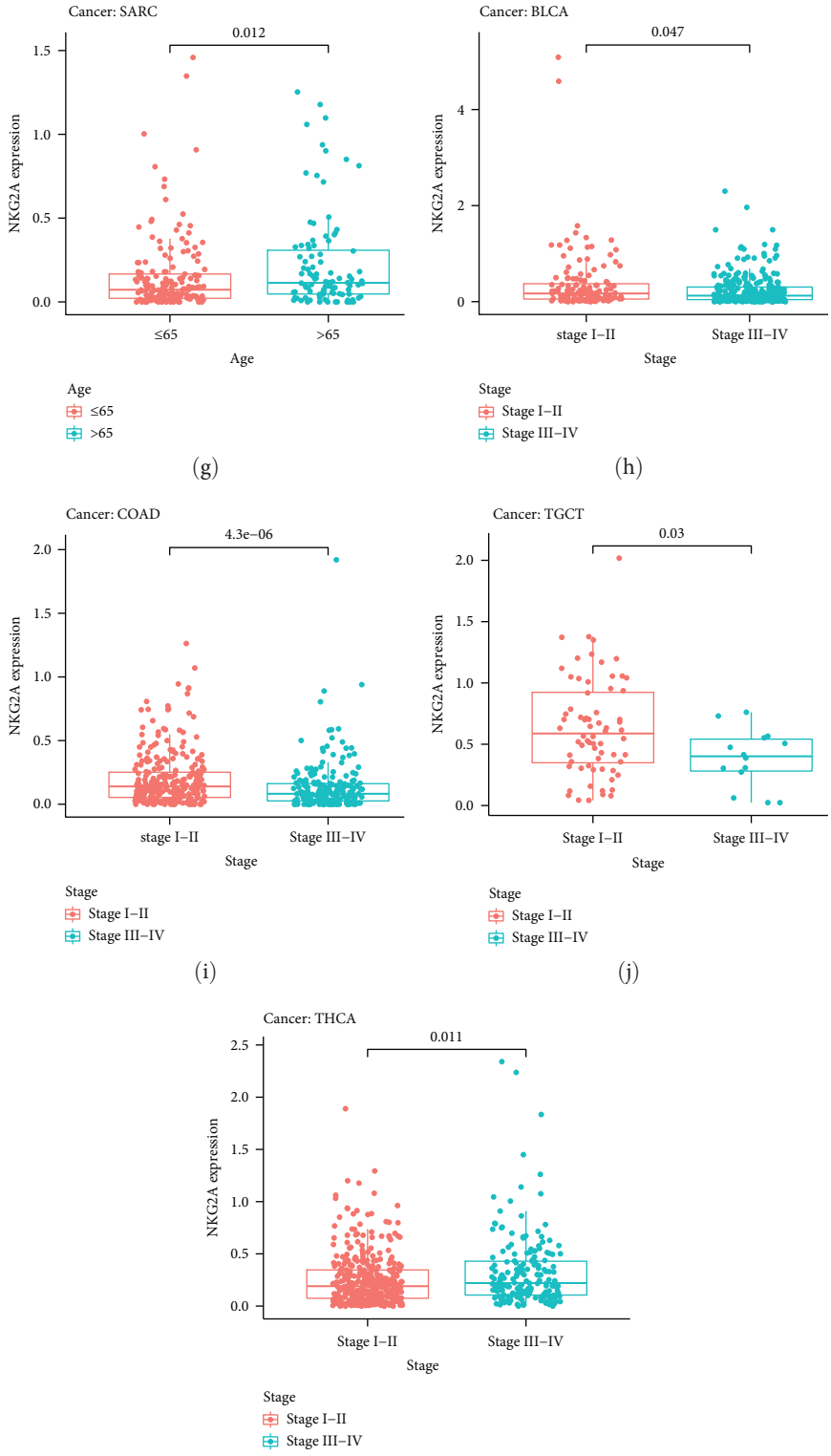


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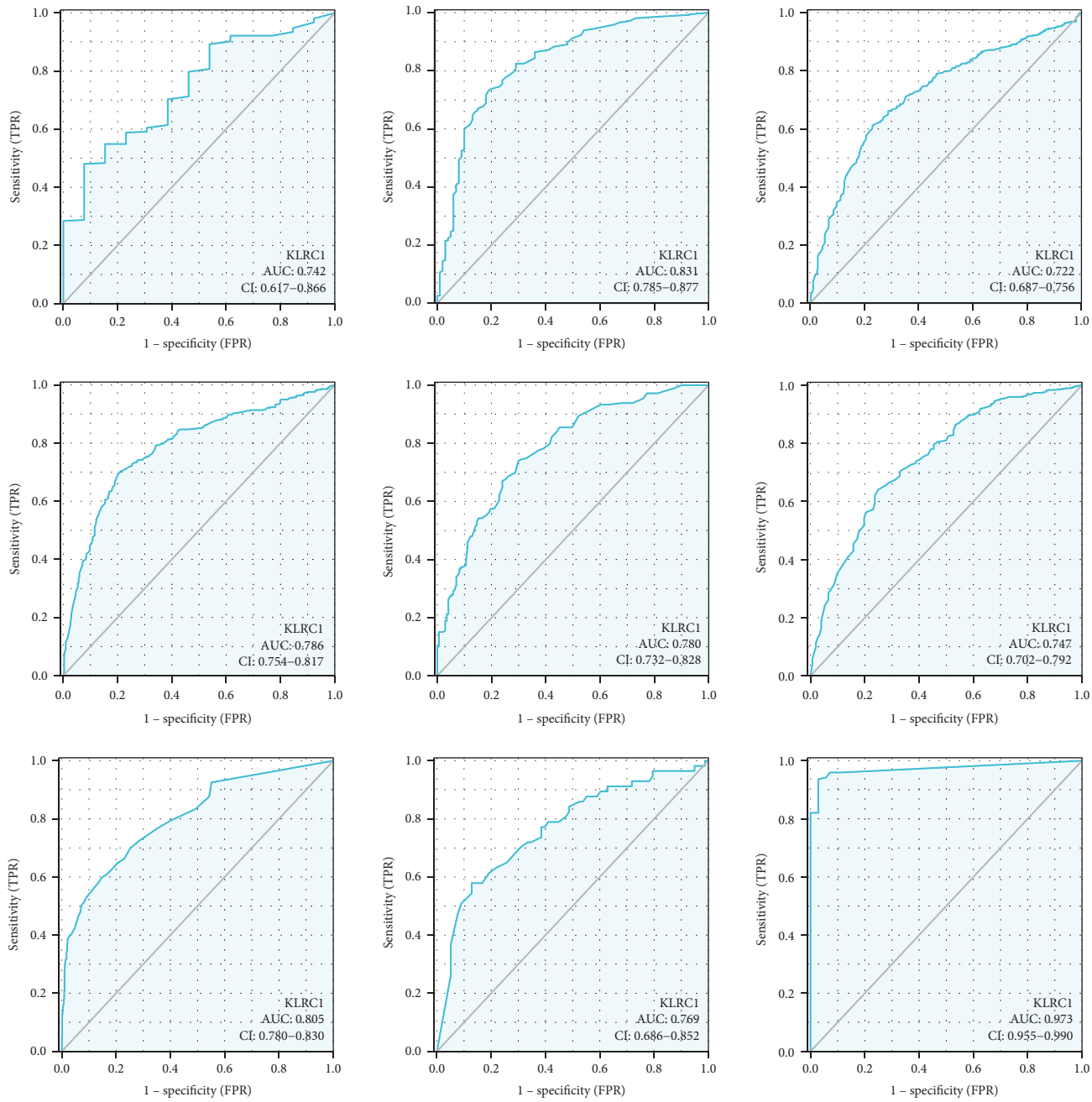



(l)

Figure 5: Correlation analysis of NKG2A expression with age and pathological stage. NKG2A gene expression correlated with age in (a) BLAC, (b) BRCA, (c) KIRP, (d) LAML, (e) OV, (f) SARC, and (g) SKCM. NKG2A gene expression correlated with pathological stage in (h) BLCA, (i) THCA, (j) TGCT, and (k) COAD. ROC analysis of NKG2A genes in the TCGA database (l). CESC, KIRC, LUAD, LUSC, PAAD, PRAD, SKCM, USC, LAML.

activation of immune checkpoints during tumor development and regulates multiple signaling pathways (Figure 6(c)).

To determine the correlation between NKG2A expression and immune/stromal scores in different cancers, we evaluated immune scores and stromal scores in 33 cancers, respectively. The 21 cancers found by the results to be positively correlated between NKG2A expression and immune score or stromal score were ACC, BLCA, BRCA, COAD, LUAD, HNSC, KIRC, KIRP, LIHC, LUSC, OV, PAAD, PCPG, PRAD, SARC, SKCM, STAD, THCA, UCEC, and UVM (Figures 7(a) and 7(b)). NKG2A expression was only negatively correlated with the immune or stromal scores of GBM (Figures 7(a) and 7(b)).

*3.5. Correlation Analysis of NKG2A Expression with Immune Subtypes and Molecular Subtypes.* It is widely recognized that different immune subtypes have important clinical value in a variety of tumors, and we focused on the correlation of NKG2A expression with immune subtypes and molecular

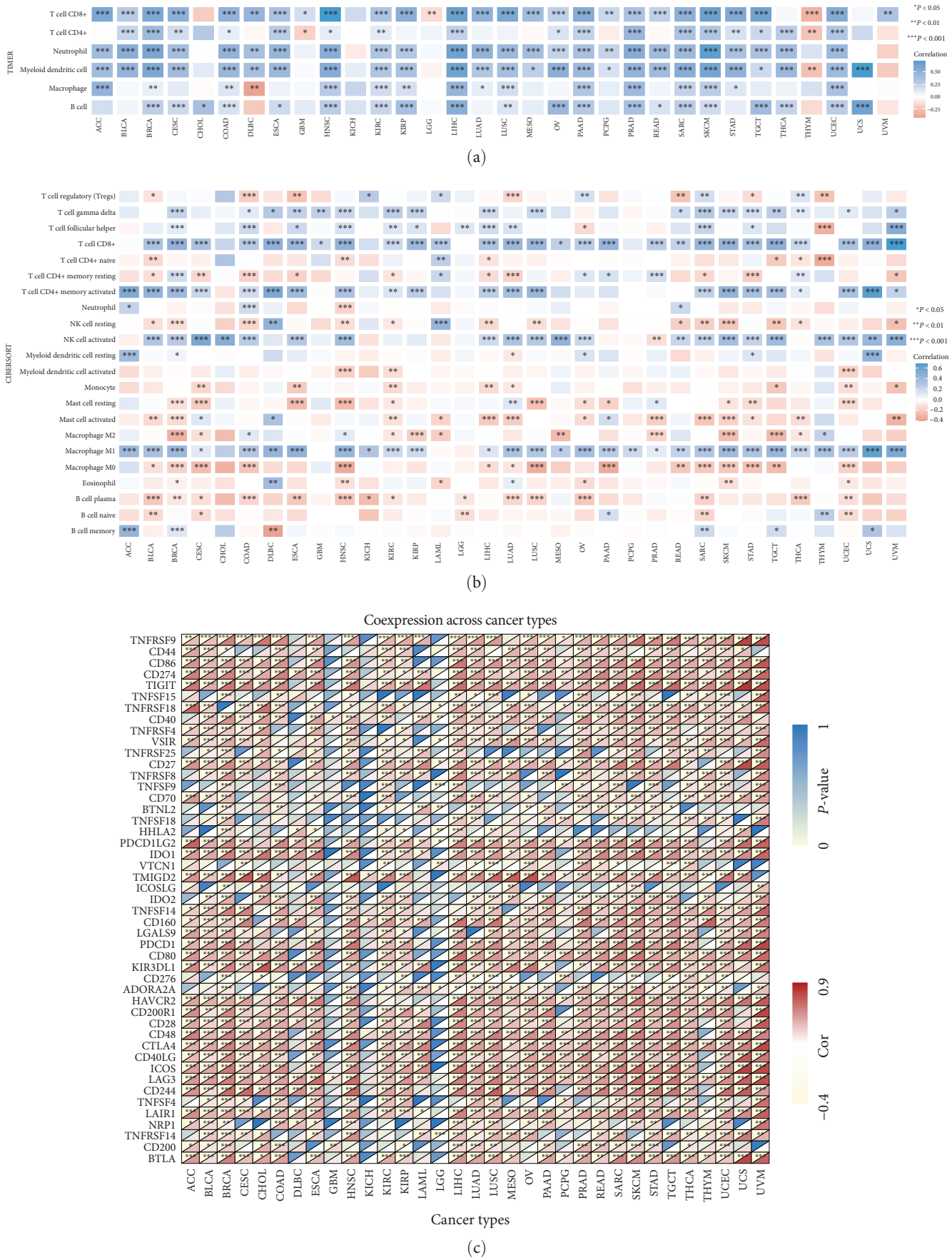



Figure 6: Correlation between NKG2A and the abundance of immune cell infiltration and with immune checkpoints. (a) Relationship between NKG2A gene expression and the level of infiltration of six types of immune cells in pan-cancer ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

(b) Relationship between NKG2A gene expression and the level of infiltration of 22 immune cells in pan-cancer ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). (c) Correlation of NKG2A with confirmed immune checkpoints in multiple cancers ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

subtypes of different cancers. As shown in Figure 8(a), NKG2A expression was significantly correlated with six immune subtypes among LUSC, MESO, BLCA, BRCA, COAD, KIRC, LUAD, SARC, SKCM, and STAD. Notably, the expression of NKG2A was also variable among different immune subtypes of the same cancer, such that NKG2A expression was higher in most C2 cancer types than in other immune subtypes. Furthermore, we found that NKG2A expression was closely associated with several molecular tumor subtypes in BRCA, COAD, ESCA, HNSC, LUSC, OV, STAD, and READ (Figure 8(b)).

*3.6. Correlation of NKG2A Expression with TMB, MSI, MMR, and DMNT.* TMB and MSI are emerging as important predictive tumor biomarkers and, therefore, we correlated TMB and MSI in cancer with NKG2A. Radar plot results for TMB showed that TMB correlated with NKG2A gene expression in a variety of cancers. Among them, NKG2A expression was significantly positively correlated with TMB in eight cancers, namely THYM, UCEC, THYM, SARC, LUAD, LAML, COAD, and BLCA. Only three cancers, namely TGCT, PRAD, and KIRP, were negatively correlated with TMB (Figure 9(a)). Another result showed that NKG2A expression was significantly correlated with MSI in seven cancers, and this correlation was negative in BLCA, TGCT, OV, MESO, LUSC, and ESCA and positive only in COAD (Figure 9(b)).

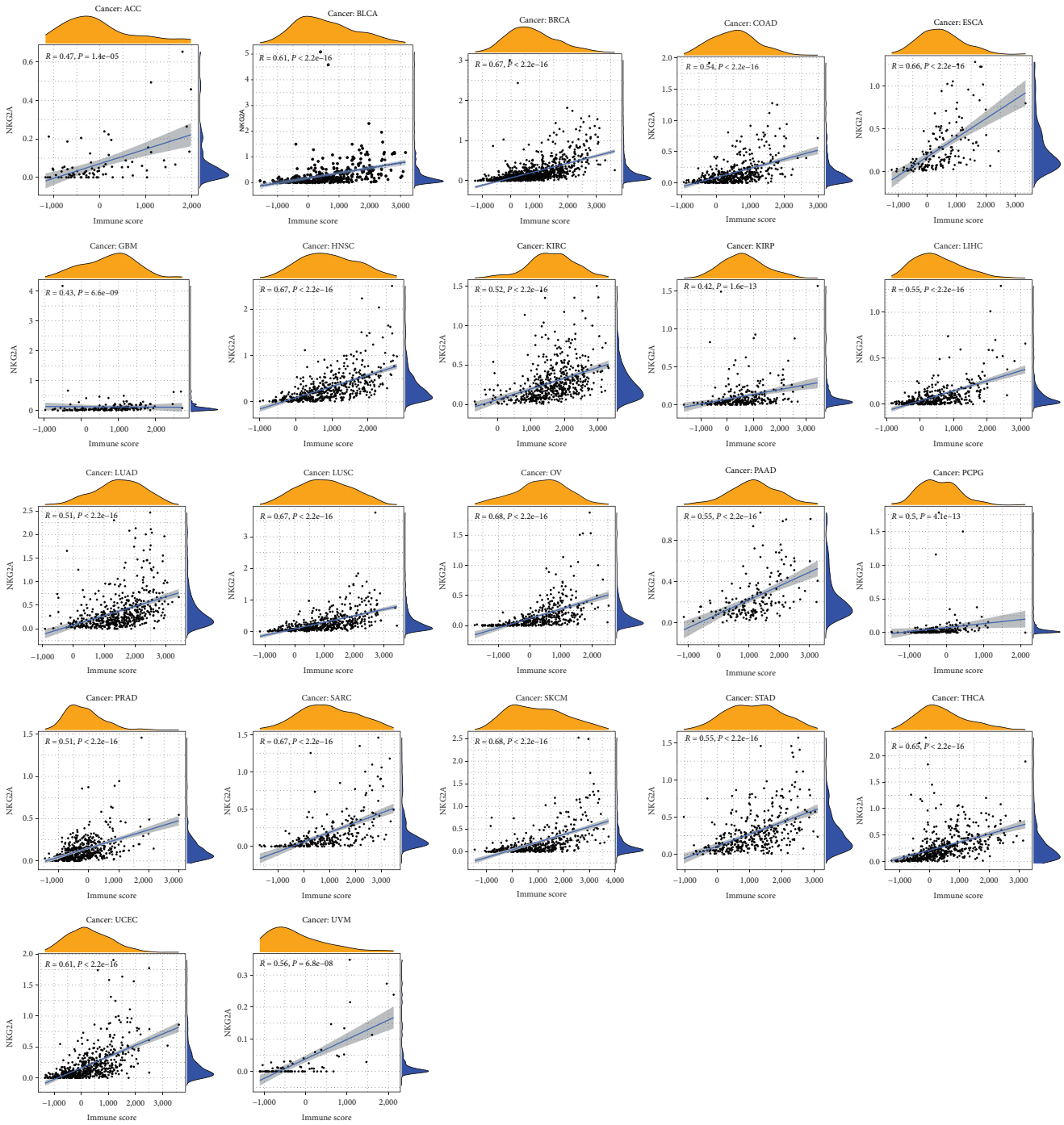
DNA methylation is an important type of epigenetic modification in tumors and its altered homeostasis is an important factor influencing tumor progression. We investigated the correlation between NKG2A and DNMT gene expression in different types of cancers. We found that the DNMT gene was significantly positively correlated with NKG2A expression in 12 out of 33 cancers. In contrast, NKG2A was significantly negatively correlated with DNMT gene expression in 10 cancers, including CESC, LUSC, and MESO (Figure 9(c)). MMR is a way to repair DNA damage, and when the function of its important components is abnormal, it will lead to DNA replication errors, which in turn will promote mutations in tumor cells. We analyzed the expression of NKG2A compared to the levels of MMR mutations in pan-cancers and showed that NKG2A expression in pan-cancers was associated with most MMR mutations. In 14 cancers, NKG2A was positively associated with at least one MMR gene expression, while it was negatively associated with MMR gene expression in eight cancers (Figure 9(d)).

*3.7. Enrichment Analysis of NKG2A-Related GO and KEGG Pathways in Pan-Cancer.* We performed GO functional annotation and KEGG pathway enrichment analysis of the NKG2A gene in TCGA cancers and selected cancer types with correlation between NKG2A and prognosis in the survival analysis step as observations. The results of GO functional analysis showed that (i) in BRCA, NKG2A positively regulates adaptive immune responses, antigen receptor-mediated signaling pathways, B-cell activation, B-cell mediated immunity; (ii) in KIRC, NKG2A positively regulates the production of T-cell receptor complexes; (iii) NKG2A is involved in the positive regulation of adaptive immune responses, humoral immune responses, and leukocyte adhesion in PCPG; (iv) NKG2A positively regulates cell surface receptor signaling pathways and leukocyte migration in SKCM; and (v) in UCEC, NKG2A positively regulates antigen binding, complement activation, and other immune functions (Figure 10(a)–10(e)). In contrast, KEGG pathway enrichment analysis revealed that (i) NKG2A positively regulates the interaction of cytokine receptors with the RNA degradation pathway in ACC; (ii) NKG2A positively regulates primary immunodeficiency in BLCA; (iii) NKG2A positively regulates antigen processing and presentation pathways and natural killer cell-mediated cytotoxicity in BRCA; (iv) NKG2A positively regulates the B-cell receptor signaling pathway in PCPG; and (v) NKG2A positively regulates NK cell-mediated cytotoxicity in SKCM (Figures 10(f) and 10(g)).

*3.8. Expression Validation of NKG2A.* Differences in expression of NG2A between normal gastric mucosal cells (GES-1) and gastric cancer cells (HGC-27, MGC-803, MKN-45), normal colonic epithelial cells (NCM460) and colon cancer cells (SW450, SW620, HCT116), normal hepatocytes (LO-2), and hepatoma cells (SMMC-7721, HUh7, H-97) were verified (Figure 11). The results showed that NKG2A expression in colon cancer cells (Figure 10(a)), gastric cancer cells (Figure 11(b)), and liver cancer cells (Figure 11(c)) was significantly lower than in normal cells. This is consistent with the results of bioinformatics analysis ($P<0.05$). Tumorigenesis is caused by a cascade of various factors, so genes are selectively and differently expressed under different growth backgrounds and conditions.

## 4. Discussion

Based on expression analysis of the TCGA and GTEX databases, we found that the expression profile of NKG2A at the transcriptional level differs among different cancer types, suggesting that NKG2A may have different mechanisms and functions in cancer. Our PCR results were consistent with the bioassay results that NKG2A was expressed at low levels in gastric, hepatocellular, and colorectal cancer cells. Indeed, NKG2A is mainly highly expressed in NK and $CD8^{+}$ T cells at the site of tumor infiltration and plays a regulatory role in the TME. NKG2A is an inhibitory receptor for NK cells and its ligand HLE-A is widely overexpressed in a variety of cancers, and the combination of the two greatly limits the effector function of tumor infiltrating lymphocytes (TIL) [25]. Therefore, the mechanisms underlying the bidirectional effects of NKG2A expression on cancer in different cancers need to be further explored. We analyzed the genetic alterations of NKG2A using the cBioPortal database and showed that the mutation rate of NKG2A in testicular germ cell tumors was more than 6%, and the most common type of mutation of NKG2A in pan-cancer was amplification,




(a)

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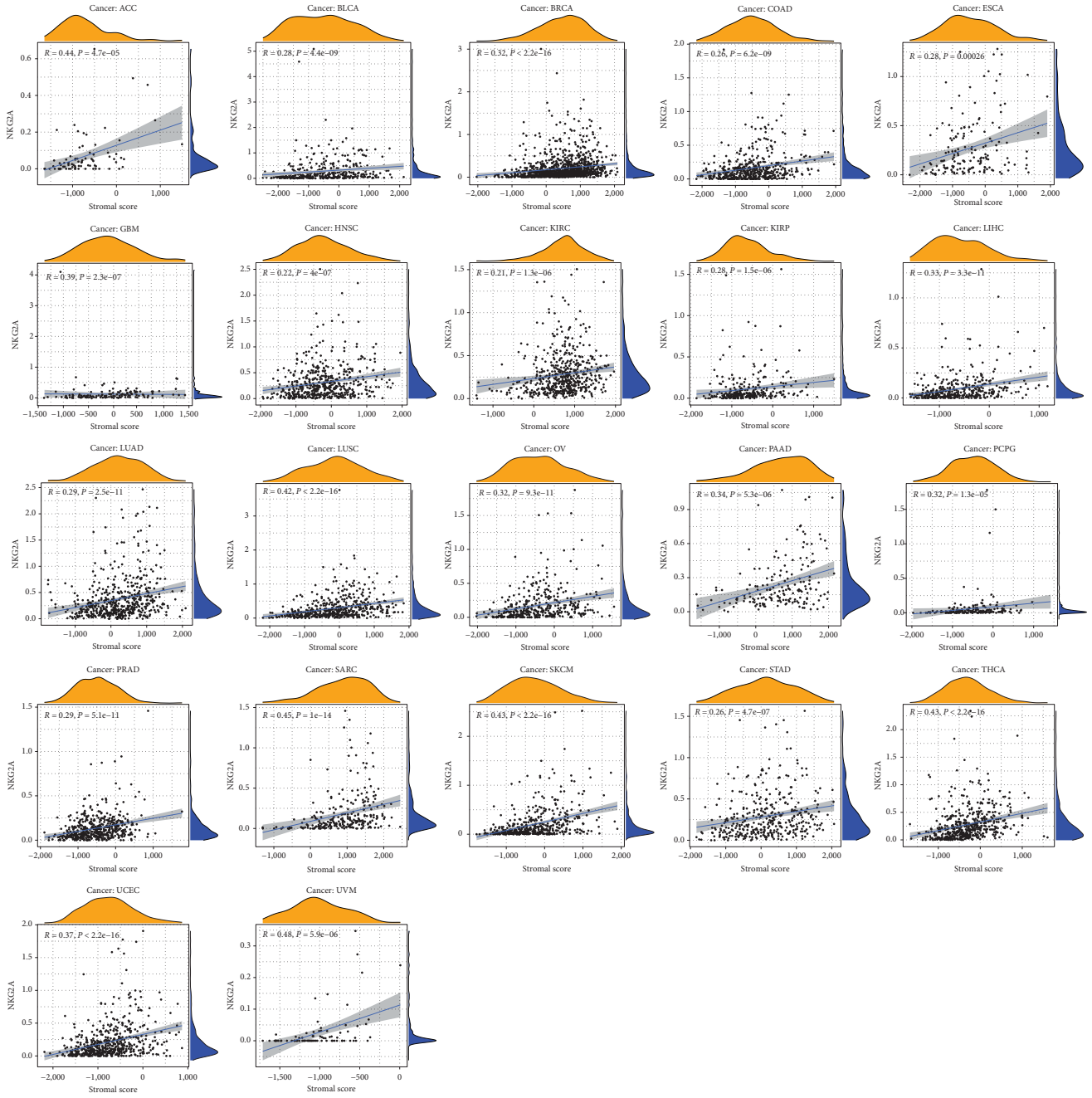
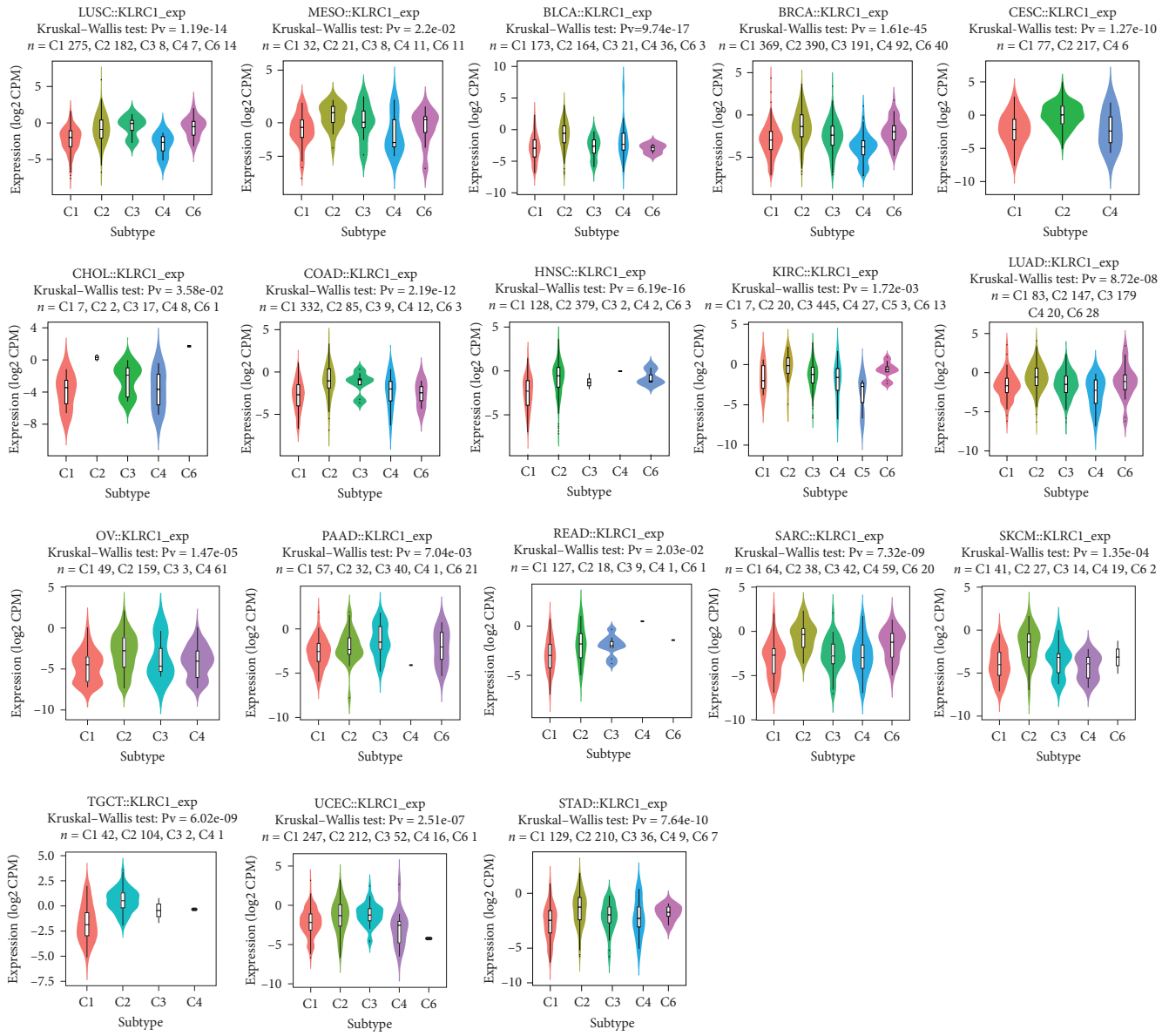



(b)

Figure 7: Correlation of NKG2A gene expression with stromal score and immune score in pan-cancer. Gene expression has a significantly correlation with the stromal score (a) and immune score (b) in ACC, BLCA, BRCA, COAD, ESCA, GBM, HNSC, KIRC, KIRP, LIHC, LUAD, LUSC, OV, PAAD, PCPG, PRAD, SARC, SKCM, STAD, THCA, UCEC, and UVM.

suggesting that some tumorigenesis was associated with mutations in NKG2A.

In the analysis of the prognostic relevance of NKG2A to patients with different cancers, the impact of NKG2A on the survival of cancer patients was assessed in four main aspects, namely OS, DSS, DFI, and PFI [26]. Survival analysis showed that the mRNA expression level of NKG2A was downregulated in PCPG, UCEC, ACC, COAD, KIRC, BRCA, LIHC, READ, and LUSC, which functioned as a tumor suppressor gene to protect the prognosis of patients. In contrast, high expression of NKG2A predicted a poor prognosis for patients with THYM, HNSC, KIRP, THCA, and PAAD. Notably, in combination with the cancer expression profile analysis of NKG2A, high expression of NKG2A in LGG, CESC, SKCM, and OV also played a protective prognostic role for patients, while low expression in THCA played a procancer role. This may be due to the fact that the expression level of NKG2A varies in different cancers, and when it is mutated or missing

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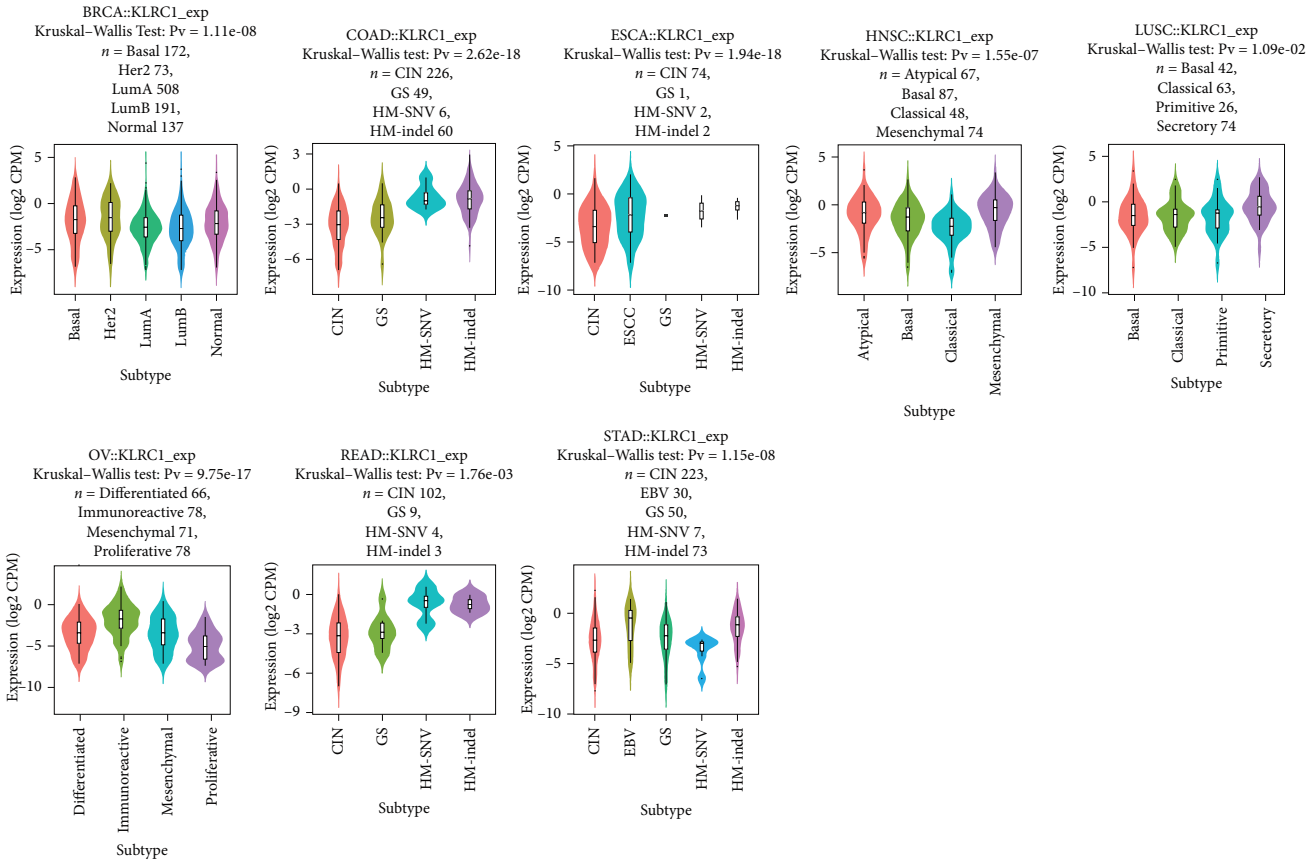



(b)

Figure 8: Correlation of NKG2A expression with immune and molecular subtypes of pan-cancer. (a) Correlation of NKG2A expression with immune subtypes of LUSC, MESO, BLCA, BRCA, CESC, CHOL, COAD, HNSC, KIRC, LUAD, OV, PAAD, READ, SARC, SKCM, TCGT, UCEC, and STAD. (b) Correlation of NKG2A expression with molecular subtypes in BRCA, COAD, ESCA, HNSC, LUSC, OV, READ, and STAD. C1: wound healing, C2: IFN-$\gamma$ dominant, C3: inflammatory, C4: lymphocyte depleted, C5: immune quiet, and C6: TGF-b dominant.

or influenced by genetic environmental factors, the cancer-promoting effect of NKG2A is silenced or upregulated. Therefore, more NKG2A studies are needed to gain insight into the specific mechanisms of this phenomenon and to develop more precise therapeutic strategies to target different types of tumors. A study from Sun et al. [22] showed that NKG2A was highly expressed in tumor-infiltrating NK cells and was associated with poor prognosis in hepatocellular carcinoma. ROC curves showed that NKG2A was a valid predictive markers of prognosis for CESC, KIRC, LUAD, LUSC, PAAD, PRAD, SKCM, UCS, and LAML. In conclusion, survival analysis and ROC curve analysis indicated that NKG2A is a potential prognostic biomarker for a variety of cancers.

The relevance of NKG2A to the TME in pan-cancer was an important finding of this study. Expression of NKG2A was highly positively correlated with infiltration of six immune cell types in 31 cancer TMEs, including $CD8^+$ T cells, neutrophils, $CD4^+$ T cells, myeloid dendritic cells, macrophages, and B cells. Through previous studies, we are familiar with the fact that NKG2A is a major regulator of tumor-infiltrating NK cells and is widely expressed in NK cells, one of the immunoregulatory pathways being the NGK2A/HLA-E axis [27]. Notably, HLE-A expression is upregulated in most cancers, NKG2A binds to HLE-A in the presence of certain peptides and allows tumor cells to evade immune recognition by NK and $CD8^+$ T cells, and the presence of HLE-A reduces the killing activity of NK cells [28, 29]. Therefore, blocking the binding of NKG2A to its ligand HLE-A is the main value of NKG2A as an immune target. However, due to the uncertainty in the presence of the peptide, this phenomenon does not seem to be invariant in different types of cancer and the mechanism of action of this variability is complex and needs to be further explored. Coexpression analysis of NKG2A with immune-related cells in TME showed that in most cancers, NKG2A was significantly associated with immune cells such as T cells, B cells, and NK cells. Indeed, NKG2A is able to be selectively expressed on cytotoxic lymphocytes, including NK cells and $CD8^+$ T cells, and T cells expressing NKG2A preferentially reside in the TME [30]. NKG2A is endowed with immunomodulatory capacity in cancer, and its aberrant expression may influence the prognosis of different tumor types by regulating immune cell infiltration in the TME. The success of immune checkpoint blockade therapies has opened new doors for cancer treatment and NKG2A is gaining attention as a new gene in this field. Monalizumab is a humanized anti-NKG2A antibody that enhances the killing

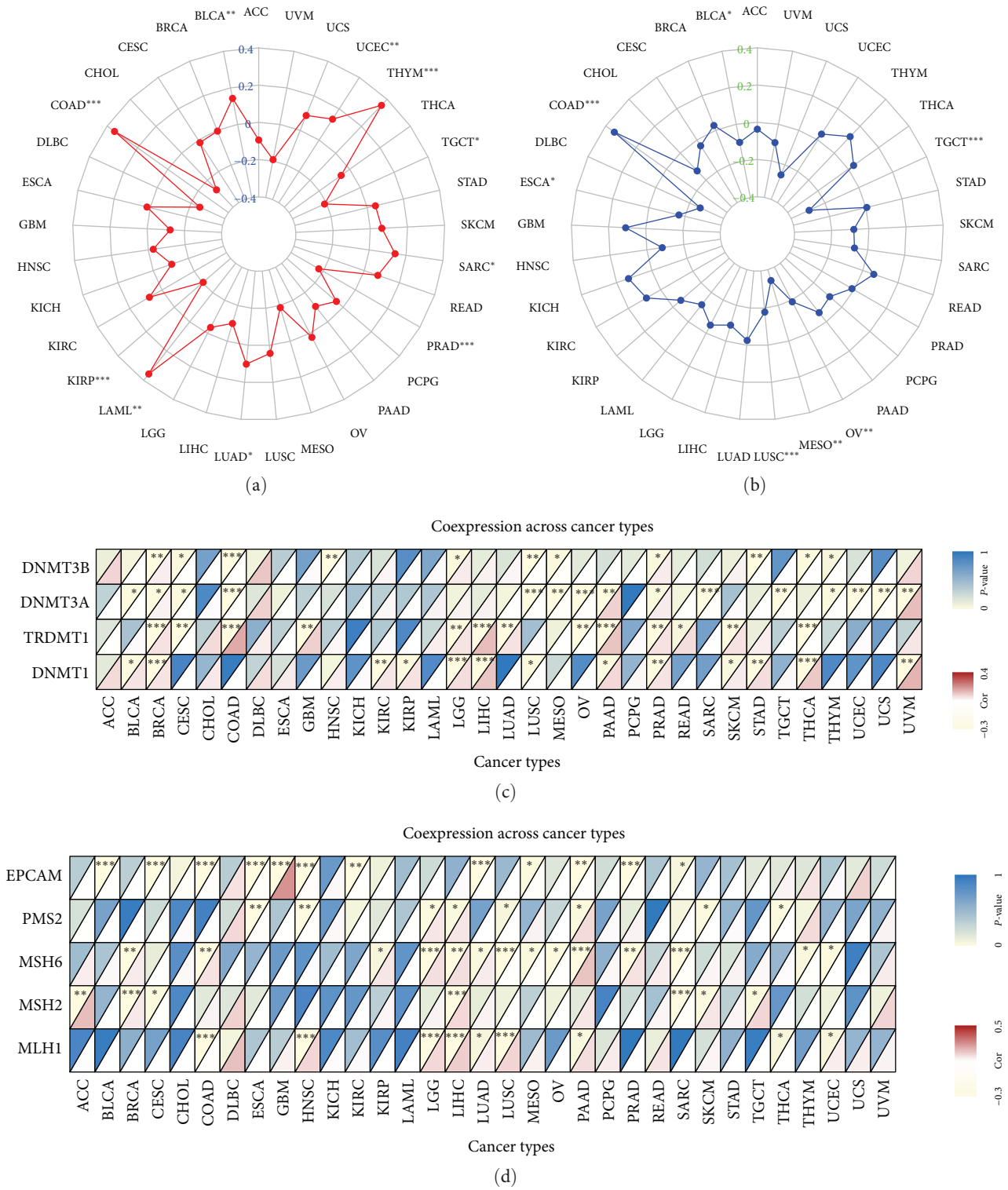
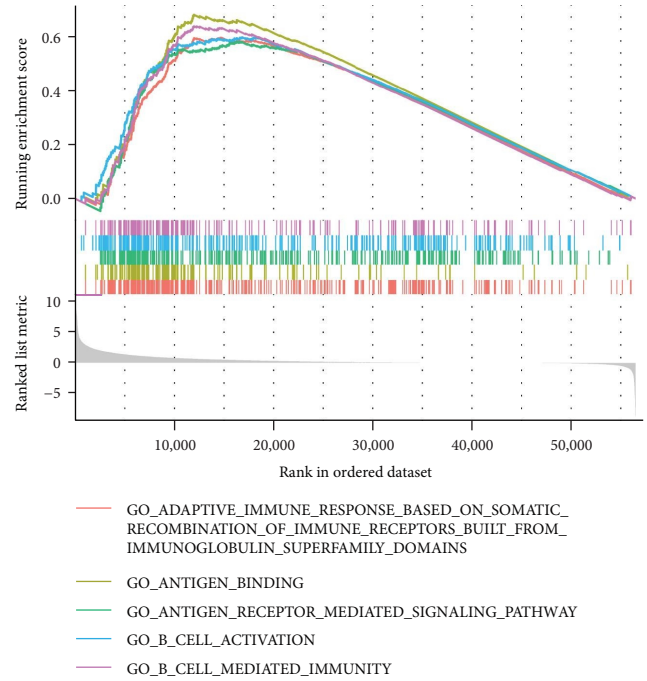



Figure 9: Association of NKG2A gene expression with TMB, MSI, and MMR gene mutation levels and DNA methyltransferase expression in pan-cancer. (a) The radar diagram demonstrated the relationship between the expression of the TMB and NKG2A genes in various malignancies. The correlation coefficient is shown by the red curve, and the range is shown by the blue value ($^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$). (b) The radar diagram showed how the expression of the MSI and NKG2A genes relates to various malignancies. The correlation coefficient is shown by the blue curve, and the range is shown by the green value ($^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$). (c) DNA methyltransferase (DNMT1, DNMT2, DNMT3A, and DNMT3B) expression was correlated with NKG2A expression ($^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$). (d) The association of NKG2A with MMR (MLH1, MSH2, MSH6, PMS2, EPCAM) gene expression in pan-cancer ($^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$).

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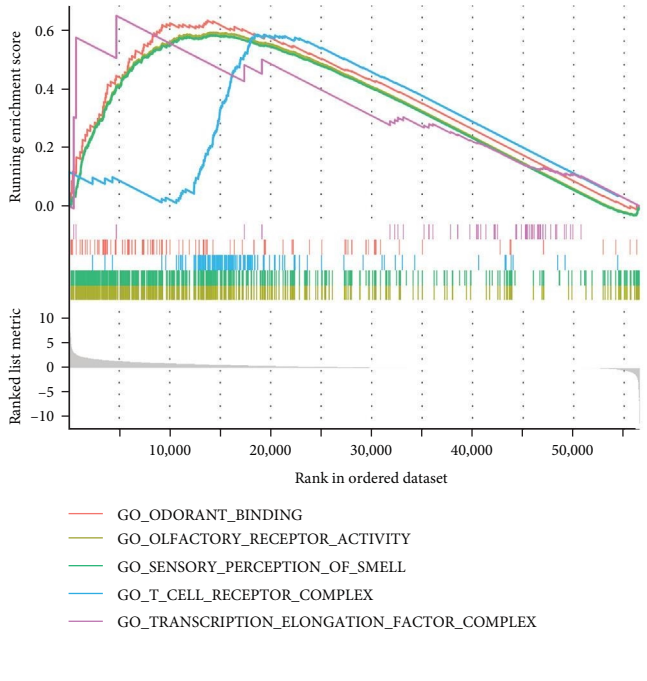


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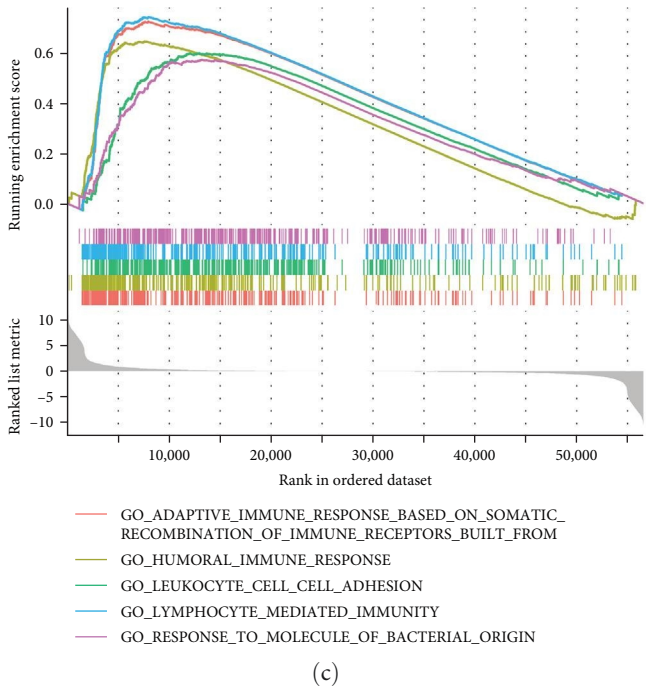


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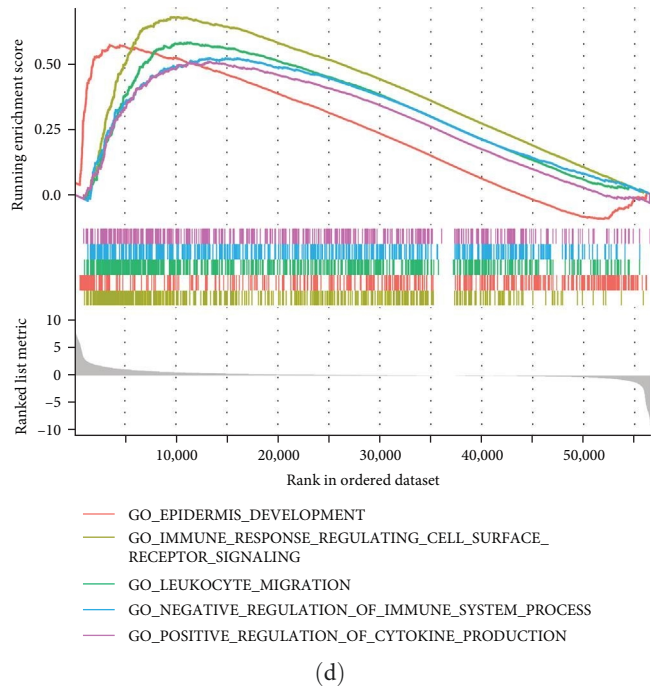


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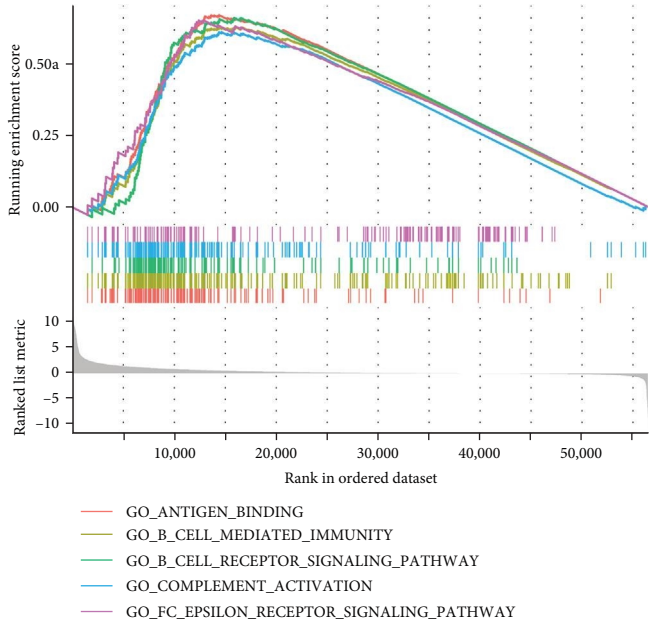


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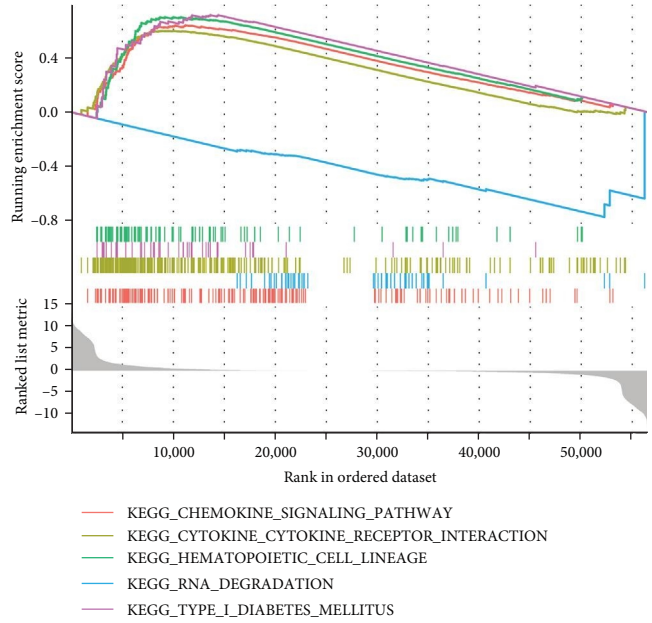


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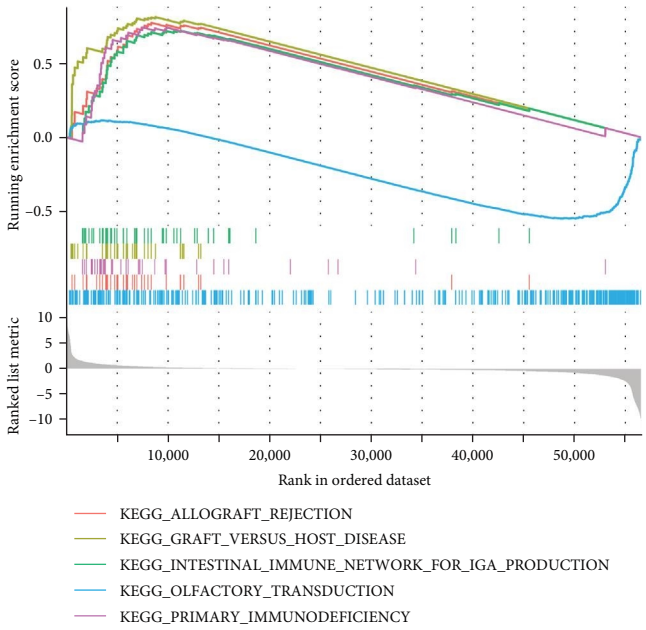


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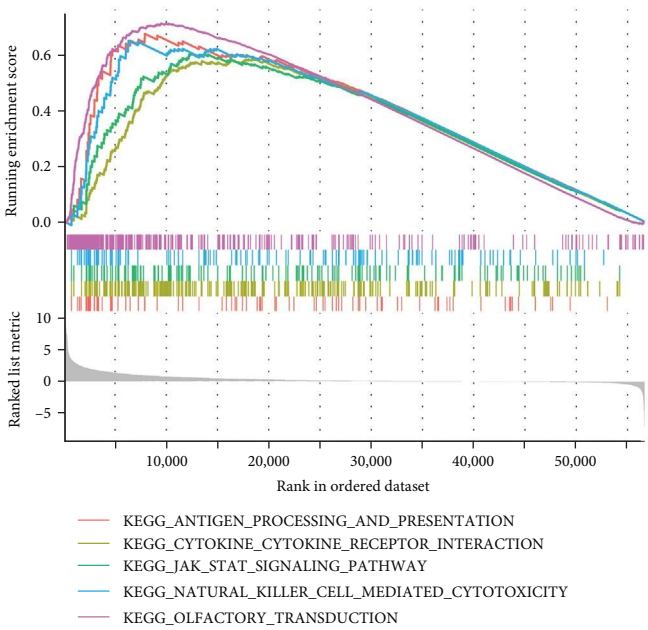


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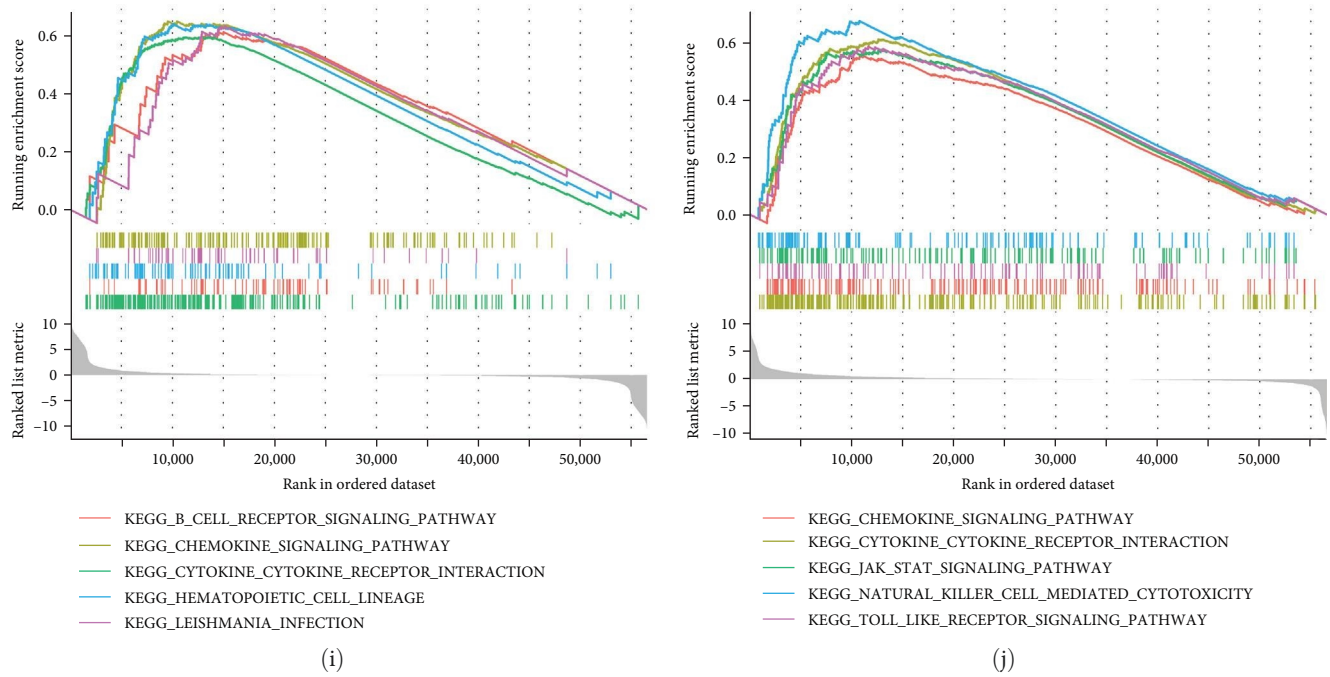


Figure 10: Enrichment analysis of NKG2A with GO functional annotation and KEGG pathway in prognosis-related cancers. (a–e) Functional annotation of GO for NKG2A in prognosis-related cancers. (f–j) Analysis of the KEGG pathway of NKG2A in prognosis-related cancers.

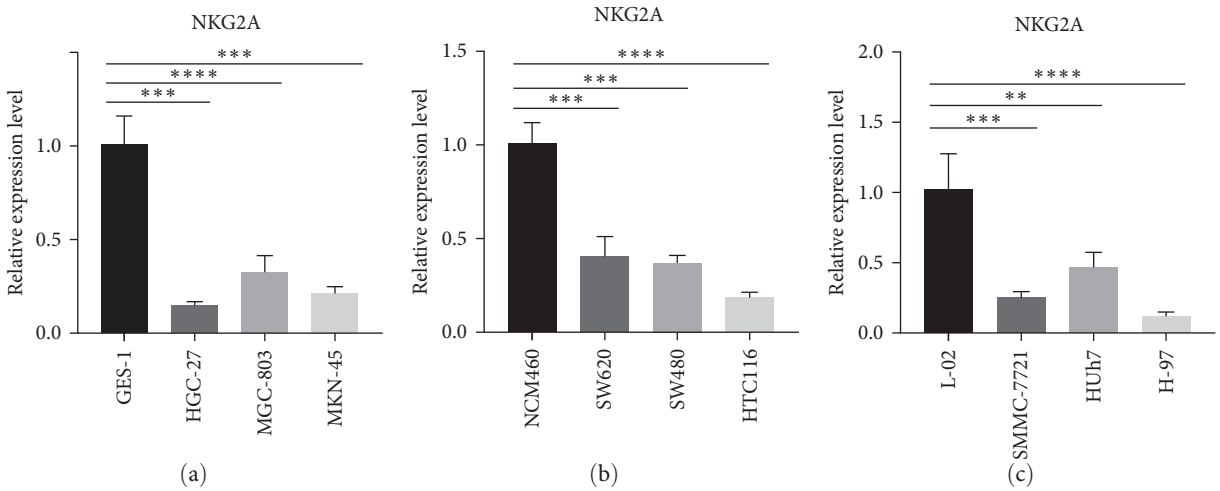


Figure 11: Results of NKG2A expression validation. (a) NKG2A expression in human normal gastric mucosal cell line (GES-1) and human gastric cancer cell lines (HGC-27, MGC-803, MKN-45). (b) NKG2A expression in human normal colonic epithelial cell line (NCM460) and human colon cancer cell lines (SW620, SW480, HCT116). (c) NKG2A expression in human normal hepatocyte line (L-O2) and human hepatoma cell lines (SMMC-7721, HUh7, H-97). $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

effect of NK cells and $CD8^+$ T cells on various tumor cells [20]. We used R software to visualize the relationship between known ICP genes and NKG2A expression in pan-cancer. We found significant positive correlations between NKG2A and multiple ICP genes in almost all cancer types, including PDCD1/PD-L1, CD274, TIGIT, TMIGD2, LAG3, CD244, BTLA, HAVCR2, KIR3DL1, and others. A trial derived from the efficacy of ICI in treating colon cancer showed that the combination of monalizumab with duvalumab (anti-PD-L1) refined innate and adaptive immunity and repaired antitumor immune function, showing higher efficacy and stability compared to PD-1/PD-L1-based monotherapy [31]. Yan et al. [32] reported that NKG2A and PD-L1 coexpression was associated with a higher immune effect on the survival of immunologically active cells in the tumor microenvironment, suggesting that the emergence of anti-NKG2A will probably compensate for the first generation of cancer immunotherapies. Interestingly, ICP genes such as LAG3, TIGIT, and BLTA have been shown to be expressed on NK cells, and these inhibitory receptors possess a role in regulating NK cell function in tumors, suggesting a corresponding link between NKG2A and their role in cancer immunotherapy

[33]. In addition, a higher immune or stromal fraction of the tumor in TME indicates a higher immune or stromal component. There was a strong positive correlation between NKG2A expression and stromal/immune score in numerous cancers such as ACC, BLCA, BRCA, COAD, and LUAD. In conclusion, our study found that NKG2A affects the immune microenvironment of tumors in an immune-dependent manner. Although tissue and cellular heterogeneity resulted in different NKG2A immunoassay results in different cancers, there is good reason to believe that NKG2A possesses an immunomodulatory role in cancer and that antagonizing NKG2A expression exerts an immune checkpoint suppressive effect.

Because of the large heterogeneity between different cancers, the existence of immune and molecular subtypes can deepen understanding and thinking about cancer. Thorsson et al. [34] classified all samples from 33 cancer types into six immune subtypes, namely wound healing (C1), IFN-g dominant (C2), inflammation (C3), lymphocyte depletion (C4), immune calm (C5) and TGF-b dominant (C6). Our study showed that NKG2A expression in most cancers is dominated by the C2 immune subtype, i.e., IFN dominant. It has been shown that the liver contains a large number of poorly functioning NK cells and that NKG2A is highly expressed in these hepatic NK cells and that they exhibit an inhibitory IFN $\gamma$ response to IL-12/IL-18 stimulation [35].

We investigated the association between NKG2A and TMB, MSI, MMR genes, and DNMTs to further explore the possible mechanisms of association between NKG2A and tumors. The amount of nonsynonymous variants within the genomic region of somatic cells in 33 malignancies is known as the tumor mutational load (TMB) of pan-cancer [36]. Alterations in microsatellite sequence length in tumors due to insertions or deletions of mutations during DNA replication are known as microsatellite instability (MSI), which is mainly caused by defects in mismatch repair (MMR) function [37]. Chalmers et al. [38] showed that tumor samples with high MSI usually also have a high TMB phenotype. TMB and MSI have gradually entered clinical applications as potential markers for predicting immune checkpoint inhibitors. Some studies have reported higher levels of TMB and MSI in gastrointestinal tumors predominantly gastric adenocarcinoma [39]. Our results showed that NKG2A was significantly correlated with TMB levels in UCEC, THYM, SARC, LUAD, LAML, COAD, BLCA, TGCT, PRAD, and KIRP, and the expression levels of NKG2A in BLCA, TGCT, OV, MESO, LUSC, ESCA, and COAD were significantly correlated with MSI. This implies that NKG2A expression can regulate the levels of TMB and MSI in cancer and affect the efficacy response of patients to immune checkpoint therapy. By the inconsistent expression of NKG2A in TMB and MSI in same cancer, it may be attributed to the characteristics and differences used in the dataset. MMR genes include MLH1/PMS2, MSH2/MSH6, and EPCAM, which function to correct base insertions, substitutions, deletions, or mismatches that occur during DNA replication, thus ensuring DNA stability [40]. The results of the analysis revealed that the expression of NKG2A was closely associated with the levels of five MMR genes in LGG, LIHC, PAAD, LIHC, BRAC, and GBM. DNA methylation is a common modification of epigenetics and its altered status is also an important factor driving cancer progression [41]. In the present study, we found that NKG2A expression was significantly associated with the four DNA methyltransferases DNMT3B, DNMT3A, TRDMT1, and DNMT1, especially in cancers such as BRCA, LGG, LIHC, PAAD, PRAD, SKCM, CESC, COAD, LUSC, MESO, and THYM. Taken together, alterations in immune cells such as cytotoxic T cells and NK cells in malignancies may be caused by aberrant NKG2A expression and epigenetic alterations. Furthermore, GO functional annotation and KEGG pathway analysis revealed that NKG2A expression in pan-cancer can drive several immune-related functions and pathways. Such as adaptive immune responses, B-cell activation, B-cell-mediated immunity, regulatory lymphocyte activation, leukocyte adhesion, humoral immune responses, T-cell receptor complex production, lymphocyte differentiation, natural killer cell-mediated immunity, negative regulation of immune system processes and natural killer cell-mediated cytotoxic expression pathways, and T/B cell receptor signaling pathways. Meanwhile, these immune-related functions or pathways were correlated with the prognosis of certain cancers, indicating that NKG2A can influence the prognosis of cancer patients by regulating some immune functions and pathways, which further suggests that NKG2A is a multifunctional immune-dependent factor.

## 5. Conclusion

Overall, we explore the cancer expression profile of NKG2A based on bioinformatics methods, describe the role of NKG2A in pan-cancer development, and discuss the potential of NKG2A as a novel cancer prognostic marker and its role in pan-cancer immune regulation. However, the study had some limitations. First, due to limited conditions, we only used PCR to verify the expression of NKG2A in three cancer types and nine cancer cells; second, we failed to explore the specific immunomodulatory mechanisms of NKG2A in different types of cancer. Therefore, the subsequent results of this study need to be verified by a large number of samples and more relevant experiments. This study contributes to the understanding of the potential carcinogenic effects of NKG2A from multiple perspectives.

## Data Availability

The data generated and analyzed during the current study are available in the TCGA Research Network (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga), GTEx (http://commonfund.nih.gov/GTEx/), cBioPortal (https://www.cbioportal.org/), and Kaplan–Meier plotter (https://kmplot.com).

## Ethical Approval

Databases such as TCGA and GEO are public databases. The patients involved in the database have obtained ethical approval. Users can download relevant data for free for research and publish relevant articles. Our study is based

on open source data, so there are no ethical issues and other conflicts of interest.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

YR, YW, and HC conceived and designed the study. YY reviewed and directed the revision of the manuscript. YR wrote drafts and revised manuscripts. YW completed the method part and made all the numbers and tables. MT completed the experimental part and participated in the revision of the manuscript. GZ, FD, GM, and ZW collected and summarized relevant literature, as well as some data analysis. SL and XZ completed the data analysis. All authors read and approved the final manuscript.

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