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Comprehensive analysis of PILRA's association with the prognosis, tumor immune infiltration, and immunotherapy in pan-cancer

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Paired immunoglobulin-like type 2 receptor alpha (PILRA) plays a vital role in regulating broad immune responses. However, the roles of PILRA in cancer immunity remain unexplored yet. In the current study, we comprehensively analyzed the oncogenic and immunologic roles of PILRA at a pan-cancer level based on the Cancer Genome Atlas and Gene Expression Omnibus datasets. PILRA was significantly dysregulated and frequently mutated in pan-cancer. Its expression and mutation status significantly impacted patient prognosis in several cancers. Besides, PILRA expression was positively correlated with ESTIMATE scores and the abundances of tumor-infiltrating immune cells. Concurrently, PILRA expression was significantly associated with predictive biomarkers of cancer immunotherapy, and positively correlated with the prognostic outcomes of cancer patients receiving immunotherapy. Mechanistically, enrichment analysis implied that PILRA might be involved in the regulation of immune response and metabolic process. This study uncovered the immunological roles of PILRA in cancers and its potential as a novel biomarker and therapeutic target for cancer immunotherapy.

Abbreviations

PILRA	Paired immunoglobulin-like type 2 receptor alpha
ITIMs	Immunoreceptor tyrosine-based inhibition motifs
BRCA	Breast invasive carcinoma
CHOL	Cholangiocarcinoma
ESCA	Esophageal carcinoma
GBM	Glioblastoma multiforme
HNSC	Head and neck squamous cell carcinoma
KIRC	Kidney renal clear cell carcinoma
KIRP	Kidney renal papillary cell carcinoma
STAD	Stomach adenocarcinoma
THCA	Thyroid carcinoma
UCEC	Uterine corpus endometrial carcinoma
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
PAAD	Pancreatic adenocarcinoma
LAML	Acute myeloid leukemia
DLBC	Diffuse large B-cell lymphoma
THYM	Thymoma
HCC	Hepatic cell carcinoma
OS	Overall survival
DSS	Disease specific survival
GBMLGG	Glioma
LGG	Brain lower grade glioma
TGCT	Testicular germ cell tumors
SKCM	Skin cutaneous melanoma

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CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma
UVM	Uveal melanoma
OV	Ovarian serous cystadenocarcinoma
SARC	Sarcoma
ACC	Adrenocortical carcinoma
UCS	Uterine carcinosarcoma
READ	Rectal cancer
COADREAD	Colon adenocarcinoma/rectum adenocarcinoma esophageal carcinoma
COAD	Colon adenocarcinoma
ICGs	Immune check point genes
MMR	Mismatch repair
TMB	Tumor mutation burden
MSI	Microsatellite instability
NEO	Neoantigen
NF1	Neurofibromatosis 1

Cancer remains to be an insurmountable challenge that threatens human life in spite of the rapid advancement of cancer therapies¹. As compared to chemotherapy and targeted therapy to which drug resistance is inevitable, immunotherapy is emerging as a novel therapeutic approach with considerable potential in effectively relieving and even curing previously untreatable malignancies^{2,3}. However, a substantial proportion of cancer patients still respond poorly to immunotherapy^{4,5}. Of note, tumors undergo genetic alterations to elude the immune systems, largely undermining therapeutic efficacy⁶. Therefore, clarifying the molecular determinants that govern the immune evasion of cancers is important to optimize therapeutic outcomes.

The inhibitory receptor paired immunoglobulin-like type 2 receptor alpha (PILRA), along with the activating receptor PILR β belongs to the PILR family⁷. Bearing two immunoreceptor tyrosine-based inhibition motifs (ITIMs) in its cytoplasmic domain, PILRA functions to transmit inhibitory signals by recruiting SHP-1 and SHP-2, upon recognition of ligands such as CD99, neuronal differentiation proliferation factor-1 and CD8A, etc^{8,9}. PILRA, which is mainly expressed on immune cells, including macrophages, dendritic cells and granulocytes, is related to regulation of broad immune responses^{10,11}. For example, it has been reported that PILRA negatively regulates neutrophil infiltration via modulation of integrin activation during inflammation¹². Besides, PILRA controls monocyte mobility through regulating integrin signaling and inhibiting CD99–CD99 binding¹³. Furthermore, Zheng L and colleagues lately found that PILRA could interact with CD8A and keep CD8 T cell in a quiescent state, thus maintaining the T cell pool size in the absence of antigen exposure¹⁴. However, the involvement of PILRA in anticancer immunity remains poorly understood. Thus, elucidation of the immunologic roles of PILRA in cancers might offer a novel perspective for cancer immunotherapy.

In the present study, we sought to explore the expression profiles, and the prognostic and diagnostic value of PILRA in pan-cancer by analyzing the multi-omics data sourced from multiple online databases, and immunohistochemistry analysis was performed to verify its expression status in non-small cell lung cancer (NSCLC). Furthermore, we conducted an integrative bioinformatics analysis on the association of PILRA with tumor immune landscape and tumor immunotherapy. Finally, we investigated the functional mechanisms underlying the oncogenic and immunologic roles of PILRA across various cancers. Our study offers a comprehensive insight into the prognostic and immunological roles of PILRA across various cancers, and might facilitate the discovery of novel biomarkers and genetic drivers regarding cancer immunity.

Results

The aberrant expression profiles of PILRA in pan-cancer. To evaluate the differential expression of PILRA between tumorous and normal tissues, we investigated the expression level of PILRA mRNA using TIMER2.0 database. According to the results, PILRA expression was significantly upregulated in breast invasive carcinoma (BRCA), cholangiocarcinoma (CHOL), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), stomach adenocarcinoma (STAD), thyroid carcinoma (THCA) and uterine corpus endometrial carcinoma (UCEC) while downregulated in only three types of cancer including lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC) and pancreatic adenocarcinoma (PAAD) as opposed to their normal tissues (Fig. 1A). Of note, the number of TCGA normal samples was relatively small for convincing statistical analysis, and several cancers are even lack of matched normal tissues. Therefore, we performed a complementary analysis by comparing the expression level of PILRA in tumor tissues with that in the GTEx normal tissues. According to the results, PILRA was differentially expressed in most of the cancers analyzed (Fig. 1B). Besides, we further confirmed that the PILRA protein expression was significantly elevated in breast cancer, PAAD, KIRC, and GBM, while decreased in LUAD and hepatic cell carcinoma (HCC) as compared to their corresponding normal tissues (see Supplementary Fig. S1 online). To verify the decreased expression of PILRA in NSCLC, immunohistochemistry (IHC) staining was performed in tumor and adjacent normal tissues, and the results confirmed that PILRA was significantly downregulated in NSCLC (Fig. 1C). Collectively, these data imply that PILRA gene is differentially expressed in multiple cancers as compared to normal counterparts.

Correlation of PILRA expression with patients' prognosis and diagnosis. To evaluate the prognostic value of PILRA in pan-cancer, the relationship between PILRA expression and patients' clinical outcomes was assessed by performing univariate cox regression analysis of TCGA data. As shown in Fig. 2A, B, high expression of PILRA was inversely correlated with both overall survival (OS) and disease specific survival

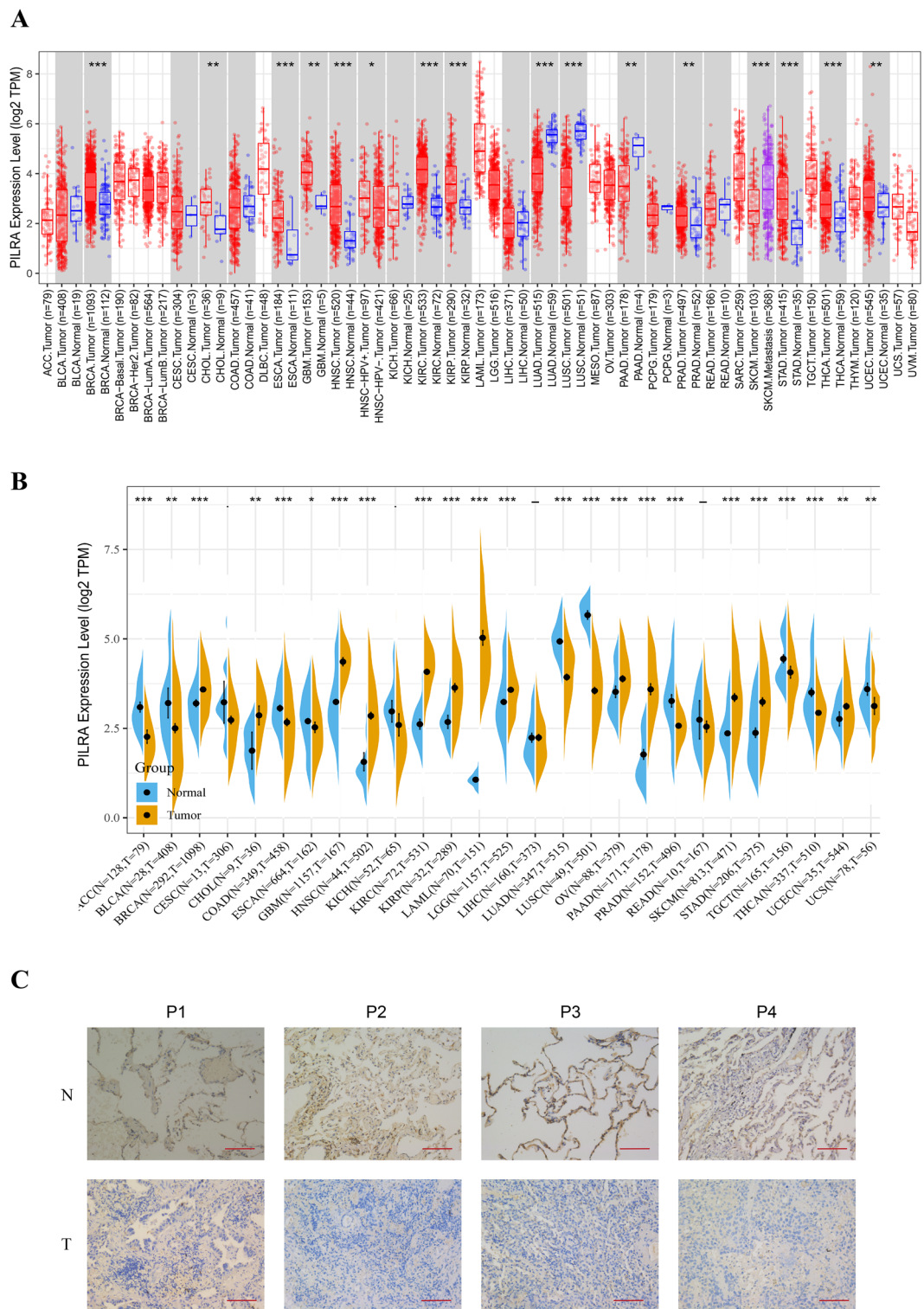


Figure 1. Analyses on the differential expression of PILRA in pan-cancer. **(A)** The differences of PILRA mRNA expression between tumor and normal tissues in 33 cancer types were analyzed by TIMER2.0 online tool based on TCGA datasets. Distributions of gene expression levels were displayed using box plots. Red and blue color represent cancerous and normal tissues, respectively. SKCM metastasis tissues are marked in purple. **(B)** The violin plots were plotted to compare PILRA expression in tumor tissues from TCGA datasets with that in normal tissues from GTEx datasets. **(C)** PILRA protein levels in tumor and corresponding normal tissues of NSCLC patients were evaluated by IHC (Scale bars, 100 μ m). *, **, and *** correspond to $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. $P < 0.05$ was considered significant.

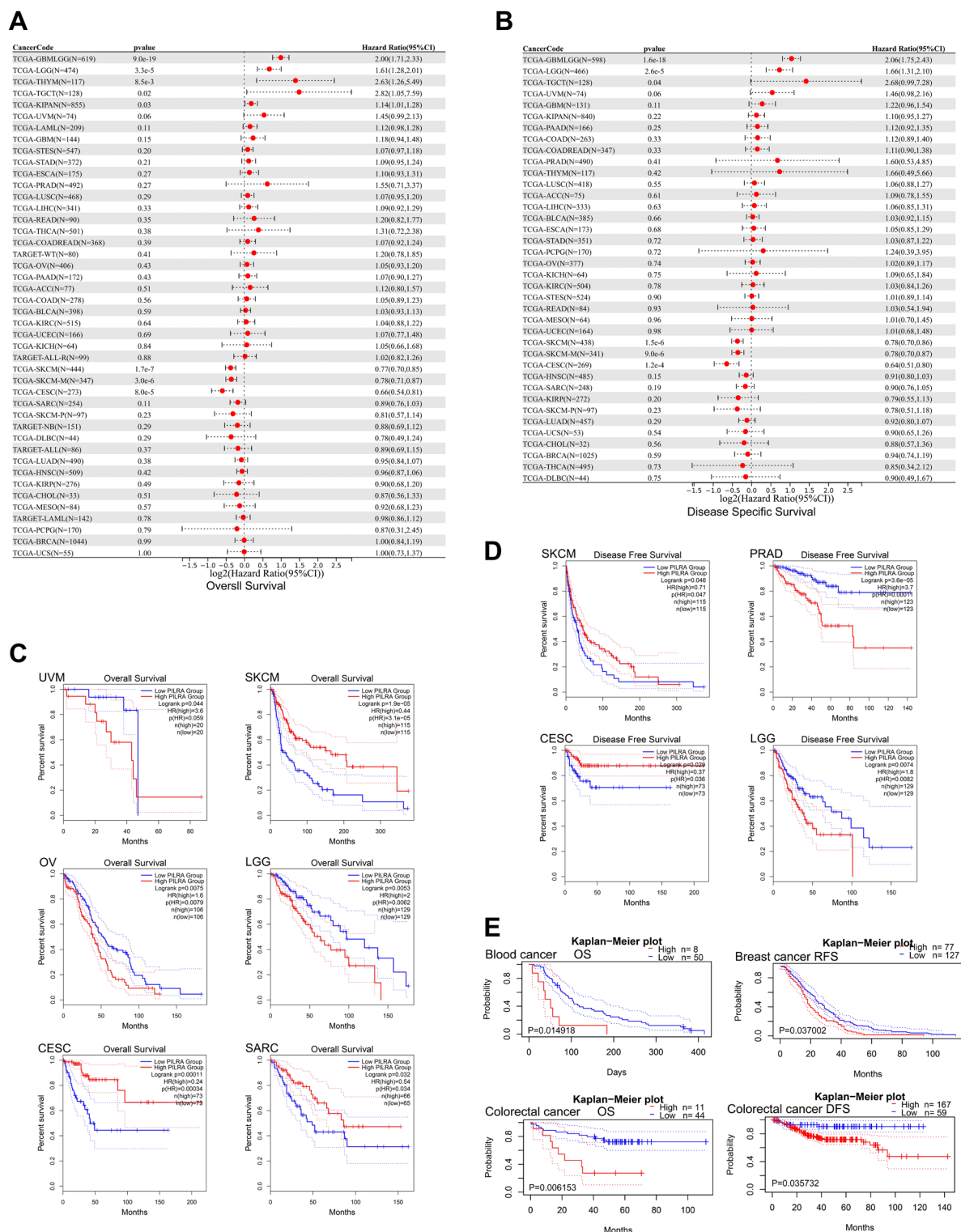


Figure 2. Survival analysis according to the high and low expression of PILRA in pan-cancer. (A,B) The forest plots showing the relevance of PILRA expression with the overall survival (A) and disease specific survival (B) of different cancers in the TCGA cohort. (C,D) Kaplan-Meier curves for the prediction of patients' overall survival of UVM, SKCM, OV, LGG, CESC and SARC (C), and disease-free survival of SKCM, PRAD, CESC and LGG (D) based on PILRA expression via GEPIA2.0. (E) Kaplan-Meier plots showing the association between PILRA expression and the survival of Blood, breast and colorectal cancer based on PILRA expression using Prognoscan websites. $P < 0.05$ was considered statistically significant.

(DSS) in glioma (GBMLGG), brain lower grade glioma (LGG) and testicular germ cell tumors (TGCT) whereas significantly positive correlation was observed in skin cutaneous melanoma (SKCM) and cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC). Besides, we employed GEPIA2.0 for Kaplan–Meier survival analysis, the results of which showed that PILRA expression was significantly associated with poorer OS in patients with uveal melanoma (UVM), ovarian serous cystadenocarcinoma (OV) and LGG while correlated with significantly prolonged survival in SKCM, sarcoma (SARC) and CESC (Fig. 2C). Moreover, the results of Kaplan–Meier survival analysis further demonstrated that PILRA expression was in significantly negative correlation with DFS in patients with PRAD and LGG whereas positive association of PILRA expression with DFS was identified in patients with CESC and SKCM (Fig. 2D). Finally, the relationship between PILRA expression and patients' prognosis in each cancer type was further analyzed using Prognoscan website. According to the results, PILRA expression level was negatively associated with patients' prognosis in three types of cancer, including: blood cancer (OS: $p = 0.014918$), colorectal cancer (OS: $p = 0.006153$; DFS: $p = 0.035732$) and breast cancer (RFS: $p = 0.037002$) (Fig. 2E).

Next, the diagnostic value of PILRA in pan-cancer was explored through analysis of TCGA dataset. As visualized by the ROC curves, PILRA expression levels exhibited strong diagnostic potency ($AUC > 0.9$) in LAML, LUSC, LUAD and KIRC, and moderate diagnostic potency ($0.9 > AUC > 0.7$) in 18 types of cancer, including: THYM, PAAD, CHOL, HNSC, adrenocortical carcinoma (ACC), ESCA, KIRP, uterine carcinosarcoma (UCS), STAD, rectal cancer (READ), DLBC, BRCA, TGCT, GBM, THCA, colon adenocarcinoma/rectum adenocarcinoma esophageal carcinoma (COADREAD), colon adenocarcinoma (COAD) and CESC (Fig. 3). The ROC curves for the remaining 8 cancers for which PILRA only exhibited a weak diagnostic potential ($AUC < 0.7$) can be found as Supplementary Fig. S2 online. Taken together, these data indicate that PILRA is a potential diagnostic factor in various cancers.

Genetic alteration and genome-wide association of PILRA in pan-cancer. Cancers feature a stark rise in genomic alterations across the genome¹⁵. In order to inspect the PILRA mutation profiles in different cancers, three main genetic alteration categories of PILRA, including amplification, deep deletion and mutation, was assessed based on analysis of TCGA datasets using cBioPortal. As shown in Fig. 4A, the alteration frequency of PILRA was the highest in esophageal adenocarcinoma ($> 8\%$), followed by STAD, CHOL, LUSC and DLBC. Notably, the most common alteration type of PILRA was “Amplification”, of which the frequency markedly exceeded that of “Mutation” and “Deep deletion” in various cancers (Fig. 4A). Besides, as illustrated by the mutation diagram, a total of 53 mutation sites were identified in PILRA, among which R236M was the dominant mutation spot (Fig. 4B). Genetic alterations might be associated with patients' clinical survival outcomes in various cancer types¹⁶. Therefore, we compared survival differences between PILRA-altered and -unaltered groups in pan-cancer using cBioPortal, and found that the presence of PILRA alterations was associated with better prognosis in LUAD, SKCM and BRCA, while inversely related to patients' survival in KIRC, CHOL, OV and PRAD (Fig. 4C).

Additionally, we analyzed the genome-wide associations of PILRA expression with several molecular features, including gene expression, DNA methylation, somatic copy number, microRNA expression, somatic mutation and protein level-RPPA on Cancer Regulome Tools. As illustrated by circus plots, associations between PILRA and other molecular signatures were observed and visualized according to genomic coordinates in SKCM, PRAD, ACC, BLAC, LIHC, STAD, UCEC, THCA, KIRC, LUAD, LGG, LUSC, GBM, ESCA and STAD, CRC, BRCA, HNSC and OV (Fig. 5).

The relationship between PILRA expression and tumor immune infiltration in cancers. Cancer immunotherapy largely depends on the accumulation and activity of immune effector cells within the TME, since increased infiltration of immune cells into tumors is associated with an immuno-supportive TME¹⁷. To understand the immunological roles of PILRA within the cancer microenvironment, we employed “estimate” R package to estimate the abundance of stromal cells and immune cells based on the PILRA expression in a total of 10,180 tumor samples. According to the results, PILRA expression showed a positive correlation with immune score (Fig. 6A), stromal score (Fig. 6B) and ESTIMATE score (Fig. 7A) in 35 types of cancer in a consistent manner. Notably, the strongest positive association of PILRA expression with immune infiltration was observed in several cancers including SARC, SKCM and COADREAD (Fig. 7B). Additionally, we evaluated the relationship between PILRA expression and infiltration scores of six main immune cell subtypes (including B cell, CD4 T cell, CD8 T cell, Neutrophil, Macrophage, dendritic cell) via TIMER2.0. As depicted in Fig. 7C, PILRA expression significantly correlated with immune infiltration in various cancers (Fig. 7C). Thus, these data indicate that PILRA is putatively implicated in tumor immune infiltration.

Analysis of the relationship between PILRA expression and response to immunotherapy in pan-cancer. The genetic landscape of tumors has been shown to be robust indicators of tumor immunity and might be used as predictors of immunotherapy¹⁸. Therefore, we sought to explore the correlation of PILRA expression with immune check point genes (ICGs), mismatch repair (MMR)-related genes, neoantigen load, and genomic instability as defined by TMB and MSI status, all of which are important predictive biomarkers of the efficacy of immunotherapies across various cancers. As shown in Fig. 8A, PILRA expression had a close and positive correlation with three ICGs including LAIR1, HAVCR2 and CD86 in almost all cancer types. Notably, PILRA expression was significantly associated with key members of the MMR system (EPCAM, MLH1, MSH2, MSH6, and PMS2) (Fig. 8B). With respect to neoantigen, PILRA was in significantly positive correlation with it in two types of cancer, including COAD and COADREAD while negatively correlated to it in three types of cancer, including GBM, KIRC and UCS (Fig. 8C). In addition, positive correlation between PILRA expression

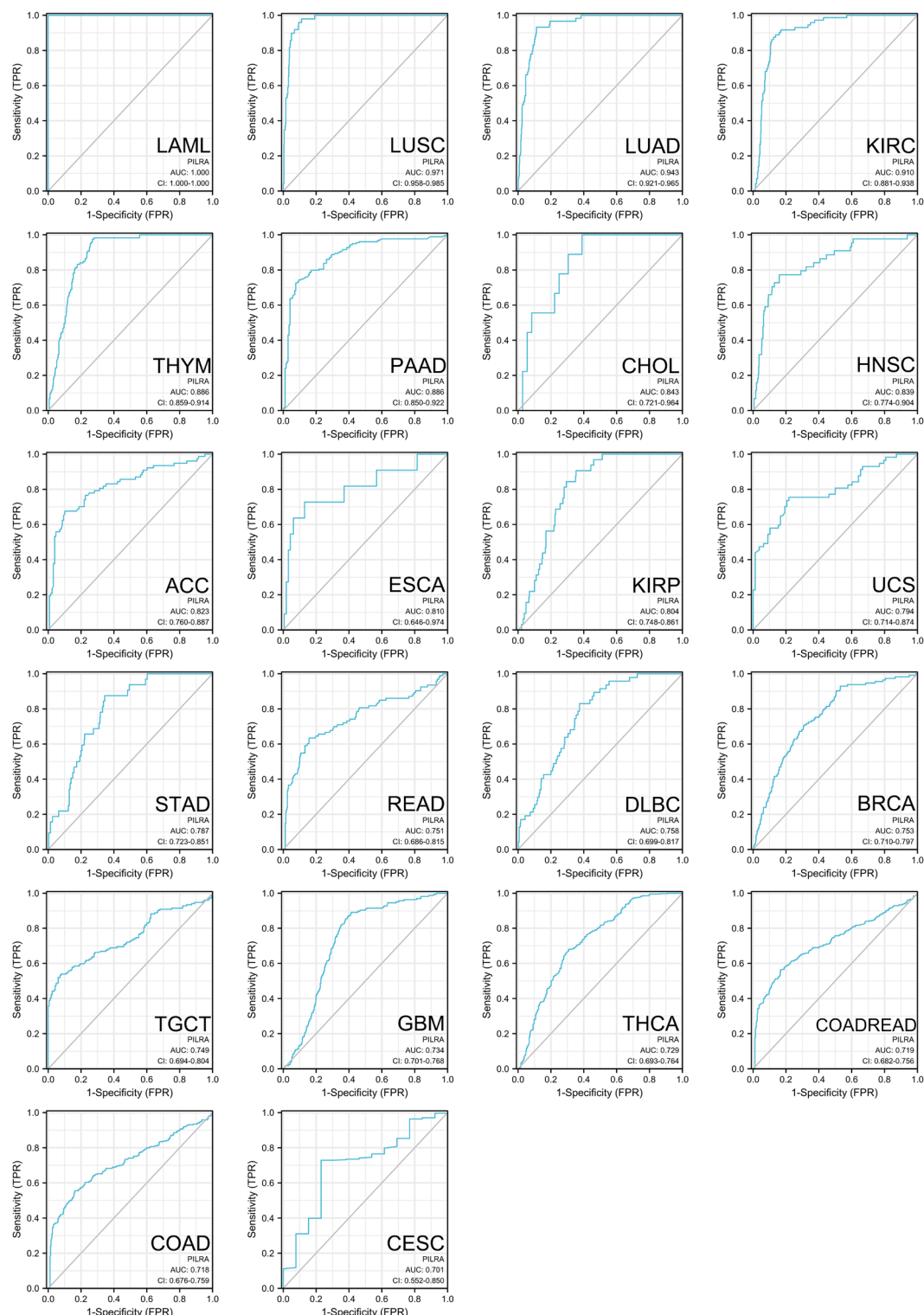


Figure 3. ROC curve analysis evaluating the diagnostic potency of PILRA in pan-cancer based on TCGA datasets. 22 cancers with AUC > 0.7 for PILRA were displayed here.

and TMB was observed in 10 types of cancer (including UVM, BRCA, CESC, COAD, LAML, OV, PAAD, SARC, SKCM and THYM) while a negative correlation was observed in only 2 types of cancer (including UVM and LAML) (Fig. 8D). Furthermore, PILRA expression was significantly associated with MSI in BRCA, COAD, HNSC, LUAD, LUSC, PCPG, PRAD, SKCM, STAD, TGCT and THCA (Fig. 8E). According to Kaplan–Meier plotter website, PILRA expression was positively associated with prognosis of cancer patients receiving

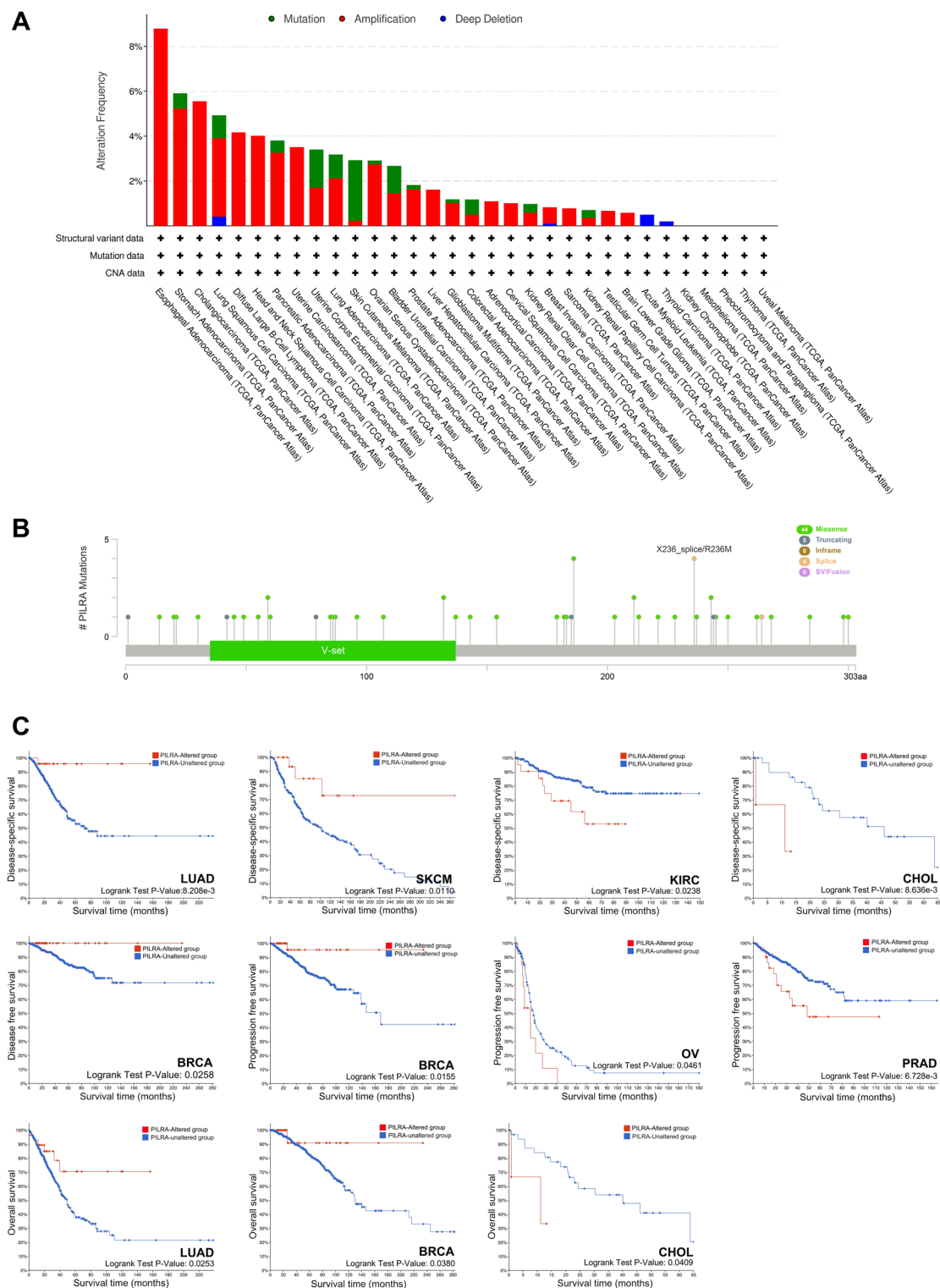


Figure 4. The mutation landscape for PILRA. (A) Bar charts showing the genetic alteration frequency of PILRA across different cancers based on cBioPortal website. Mutation, amplification and deep deletion were included as the genetic alteration. (B) The mutation sites spanning the protein domains of PILRA, containing Missense, Truncating, Inframe, Splice and SV/Fusion. (C) Associations of PILRA genetic alterations with prognosis in pan-cancer.

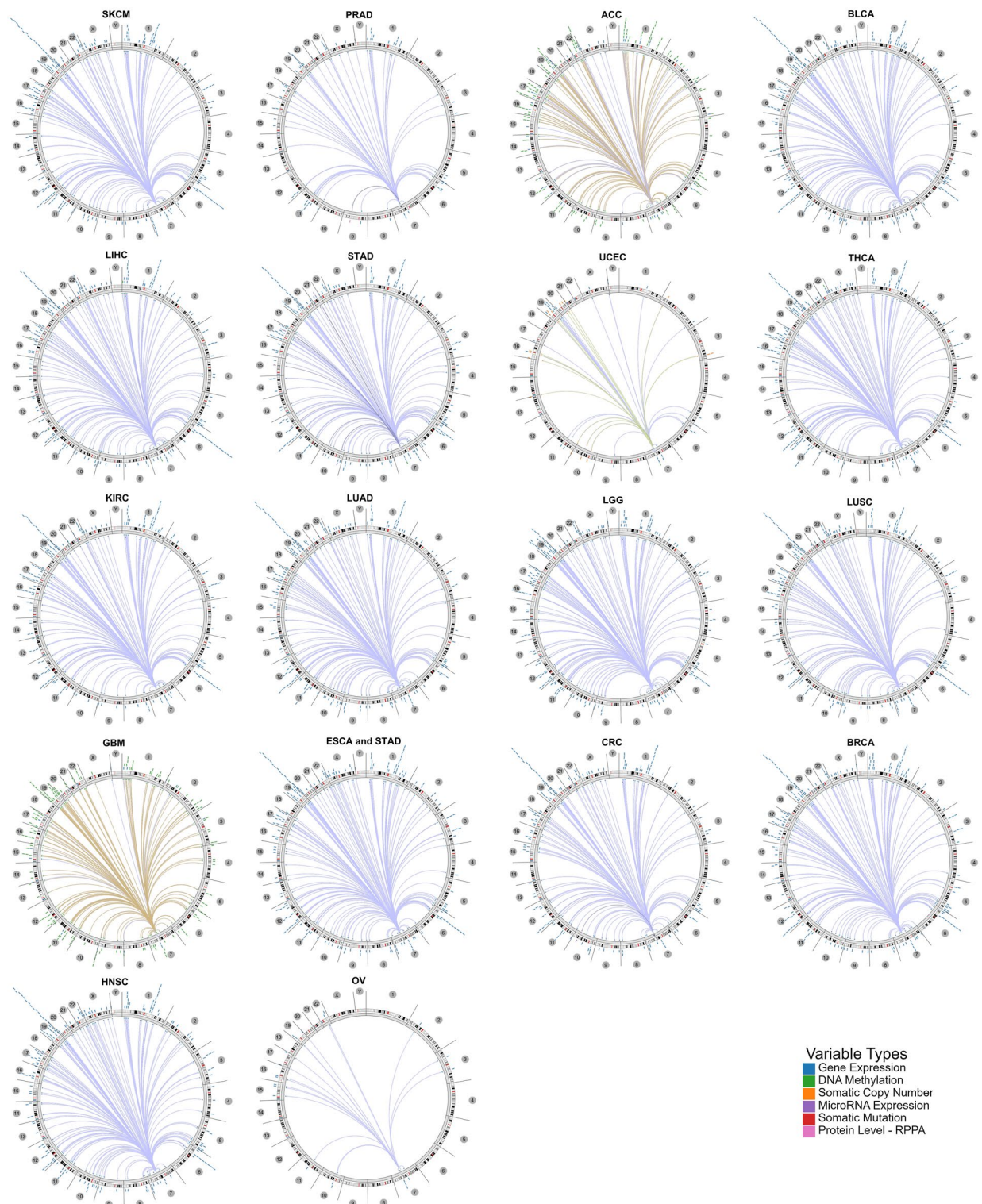


Figure 5. Genome level view of the association between PILRA and other molecular signatures within the context of genomic coordinates using Cancer Regulome website.

immunotherapy, including anti-PD1, anti-PD-L1 and anti-CTLA-4 (Fig. 8F-G). Collectively, these results indicate that PILRA is a potential predictive biomarker of patients' response to immunotherapy.

Analysis of PILRA's correlation with RNA modification and DNA methylation in pan-cancer. Accumulating evidence demonstrates that tumors commonly hijack epigenetic mechanisms, such as RNA modification and DNA methylation, to regulate immunity, thus evading immune suppression^{19–21}. To

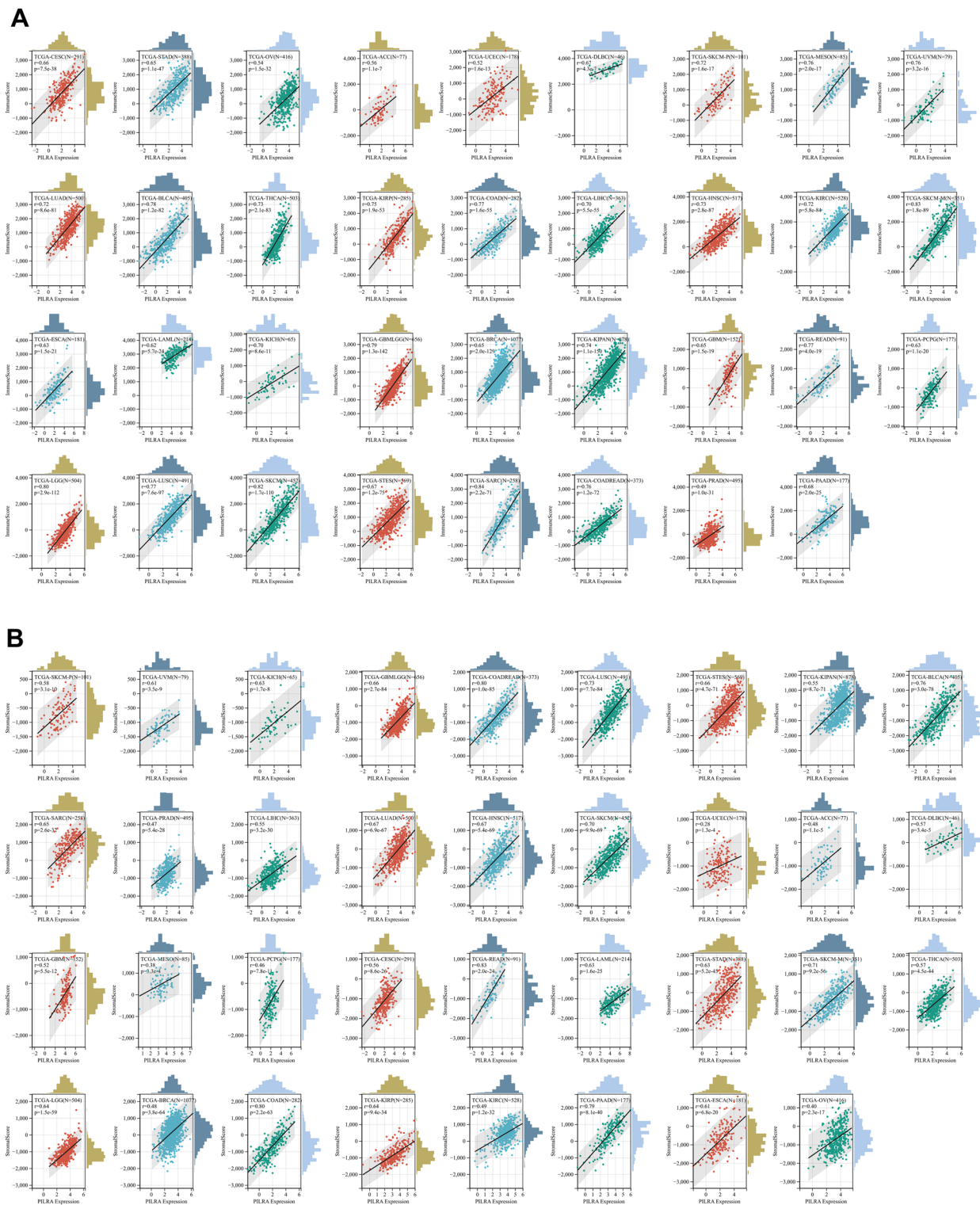


Figure 6. Associations of PILRA to immune score and stromal score. (A,B) Scatter plots showing the correlation between PILRA and immune score (A), and stromal score (B) in various cancers. $P < 0.05$ was considered significant.

to explore the relevance between PILRA and epigenetic modulations in pan-cancer, we analyzed the correlation of PILRA with 44 genes categorized into three broad types of RNA modification (m1A, m5C, m6A). As depicted in Fig. 9A, PILRA had positive correlations with a majority of RNA modulator genes in most of the cancers. Additionally, we investigated PILRA's correlation with DNA methylation status, and a significant association was observed between PILRA and four DNA methyltransferases in 23 cancers (Fig. 9B). Notably, the highest

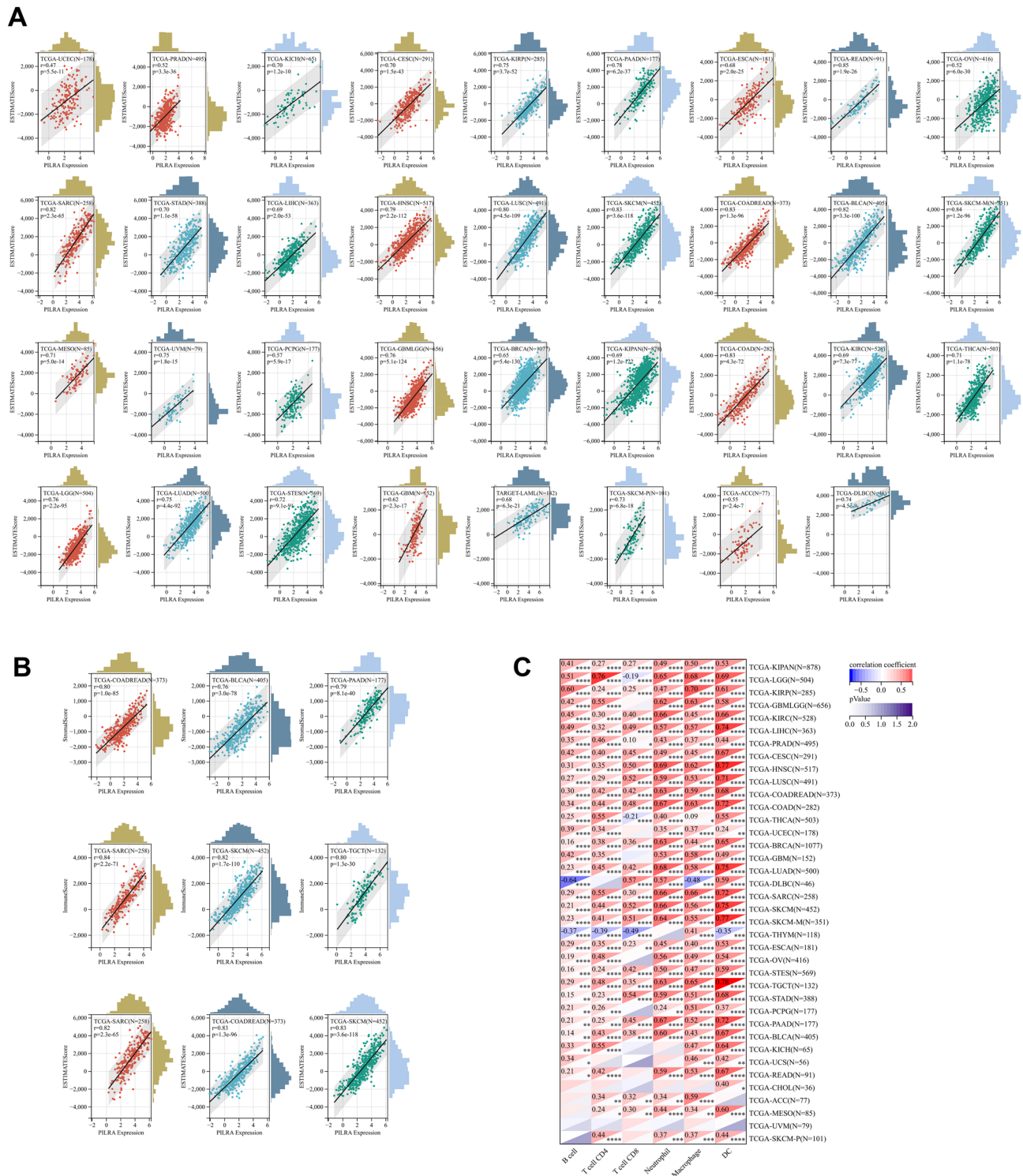


Figure 7. Associations between PILRA and immune infiltration. **(A)** The correlation between PILRA expression and ESTIMATE score in pan-cancer. **(B)** Scatter plots displaying the correlation of PILRA with Immune score, Stromal score and ESTIMATE score in the top three cancers. **(C)** The heatmap showing the relation between PILRA expression and levels of six tumor-infiltrating immune subsets in pan-cancer using TIMER. $P < 0.05$, $**P < 0.01$, $***P < 0.0011$, $****P < 0.0001$. $P < 0.05$ was considered significant.

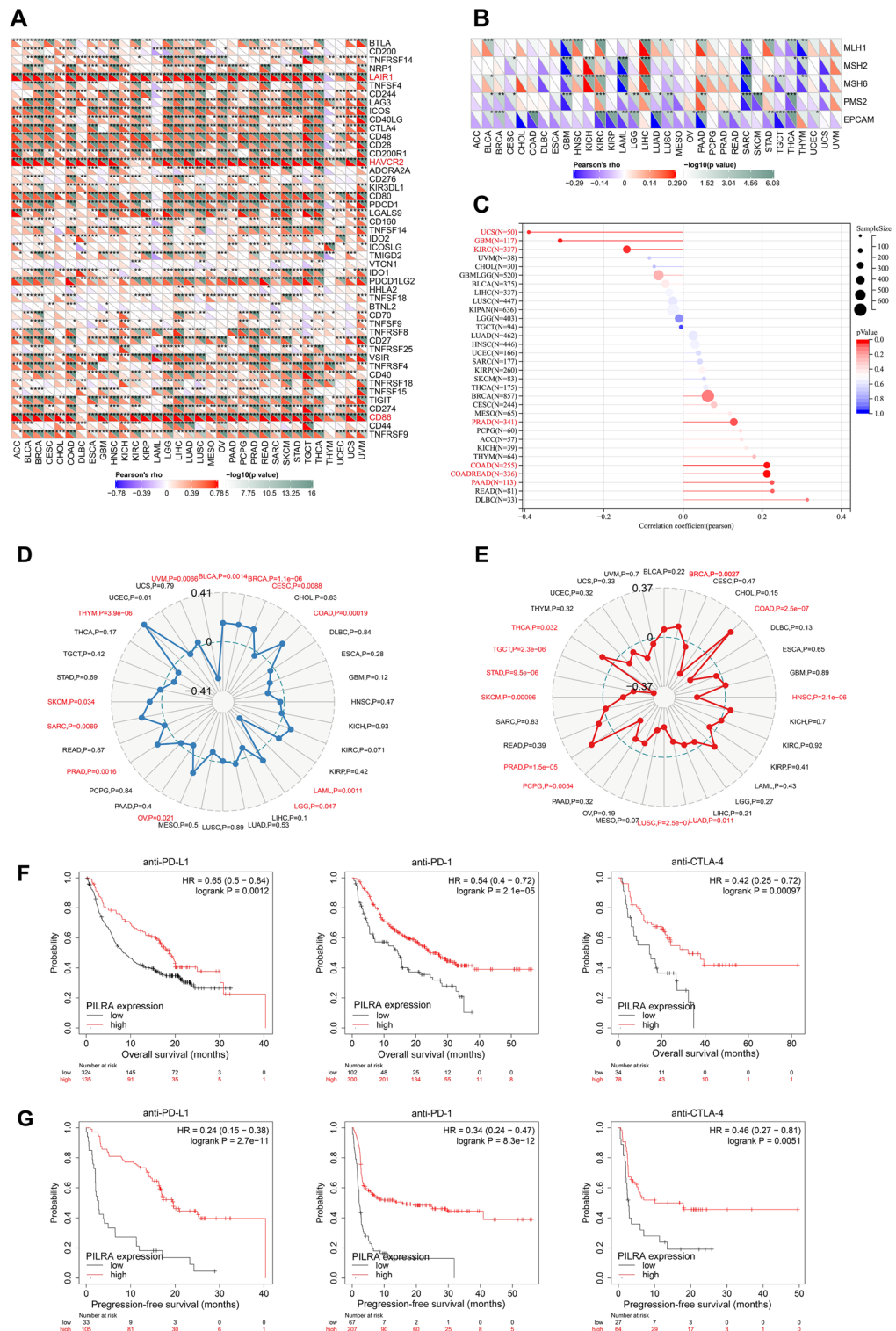


Figure 8. Associations of PILRA expression with immunity-associated markers in pan-cancer. **(A)** The heatmap exhibiting the correlation between PILRA and levels of 60 immune checkpoint genes (ICGs) in pan-cancer. ICGs with a close and positive correlation with PILRA expression were labeled in red. **(B)** The correlations between PILRA and levels of 5 genes that are key to intact MMR functions in multiple cancers. **(C)** The bar chart displaying the correlation coefficients between neoantigen levels and PILRA. Red color indicated statistical significance. **(D,E)** The radar charts display the correlations between TMB **(D)** and MSI **(E)**, and PILRA in cancers, respectively. The dotted-line circles indicate correlation coefficients, the number of which were shown on the graphs. Red color indicated statistical significance. **(F,G)** Kaplan-Meier plots predicting patients' overall survival **(F)** and progression-free survival **(G)** in cancers based on PILRA expression in Kaplan-Meier website. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $P < 0.05$ was considered significant.

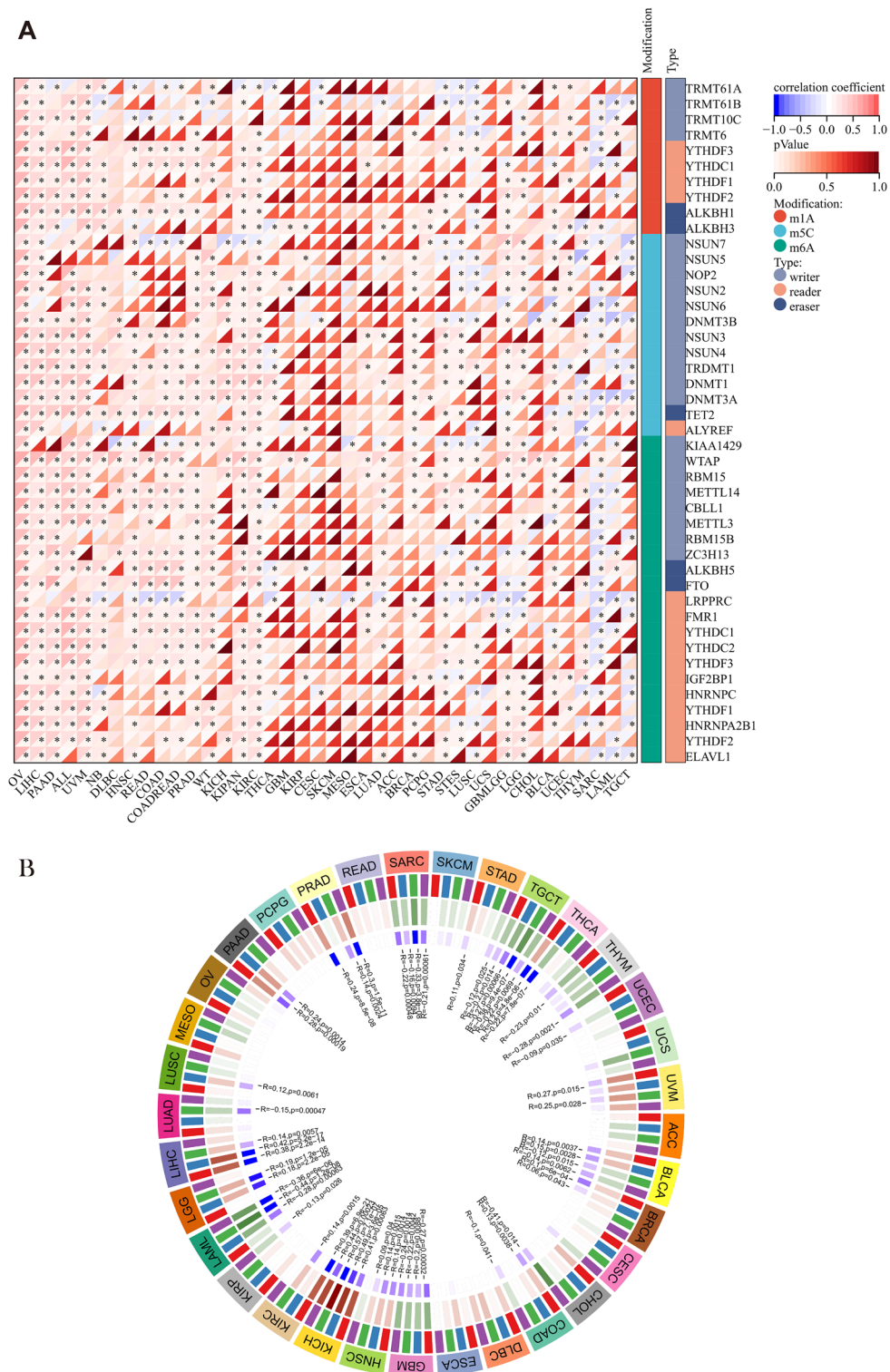


Figure 9. PILRA was implicated in epigenetic modulations in various cancers. **(A)** The heatmap exhibiting the associations of PILRA with 44 RNA-modulating genes in pan-cancer. The left and right bar indicates m⁶ARNA modification and modification enzyme types, respectively. **P* < 0.05. **(B)** The circus plot showing the correlations between PILRA expression and four methyltransferases (DNMT1, DNMT2, DNMT3A and DNMT3B), with darker shades of blue blocks in innermost layer corresponding to lower *P* value. The brown- and green-colored blocks in the second layer corresponds to positive and negative correlation coefficient values, respectively. In the third layer, DNMT1, DNMT2, DNMT3A and DNMT3B were shown in red, blue, green and purple, respectively. The outmost layer indicates cancer names. *P* < 0.05 was considered significant.

co-expression coefficients (>0.4) were observed between PILRA expression and four methylation transferases in KICH.

Enrichment analysis of PILRA-related pathways in pan-cancer. To comprehend the functional roles of PILRA in cancers, interacting proteins and most relevant genes of PILRA were identified for conduction of functional enrichment analysis. The experimentally validated PPI network of PILRA was visualized using STRING databases (Fig. 10A). Besides, the top 100 co-expressed genes of PILRA in various cancers were displayed by the correlation heatmap on GEPIA2.0 (Fig. 10B). And the strongest correlation of PILRA was noticed with 6 genes, including SPI1, TYROBP, CD300C, IGSF6, HK3 and LILRB3 (Fig. 10C). According to the GSEA results of GO and KEGG, immune response and metabolic process were enriched for PILRA (Fig. 10D). In addition, the functional enrichment of GO terms indicated that PILRA was primarily associated with neutrophil activation, neutrophil activation involved in immune response and neutrophil degranulation (Fig. 10E).

Discussion

Recent therapeutic advancement in cancer immunotherapy has conferred a remarkable survival benefit to terminal cancer patients. However, it remains to be identified regarding a novel genetic alteration that encompasses a broader spectrum of patients for cancer immunotherapy. Herein, by performing integrative bioinformatics analysis, we report a novel tumor immune-associated gene, termed as PILRA, that is aberrantly expressed and significantly relates to patients' prognosis in pan-cancer. Specifically, we unveil that PILRA is positively associated with tumor immune infiltration, and analyze the correlation between PILRA expression and tumor immune markers and immunotherapy. Moreover, we investigated the biological functions of PILRA in different cancers by performing pathway enrichment using GSEA, GO and KEGG analysis for the first time.

Cancer is a genetic disease arising from the accumulation of alterations in genes that participate in various oncogenic processes²². For example, combined Dusp4 and p53 loss with Dbf4 amplification drives tumorigenesis in breast cancer²³. Neurofibromatosis 1 (NF1) mutation drives initiation of optic glioma through mediating dysregulation of neuronal activity and stimulating of optic nerve activity²⁴. In the present study, we demonstrated that PILRA is abnormally expressed and associated with patients' survival across different cancers. In addition, we uncovered that PILRA was highly mutated in several tumors, including EA, STAD, CHOL, LUSC and DLBC. By conducting survival analysis, we unprecedentedly illustrated that genetic alteration in PILRA was associated with improved patients' survival in LUAD, BRCA and SCKM, while significantly shortened the survival in KIRC, CHOL, OV and PRAD patients. Taken together, these results uncover that both the expression and genetic alterations of PILRA correlates with patients' prognosis in various cancers.

Our current work provides the first evidence of the immunological roles of PILRA in pan-cancer. Initially, PILRA was mainly recognized for its regulatory role in immune systems. For instance, PILRA was reported to be involved in the regulation of innate immunity through recognition of a CD99-like ligand²⁵. PILRA plays an important role in HSV-1 infection of monocytes as a coreceptor that associates with gB²⁶. Moreover, PILRA G78R variant was associated with significantly decreased levels of HSV-1 infection in macrophages²⁷. However, the roles of PILRA in tumor-related immunity remain unexplored yet. Here, we uncover that PILRA is positively associated with tumor immune infiltration in most of the cancers analyzed. Additionally, we demonstrate here that the presence of TP53 mutations in PILRA high-expression group is more frequent than low ones in KICH, LGG, ACC, UCEC, PRAD, BLCA, BRCA, LIHC, PAAD, LUAD and GBM. Studies have shown that mutant TP53 are major determinants of the tumor immune composition by inducing genomic instability²⁸. The above results strongly indicate that PILRA may be implicated in regulating cancer-related immune processes, thereby potentially playing a role in immunotherapy.

The presence of certain immune cells, particularly T cells, within the tumor microenvironment has been associated with an increased likelihood of durable response to immunotherapies²⁹. Therefore, we further explored the relationship between PILRA expression and cancer immunotherapies. Currently, multiple predictive biomarkers for immunotherapies have emerged, such as NEO, TMB, MSI and MMR^{30,31}. Tumor neoantigens, which are mainly generated due to genomic instability, are tumor-specific antigens that predicted improved responses to immunotherapy in various cancers^{32,33}. By analyzing the correlation between PILRA expression data and neoantigen, we found that PILRA was positively related with neoantigen in COADREAD and COAD while negatively with it in GBM, KIRC and UCS. As the main source of neoantigens, high tumor mutational burden is an important predictor of immunotherapy responses³⁴. Besides, MSI and MMR could lead to accumulation of genetic mutations in cancers, for which they were included as main biomarkers for immunotherapy responses^{35,36}. Importantly, our results showed significant associations of PILRA with TMB, MSI and MMR, implying the potential of PILRA as a potential predictor for immunotherapy. Therefore, our study provided novel bioinformatics evidence supporting the potential involvement of PILRA in cancer immunity in various cancers.

There are some caveats to the present study. Firstly, the results concerning the oncogenic and immunologic roles of PILRA are achieved through bioinformatics analysis of multiple online repertoires, and therefore warrants further experimental validation to verify its cancer-related roles by performing *in vitro* and *in vivo* experiments. Besides, we only demonstrated positive correlations of PILRA with tumor immune infiltration, and thus the associations of PILRA with immune responses in various cancers need to be further explored and validated by experiments and analysis of clinical data to clarify the roles of PILRA in tumor-related immunity.

In summary, we conducted integrative bioinformatics analysis of the expression profiles and immunological roles of PILRA in pan-cancer. Our results demonstrated that PILRA was dysregulated in various cancers and positively mediated tumor immune infiltration, which provides a novel insight into the potential mechanism underlying the modulation of the immune landscape within tumor microenvironment. In conclusion, we unveiled that PILRA was implicated in immune responses and cancer-related immunity.

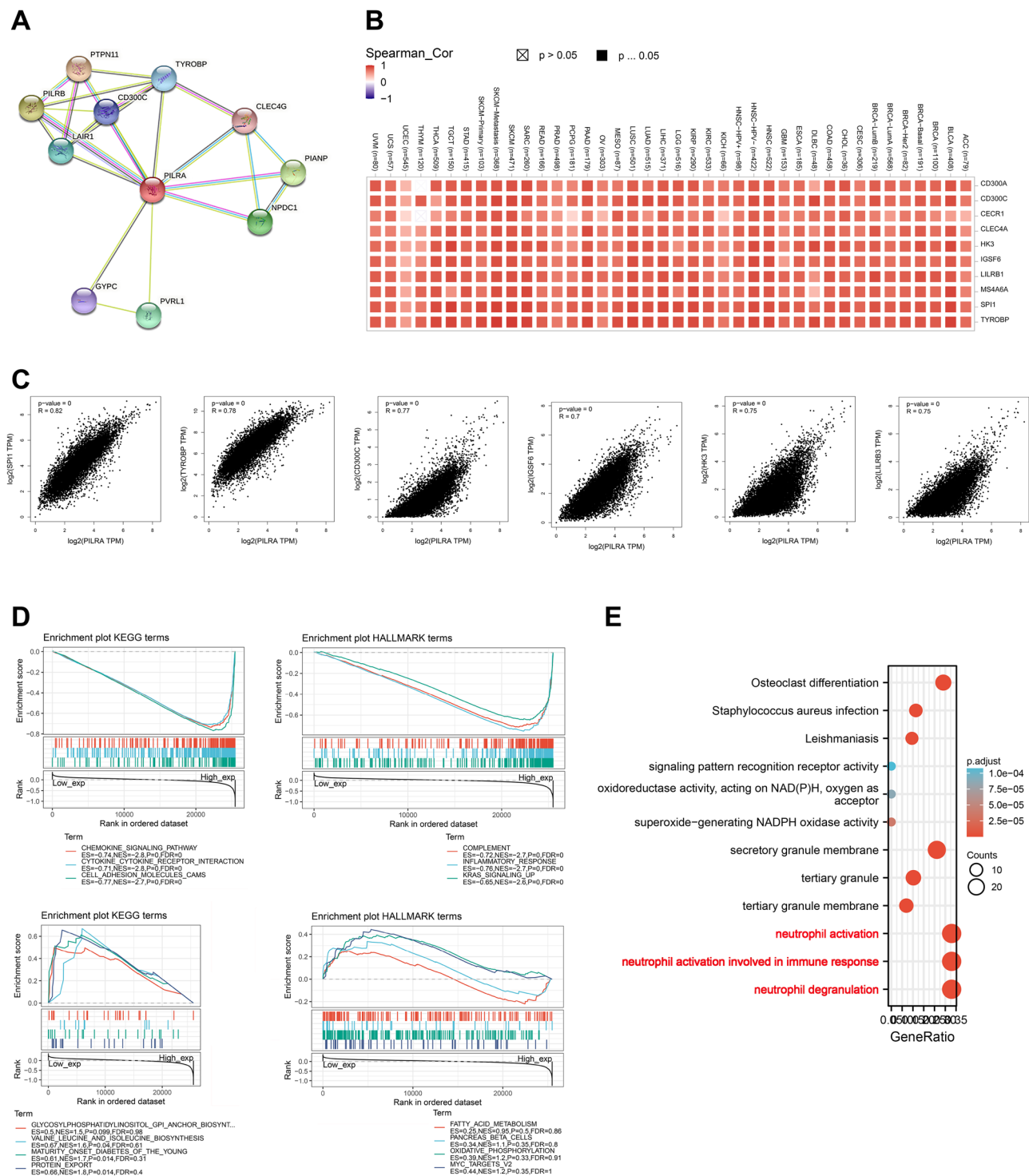


Figure 10. The function profiles of PILRA in pan-cancer. **(A)** The network of experimentally validated PILRA-interacting partners visualized via STRING web tool. **(B,C)** The heatmap **(B)** and scatter plots **(C)** showing the correlations between PILRA and its top 5 related genes in pan-cancer using GEPIA2.0. Spearman_Cor is short for Spearman correlation. **(D)** GSEA analysis of the enriched KEGG and HALLMARK terms according to the expression of PILRA in pan-cancer. Top three mostly significantly enriched pathways were visualized. **(E)** GO and KEGG pathway analysis of PILRA-related functions in pan-cancer. $P < 0.05$ was considered significant.

Materials and methods

Data acquisition and processing. The genomic and epigenomic data of PILRA and related clinical information of 33 common cancer types were downloaded from the Cancer Genome Atlas (TCGA, <https://portal.gdc.cancer.gov/>). Meanwhile, publicly available PILRA gene expression data of normal tissues were acquired from genotype-tissue expression database (GTEx, <http://commonfund.nih.gov/GTEx/>). The comparisons between cancerous and adjacent normal tissues were carried out using both TCGA and GTEx datasets. To normalize the gene expression among different samples, gene expression levels were universally presented as transcripts per million (TPM).

Clinical samples. The tissue samples used in this study were obtained from NSCLC cancer patients with no history of radiotherapy or chemotherapy at People's Hospital of Deyang City. The investigation was approved by the ethics committee of People's Hospital of Deyang City.

Comparison of differential expression of PILRA between cancerous and normal tissues. The PILRA mRNA expression differences between cancer tissues and adjacent normal tissues were compared using TIMER2.0 website (<http://timer.cistrome.org/>)³⁷. Furthermore, the differential PILRA protein expression profiles were explored through analysis of the National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium platform (CPTAC, <https://proteomics.cancer.gov/>). The cutoff values of $|\log_2FC|$ and P-value for statistical significance were set as 1 and 0.05, respectively.

Survival analysis and receiver operating characteristic (ROC) analysis. The prognostic value of PILRA, including overall survival and disease specific survival, were evaluated at a pan-cancer level by conducting univariate cox regression analysis of TCGA datasets. Besides, Kaplan–Meier (KM) curves were plotted to further confirm the relationship between PILRA expression and patients' prognosis using Gene Expression Profiling Interactive Analysis 2 (GEPIA2.0, <http://gepia2.cancer-pku.cn/>)³⁸, Prognoscan (<http://www.prognoscan.org/>)³⁹ and Kaplan–Meier plotter online database (kmplot.com/)⁴⁰. The log-rank *p* value and hazard ratio (HR) were calculated to determine the prognostic value of PILRA. A *p* value of smaller than 0.05 was considered to be of statistical significance.

The diagnostic potential of PILRA in pan-cancer was assessed utilizing area under the ROC curves (AUC) based on TCGA datasets. Specifically, $AUC > 0.9$ corresponds to a good level of prediction accuracy while $0.9 > AUC > 0.7$ is linked to a moderate level of prediction accuracy.

Analysis of the genomic alteration of PILRA in pan-cancer. The pan-cancer analysis was carried out on the PILRA genomic alteration landscape, comprising mutation, amplification and deep deletion, using the Cancer Types Summary module of the cBioPortal online web tool (<https://www.cBioPortal.org/>)⁴¹. The mutation landscape of PILRA protein was analyzed and displayed by combining the processed SNV data with the protein domains derived from the “maftools” R package in pan-cancer.

Pan-cancer analysis of the immunological roles of PILRA. To investigate the roles of PILRA expression in immune infiltration, the stromal, immune, and ESTIMATE scores of each tumor sample were calculated based on PILRA gene expression via R package “ESTIMATE” (version 1.0.13) (<https://bioinformatics.mdanderson.org/>)⁴². Next, the Pearson's correlation coefficient of PILRA expression and immune infiltration within tumor microenvironment (TME) in pan-cancer was calculated using the R package psych (version 2.1.6). In addition, the separate correlation between PILRA and infiltration of B cell, T cell CD4, T cell CD8, Neutrophil, Macrophage and dendritic cell (DC) was evaluated using TIMER online tool of the R package “IOBR” (version 0.99.9) (<http://timer.comp-genomics.org/>)⁴³.

Investigation of the correlation of PILRA with tumor antigen and epigenetic modulation. The effects of PILRA in anti-cancer immunity were analyzed based on TCGA datasets. A total of 47 immune checkpoint (ICI) genes were retrieved, and the expression correlation between these genes and PILRA was calculated and displayed in the fashion of a heatmap. Spearman's correlation coefficient for ranked data was calculated to assess the association between PILRA gene expression and microsatellite instability (MSI) and tumor mutation burden (TMB) of each tumor sample. Additionally, Pearson's correlation coefficient was employed to present the relationship between PILRA expression and the number of neo antigen count (NEO). $P < 0.05$ indicates statistical significance.

The correlation between PILRA and 44 RNA modulating genes including N1-methyladenosine (m1A), 5-methylcytosine (m5C), and N6-methyladenosine (m6A) modification genes was presented in a heatmap based on UCSC database (<https://xenabrowser.net/>)⁴⁴. In addition, the correlation between PILRA and DNA methyltransferase (including DNMT1, DNMT2, DNMT3A, and DNMT3) were analyzed.

Enrichment analysis. To observe the regulatory mechanisms of PILRA in tumor cells, tumor samples were divided into two groups according to the high and low expression of PILRA. Gene Sets Enrichment Analysis (GSEA) analysis on PILRA was conducted to enrich and visualize related gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. $|\text{NES}| > 1$ and $\text{FDR} < 0.05$ were set as cutoffs for statistical significance. Besides, the STRING platform (<https://string-db.org/>) was used to plot the protein–protein interaction (PPI) network diagrams for PILRA⁴⁵.

Immunohistochemistry (IHC) staining. To verify the protein expression of PILRA in cancer tissues relative to that in normal ones, IHC staining was performed according to the manufacturer's protocols. Four pairs of Formalin-Fixed Paraffin-Embedded (FFPE) tissue sections (cancer and adjacent normal tissues) of NSCLC patients, were sampled. The slides were first subjected to dewaxing, hydration, incubation with 3% hydrogen peroxide and antigen retrieval (AR) by heating. Next, the tissues were successively incubated with primary antibody against PILRA (1:100, abcam) at 4 °C overnight and secondary antibody for 1 h at room temperature. Then, the tissues were stained with 3,3'-diaminobenzidine (DAB) and counterstained by hematoxylin. Images were taken under using eclipse 80i (Nikon, Japan) magnified 200×.

Statistical analysis. The differences of gene expression between independent groups were compared using Student's t-test. The statistical significance of survival analysis was assessed by conducting a log rank test. The Spearman's and Pearson's correlation coefficients were applied to quantify and estimate the correlation between PILRA and other variables. *P*-value less than 0.05 was considered statistically significant.

Ethics declarations. All experiments involving human participants were performed in accordance with the guidelines and regulations of the ethics committee of People's Hospital of Deyang City (Declaration of Helsinki) (Approval number: 2022-04-102-K01). The need for informed consent was waived by the ethics committee of People's Hospital of Deyang City owing to the retrospective nature of the study.

Data availability

The datasets used in this study can be found in online repositories, the name of which could be found in the article.

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Author contributions

Q.L. and X.Y. conceived and supervised the study. Z.Y., X.H. and X.Y. collected and assembled the data. X.Y. and Z.Y. performed data analysis and drew the figures. Q.L. and X.Y. wrote and revised the manuscript. All authors participated in the production of the article and approved the submitted version.

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Competing interests

The authors declare no competing interests.

Additional information

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