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Original Article

Pan-Cancer Analysis Reveals NMDA Receptors as Prognostic and Immunotherapy Biomarkers in Cancer Patients

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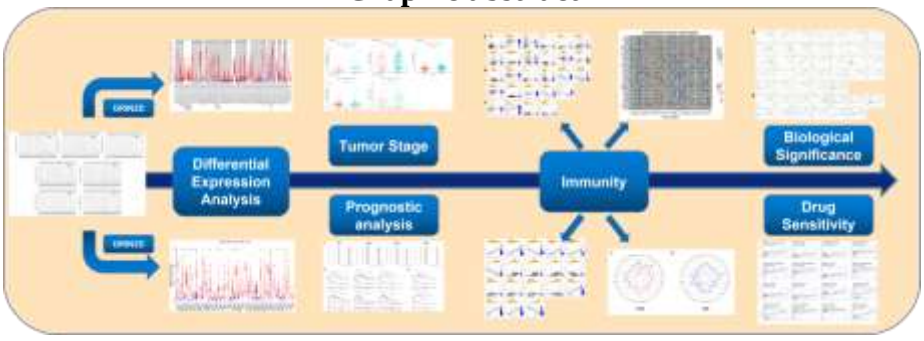
Abstract:
N-methyl-D-aspartate (NMDA) receptors, one subtype of ionotropic glutamate receptors, may combine with glutamate to further exert regulatory effects on tumors. This research first analyzed the expression levels of 7 genes expressing NMDA receptor subunits, and found that GRIN2D was expressed at higher levels in most types of tumor, while the other genes expression levels differed little. Prognostic analysis showed that GRIN2D was positively or negatively correlated with the prognosis of various tumor patients. In 14 tumor types, GRIN2D expression was discovered to be related to tumor mutational burden (TMB), and in 7 tumor types, it was strongly related to microsatellite instability (MSI). Analysis of immune cell infiltration level results showed that GRIN2D was correlated with various immune cells, especially T cells, T cells regulatory (Tregs) and tumor-associated macrophages, and GRIN2D was associated to immune score in 14 different tumor types by tumor microenvironment analysis. The study found two tumors more associated with GRIN2D, kidney renal clear cell carcinoma (KIRC) and liver hepatocellular carcinoma (LIHC), for which NMDA receptors might be more significant for their diagnosis, prognosis and immunotherapy. Furthermore, enrichment analysis of GRIN2D revealed that some immune processes were activated. According to the drug sensitivity analysis, GRIN2D was sensitive to 34 drugs, and it might be a potential target of these drugs against tumors. These studies suggested that GRIN2D was used as a potential tumor marker to evaluate the prognosis of tumor patients and might become a key immunomodulatory target in tumor progression.
Keywords: NMDA, GRIN2D, tumor immunity, Pan-cancer, tumor microenvironment, biomarker

Graphic abstract



Introduction

NMDA receptors (NMDARs) are ligand-gated ion channels induce by glutamate. NMDA receptor subunits can be divided into three major categories, including the glycine / D-serine binding GluN1 and GluN3 (GluN3A and GluN3B) subunits, and the glutamate binding GluN2 subunits (GluN2A, GluN2B, GluN2C, and GluN2D) [1]. Functional NMDARs subunits are assembled as heterotetramers composed of two GluN2 or GluN3 subunits combined with two essential GluN1 subunits [2]. In addition to being distributed throughout the brain, particularly in the hippocampus, cerebral cortex, striatum, and amygdala [3], NMDARs are also expressed and play an important role in extracerebral tissues and some non-neural tissues. For example, the conversion of chondroprogenitor cells into chondroblasts, which produce the cartilage matrix, is regulated by NMDAR [4]. The development of chronic pain caused by peripheral nerve damage can enhanced by increased activation of spinal NMDA receptors [5]. NMDARs also play crucial roles in neurotransmission and are involved in many complex biological processes, including induction of long-term potentiated LTP (associated with learning and memory), control of the structure of neuronal circuits in the brain, and developmental processes such as synaptic plasticity, neurodegeneration, ischemia-hypoxia-induced excitotoxic effects, and the formation of epilepsy [1,2,6,7] .

Various researches have found that NMDA receptors were associated with the initiation, promotion and progression of different types of tumor. Synthesized and stored of glutamate in tumor cells can be secreted outside of tumor cells through the glutamate transport system [8], and NMDA receptors may combine with these released glutamate to further regulate tumors [9]. Anti-NMDA receptor encephalitis is a newly discovered most common autoimmune encephalitis closely related to autoimmunity [10, 11, 12], which is considered to be a paraneoplastic disease, and tumors are the main cause of its occurrence [13]. Studies have observed an upregulation of NMDAR at the periphery of pancreatic neuroendocrine tumors, especially in invasive areas, and notes a correlation between increased coexpression of NMDAR and glutamate exporters with poor prognosis in cancer patients. The research emphasizes the role of fluid flow-induced autologous glutamate secretion in activating NMDAR signaling, thereby promoting invasiveness through MEK-MAPK and CaMK effectors [14]. Some researchers have concluded experimentally that NMDA receptors exist in small cell lung cancer (SCLC) and might play a role in maintaining the growth and viability of tumor cells [15]. Overactivation of NMDA receptors could promote oxidative stress, aggravate inflammatory responses and induce excitotoxic lung injury [16]. The reduction or deletion of NMDAR2B was closely related to primary gastric cancer [17], whereas the NMDAR2A subunit could promote gastric cancer cell proliferation by accelerating the cell cycle, while specific receptor blockers could partially block its effect [18]. Studies have also confirmed that NMDA receptor antagonists could inhibit the proliferation through cell cycle arrest in hepatocellular carcinoma [19]. A study delves into the mechanism of breast cancer metastasis to the brain, revealing a crucial interaction between B2BM cells and neurons through the NMDAR signaling pathway, that highlights the cancer cells' utilization of pseudo-tripartite synapses to interact with glutamatergic neurons, activating the NMDAR signal and facilitating invasive growth in the brain [20]. All of the above studies have shown that NMDA receptors were tightly related to tumors. However, the research on the role of NMDA receptors in tumors was currently mostly limited to specific types of cancer, and there has been no report on the role of NMDA receptors in different cancer types.

Therefore, in this study, the expression levels of NMDA receptors and their correlation with prognosis in various tumor types were studied by using multiple databases. In 33 cancer types, the possible relationship between the expression of NMDA receptors and MSI and TMB was also investigated. Furthermore, immune correlation analysis and co-expression analysis of immune checkpoints and NMDA receptors were performed, and the biological functions of NMDA receptors in tumors by enrichment analysis. The study found two tumors more associated with GRIN2D, KIRC and LIHC, for which NMDA receptors might be more significant for their diagnosis, prognosis and immunotherapy. At last,

potential therapeutic drugs were obtained by drug sensitivity analysis. By regulating immune cells, NMDA receptors can play a significant role in tumor immunity and may be used to predict the prognosis of certain tumor types. It provided new directions and potential targets for the treatment of various tumors, and established the foundation for more thorough investigation of the development and occurrence of cancers.

2. Methods

2.1 Differential Expression Analysis

TCGA (The Cancer Genome Atlas) data(contains 11057 samples from 33 types of cancer) including RNA sequencing, clinical data and somatic mutations were obtained (https://xena.ucsc.edu/). Using the downloaded data, the expression of NMDA receptor-related genes was assessed in 24 normal tissues and 33 tumors. R software (available at https://www.R-project.org) was utilized to carry out the analysis.

The expression levels of genes containing NMDA receptors in 33 types of tumor were analyzed by using the GEPIA2.0 database (http://gepia2.cancer-pku.cn/#general) and the TIMER2.0 database (timer.cistrome.org/). Between tumor and normal tissues, $P < 0.05$ was deemed to be differently expressed.

2.2 Correlation Analysis between GRIN2D Expression and Tumor Stage

According to expression levels of NMDA receptors, we selected GRIN2D for subsequent analysis. The relationship of tumor stage with GRIN2D expression was analyzed using TCGA data. Two groups of tumor patients were formed based on their tumor stage, including tumor stage 1, 2 and stage 3, 4.. Using the R-packages "limma" and "ggpubr", clinical stage correlation analysis was conducted.

2.3 Prognostic analysis of GRIN2D in Pan-cancer

Using the survival package and the survminer package, Kaplan-Meier survival analysis was performed on 33 tumors, and the survival curve was drawn. Four indicators were selected, including overall survival (OS), disease-specific survival (DSS), disease free survival (DFS), and progression free survival (PFS). The expression of GRIN2D was regarded as a continuous independent variable, and univariate COX regression analysis was performed on 33 tumors using the survival package, and draw a forest plot. Set the filtered P value to 0.05.

2.4 Correlation of GRIN2D Expression With TMB and MSI

TMB serves as a predictive biomarker for immunotherapy of multiple tumors and indicates mutation levels in tumor [21]. Tumor MSI is due to deletions or errors in the process of mis-match repair deletion or gene replication, resulting in variations in the length of the microsomal. MSI has important implications for tumor prognosis and treatment [22]. A Perl script was used to compute TMB scores, somatic mutation data downloaded from TCGA was used to produce MSI scores for all samples, and Spearman rank correlation coefficients were utilized to examine the relationship between GRIN2D expression and TMB and MSI. Results radar charts were drawn by utilizing the R package "fmsb".

2.5 Relationship Between GRIN2D Expression and Immunity

The ESTIMATE is a method for predicting tumor purity, using gene expression data to predict the abundance of stromal and immune cells in tumors [23]. The stromal score and immune score were obtained using the ESTIMATE algorithm, and the correlation of GRIN2D expression with all sample scores was calculated in each tumor using the "limma" package.

Moreover, the CIBERSORT algorithm was used to determine the infiltration ratio of 22 types of infiltrating immune cells in the tissue of each tumor patient, and a relative score was obtained. The correlation of GRIN2D expression with immune cell scores in each tumor was then analyzed. Analysis and plotting were performed using the R packages "ggplot2", "ggpubr" and "ggExtra".

Additionally, by sorting out 49 common immune checkpoint genes, we utilized the R-package "limma" to analyze the co-expression of these genes with GRIN2D. Using the R packages "reshape2" and "RColorBreyer", the final result is displayed as a heatmap.

2.6 The Biological Significance of GRIN2D Expression in Tumors

The biological function of GRIN2D in tumors was investigated using gene set enrichment analysis

(GSEA). To explore the relationship between GRIN2D expression and biological function in tumors, use the R packages "limma" and "clusterProfiler" to perform enrichment analysis and screen out the top 5 biological functions with absolute enrichment scores and plot them.

2.7 Drug Sensitivity of GRIN2D in Tumors

RNA-seq expression profiles and NCI-60 compound activity data from the CellMiner (https://discover.nci.nih.gov/cellminer/home.do) were obtained in order to assess drug sensitivity of GRIN2D in pan-cancer [24]. The Pearson correlation coefficient between the GRIN2D expression and different drugs was calculated separately, and the analysis results were screened according to the P value < 0.05. Drugs that had received FDA approval or in clinical trials were chosen. The “impute”, “ggplot2”, and “ggpubr” R packages were then used to examine the influence of GRIN2D on drug sensitivity.

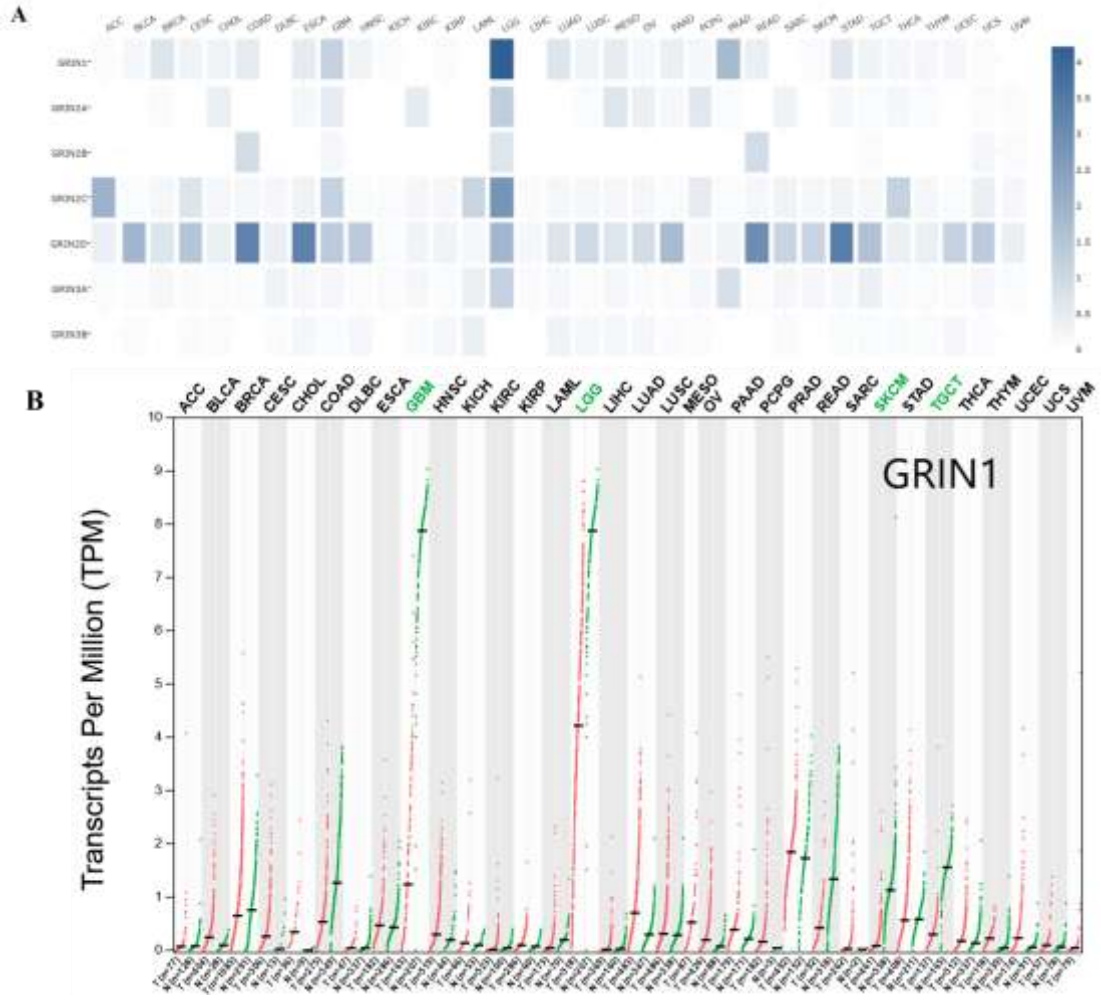
2.8 Statistical Analysis

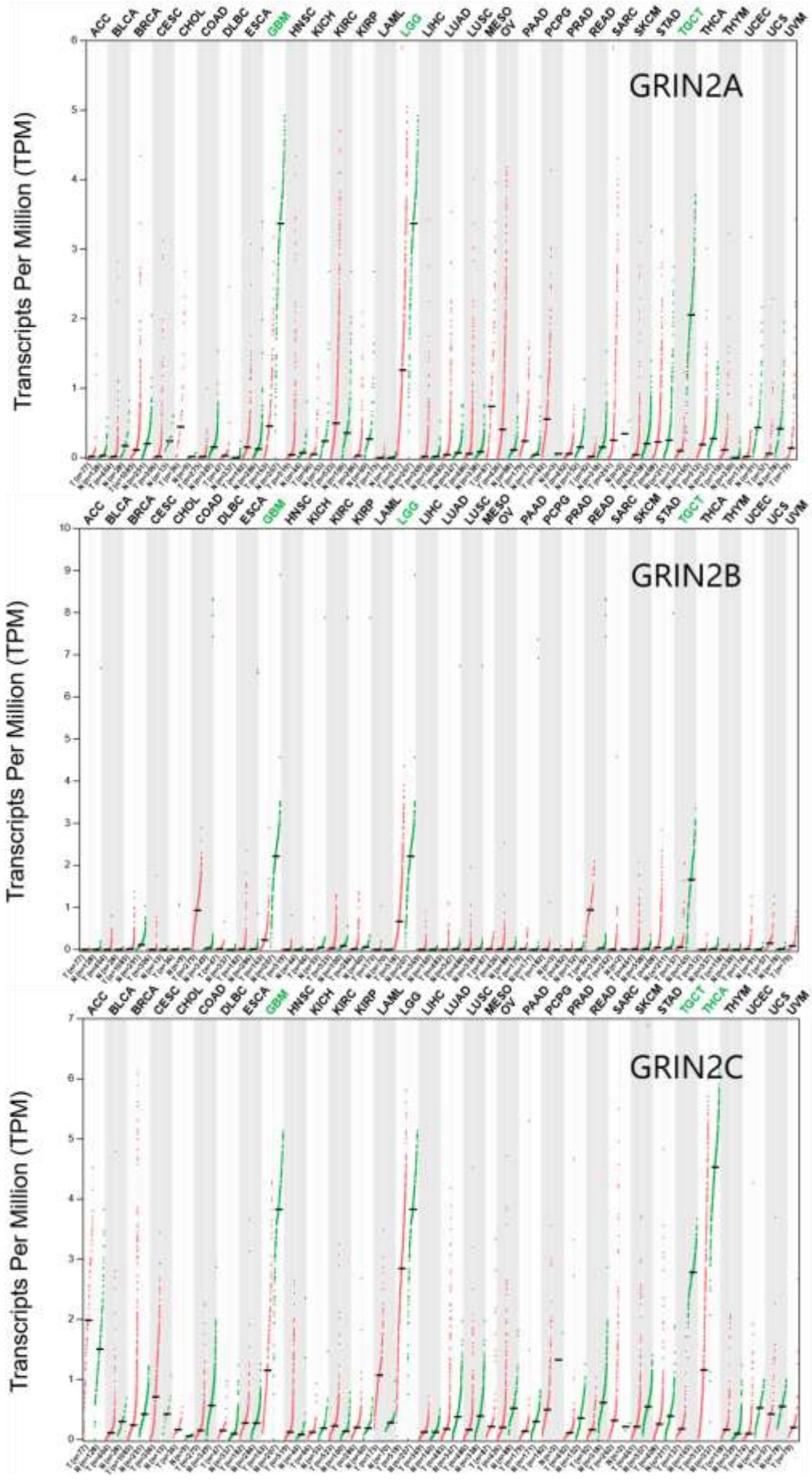
T test were utilized to compare normal tissue and tumor tissue. Using Spearman or Pearson tests, the correlation study between two variables was conducted. All statistical analyses were performed using the R package (v4.2.0), and $P<0.05$ was regarded as statistically significant.

3. Results

3.1 Expression of GRIN1, GRIN2A, GRIN2B, GRIN2C, GRIN2D, GRIN3A and GRIN3B in tumors

The expression levels of 7 genes expressing NMDA receptor subunits in tumor tissues were analyzed by using the GEPIA2.0 database. The results showed that 7 genes were differentially expressed in a variety of tumors, of which, especially GRIN2D, the tumor types with significantly differential expression are the most (Fig. 1A and B).




GRIN2A
GRIN2B
GRIN2C
Transcripts Per Million (TPM)
ACC BLCA BRCA CESC CHOL COAD DLBC ESCA GBM HNSC KICH KIRC KIRP LAML LGG LIHC LUAD LUSC MESO OV PAAD PCPG PRAD READ SARC SKCM STAD TGCT THCA THYM UCEC UCS UVM

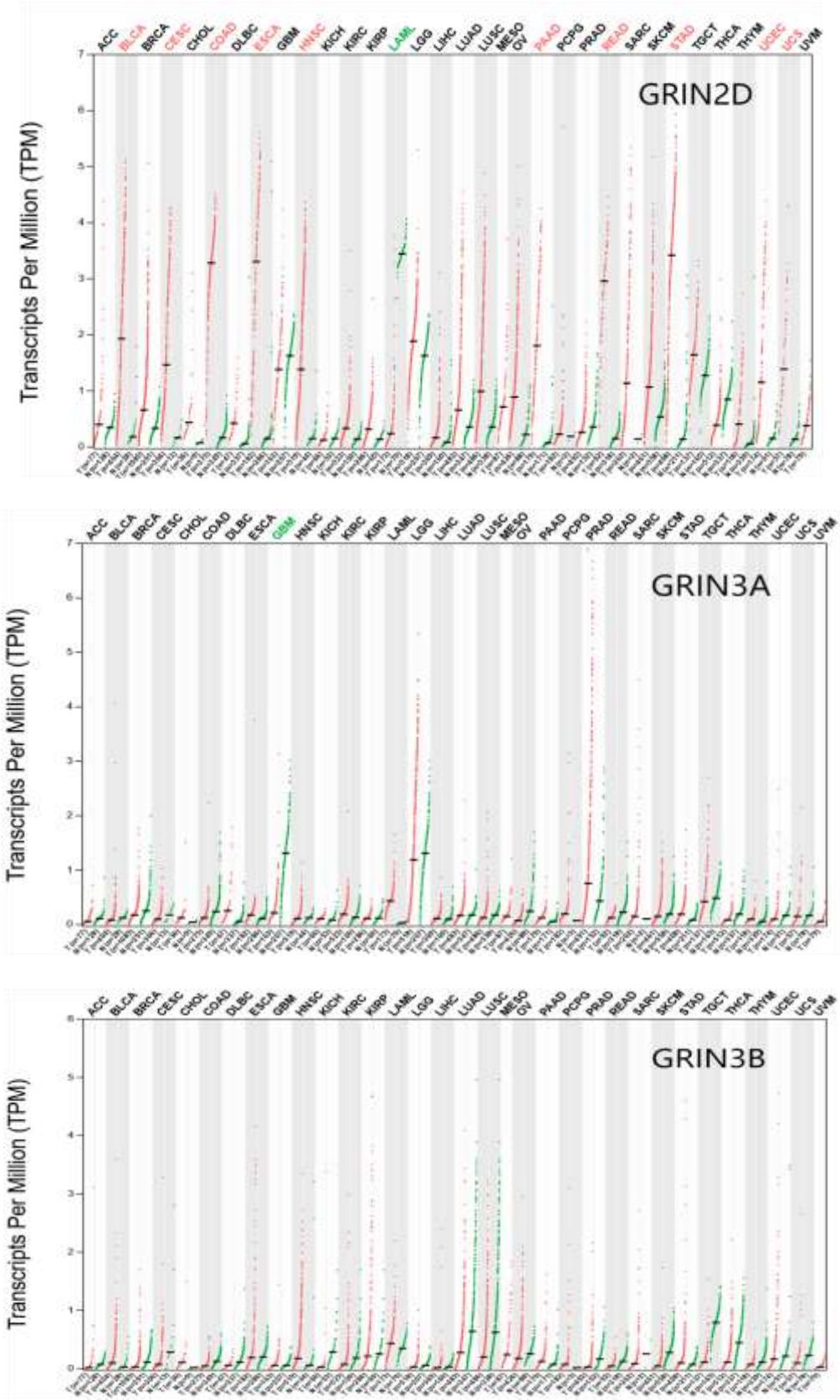


Figure 1 Differential expression of GRIN1, GRIN2A, GRIN2B, GRIN2C, GRIN2D, GRIN3A and GRIN3B. (A) Expression of 7 genes in 33 cancers. (B) Expression comparison of the 7 genes in tumor and normal tissues. Red represents high expression in tumor tissue, and green represents high expression in normal tissue. GRIN2D was more highly expressed with significant differences in many tumors compared to normal tissues, while other genes were less differentially expressed between normal and tumors.

And it could be seen that in glioblastoma multiforme (GBM), brain lower grade glioma (LGG) and testicular germ cell tumors (TGCT), the expression levels of GRIN1, GRIN2A, GRIN2B and GRIN2C were significantly lower than those in normal tissues, while GRIN2D failed to show a significant difference probably due to the amount of data. The level of GRIN2D expression in 33 tumors was further verified using the UCSC Xena and TIMER 2.0 databases. Based on the results of UCSC Xena, in colon adenocarcinoma (COAD), cholangio carcinoma (CHOL), stomach adenocarcinoma (STAD), lung squamous cell carcinoma (LUSC), esophageal carcinoma (ESCA), breast invasive carcinoma (BRCA), lung adenocarcinoma (LUAD), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), sarcoma (SARC), KIRC, LIHC, uterine corpus endometrial carcinoma (UCEC), head and neck squamous cell carcinoma (HNSC), bladder urothelial carcinoma (BLCA), kidney renal papillary cell carcinoma (KIRP) and rectal adenocarcinoma (READ), the expression of GRIN2D were significantly higher in tumor tissues than that in normal tissues (Fig. S1A). Whereas, the expression levels of GRIN2D in acute myeloid leukemia (LAML) and thyroid cancer (THCA) were significantly lower. Similar results were obtained through the TIMER2 database, expression of GRIN2D was significantly higher in KIRC, BLCA, ESCA, BRCA, LUAD, CESC, CHOL, LUSC, COAD, STAD, HNSC, UCEC, READ, KIRP and LIHC compared to normal tissues ($P < 0.05$) (Fig. S1B). In view of the differential expression of GRIN2D, it is conjectured that its expression may be significantly related to the progression and occurrence of various cancer types. Therefore, GRIN2D was selected for subsequent pan-cancer analysis.

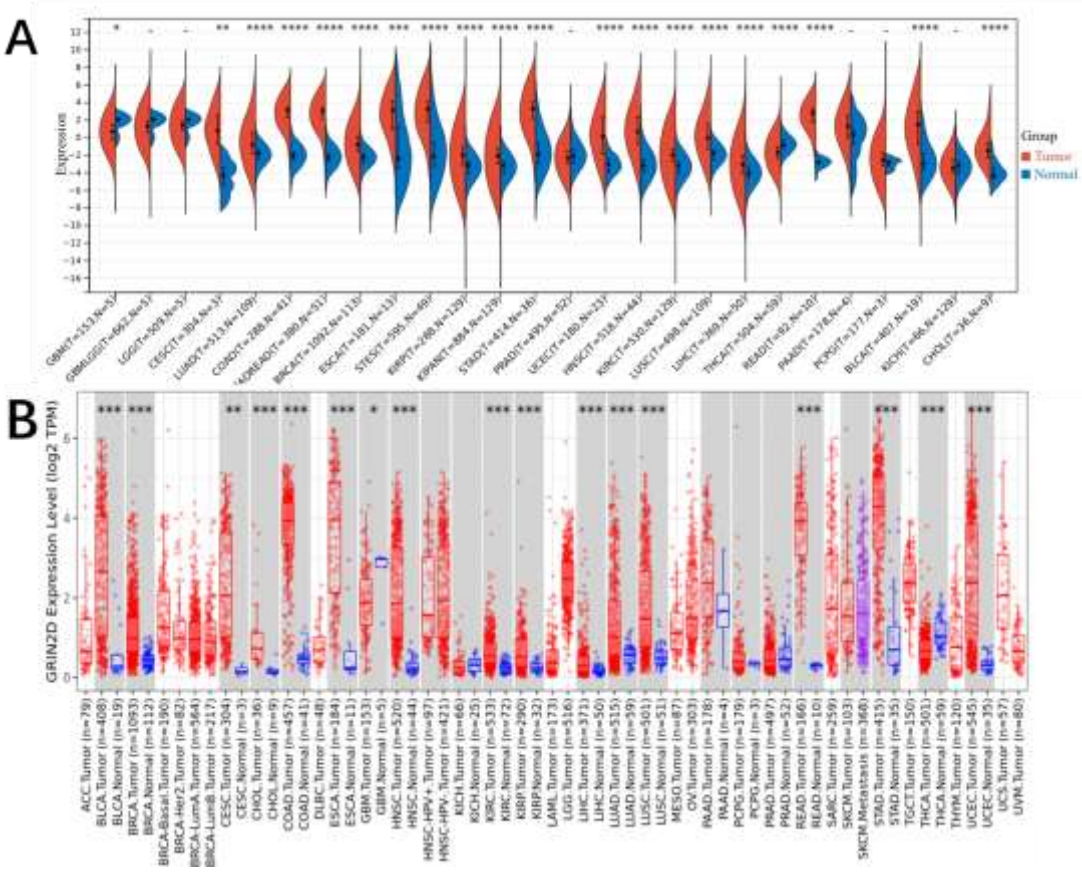


Figure S1. Differential expression of GRIN2D.

(A) Differential expression analysis of GRIN2D in 33 tumors based on UCSC xena database. (B) Differential expression analysis of GRIN2D based on TIMER 2.0 database. The abscissa in the figures represents tumors and normal tissues, corresponding to red and blue respectively. The ordinate represents the expression level of GRIN2D.

3.2 Correlation analysis between expression levels of GRIN2D and clinical stage

The correlation between the expression of GRIN2D and tumor clinical stage was investigated through UCSC Xena database. The findings demonstrated a favorable correlation between GRIN2D expression levels and the clinical stages of five different tumor types, including uveal melanoma (UVM) (P =0.046), KIRC (P =1.2e-05), adrenocortical carcinoma (ACC) (P =0.00025), LIHC (P =0.0049), BLCA (P =0.033) (Fig.2).

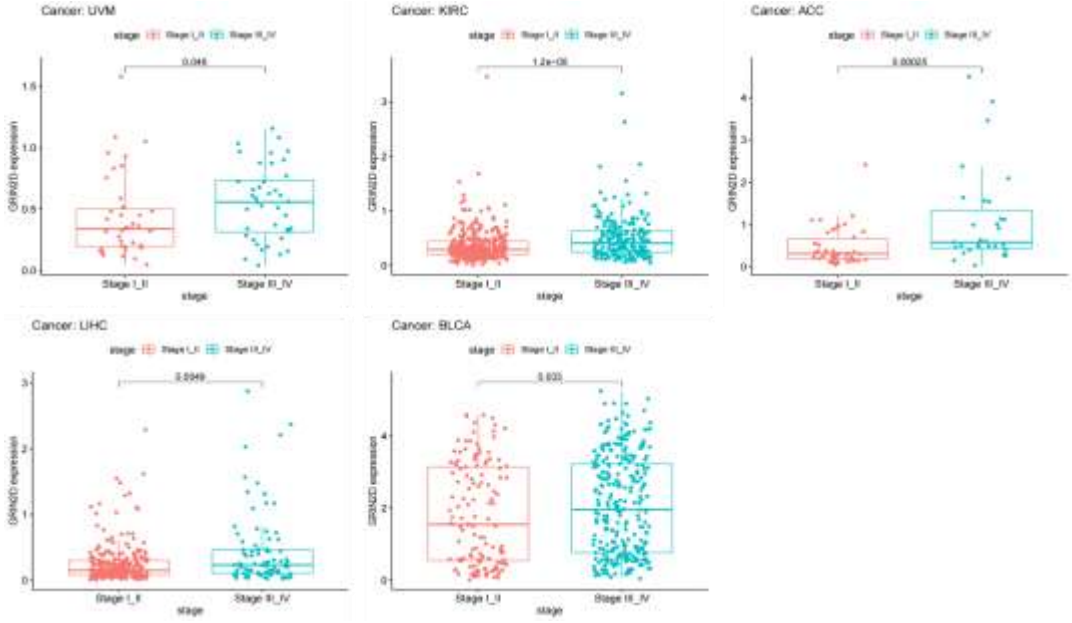
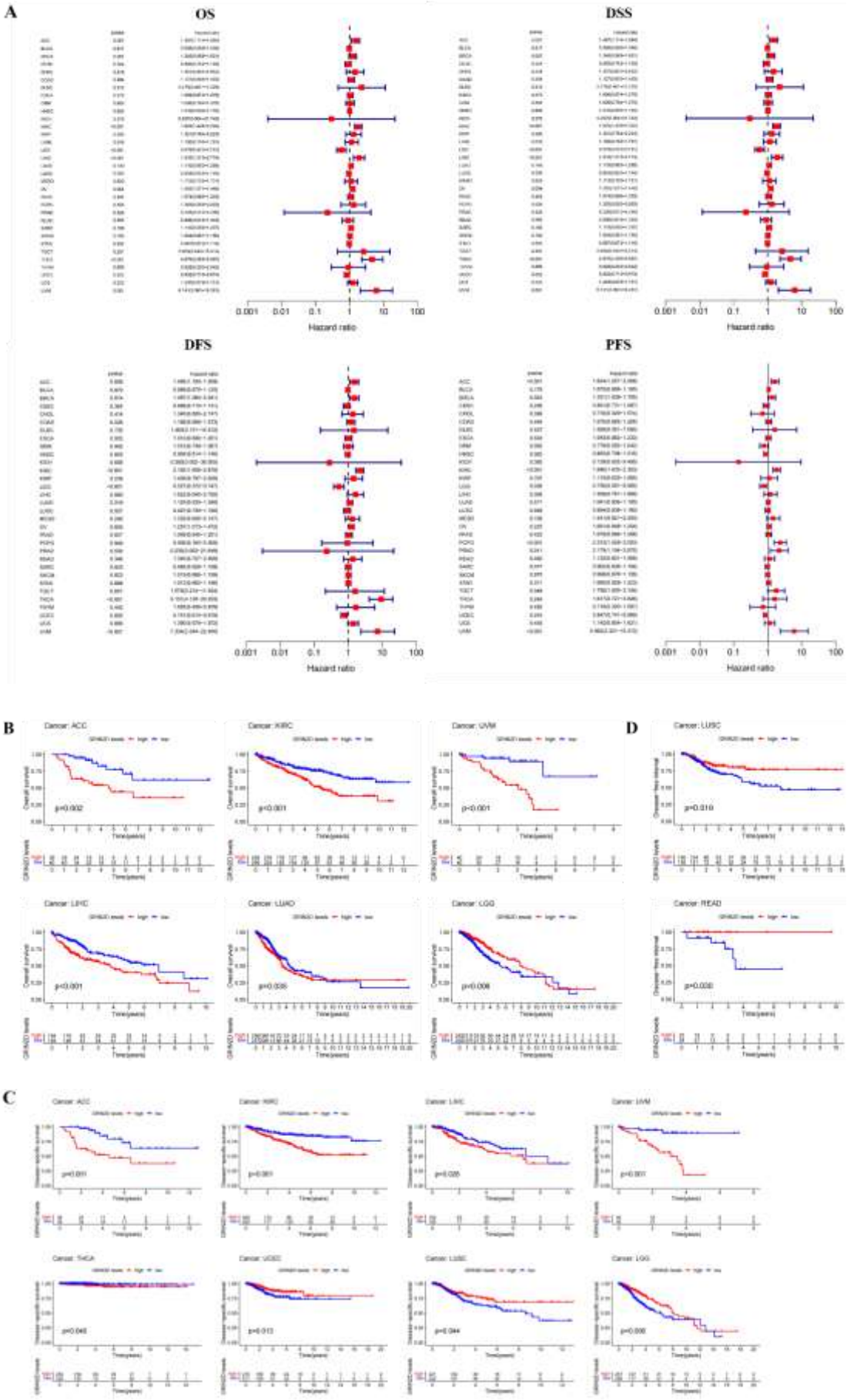


Figure 2 Association between GRIN2D expression and clinical stage of tumors in UVM, KIRC, ACC, LIHC, and BLCA. Red plots represent the expression of tumor stage 1 and 2, and blue plots represent the expression of tumor stage 3 and 4. The number on the horizontal line represents the P value.

The low expression of GRIN2D was seen in these tumors in the early stage, and the proportion of high expression in the late stage increased. Among these 5 tumors, KIRC, LIHC and BLCA had shown significant differences in differential expression analysis of GRIN2D. It was demonstrated that GRIN2D expression was positively correlated with clinical features in multiple tumors, suggesting that GRIN2D plays a certain role in promoting tumor progression.

3.3 Prognostic analysis of GRIN2D in Pan-cancer

Clinically, the prognosis of patients is an important evaluation index. The survival information from TCGA for 33 tumors was extracted, and the relationship between GRIN2D and tumor prognosis were performed. OS survival analysis results showed that in UVM (P <0.001), LIHC (P <0.001), LUAD (P =0.035), ACC (P =0.002) and KIRC (P <0.001), tumor patients have better prognosis for low expression of GRIN2D, and tumor patients have better prognosis for high expression of GRIN2D in LGG (P =0.006) (Fig. 3A and B). DSS survival analysis results revealed that in UVM (P <0.001), LIHC (P =0.026), ACC (P =0.001), THCA (P =0.040) and KIRC (P <0.001), tumor patients have better prognosis for low expression of GRIN2D, and in UCEC (P =0.013), LUSC (P =0.044) and LGG (P =0.006), tumor patients have better prognosis for high expression of GRIN2D (Fig. 3A and C). DFS analysis results revealed that in LUSC (P =0.019) and READ (P =0.030), tumor patients have better prognosis for high expression of GRIN2D (Fig. 3A and D). PFS survival analysis revealed that in ACC, KIRC, prostate adenocarcinoma (PRAD) and UVM, tumor patients have better prognosis for low expression of GRIN2D, and tumor patients with kidney chromophobe (KICH) or LGG have better prognosis for high expression of GRIN2D (Fig. 3A and E). Combined with differential expression analysis, clinical correlation analysis and prognostic analysis of GRIN2D, GRIN2D showed more significant significance in KIRC and LIHC. These investigations suggested that GRIN2D might function as a biomarker for predicting the tumor prognosis.


A
OS
DSS
DFS
PFS
Hazard ratio
B
C
D

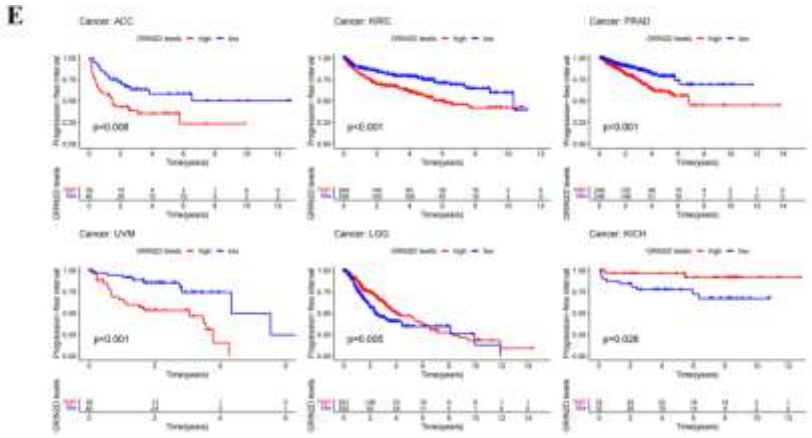


Figure 3 Association between GRIN2D expression and survival. (A) Forest plots of OS, DSS, DFS, PFS associations in 33 types of tumors. (B,C,D,E) KM analysis of the association between GRIN2D expression and OS, DSS, DFS, PFS. The red lines indicate the high GRIN2D expression group, while the blue lines indicate the low GRIN2D expression group.

3.4 Correlation of GRIN2D expression with TMB and MSI

TMB and MSI have been found to correlate with immunotherapy response and prognosis in cancer. To investigate the tumor mutation status of GRIN2D, mutation data of GRIN2D in 33 tumors were purchased from the UCSC Xena database. The analysis showed that the mRNA of GRIN2D was significantly mutated in several tumor types, including HNSC, CESC, LUSC, thymoma (THYM), ACC, pancreatic adenocarcinoma (PAAD), BRCA, UCEC, STAD, LIHC, BLCA, THCA, LUAD, ESCA (Fig. 4A). Meanwhile, GRIN2D expression was significantly correlated to MSI in tumors including skin cutaneous melanoma (SKCM), BLCA, THCA, STAD, LGG, LAML and KIRC (Fig. 4B). Among these tumor types, KIRC and LIHC also showed a significant correlation with TMB and MSI.

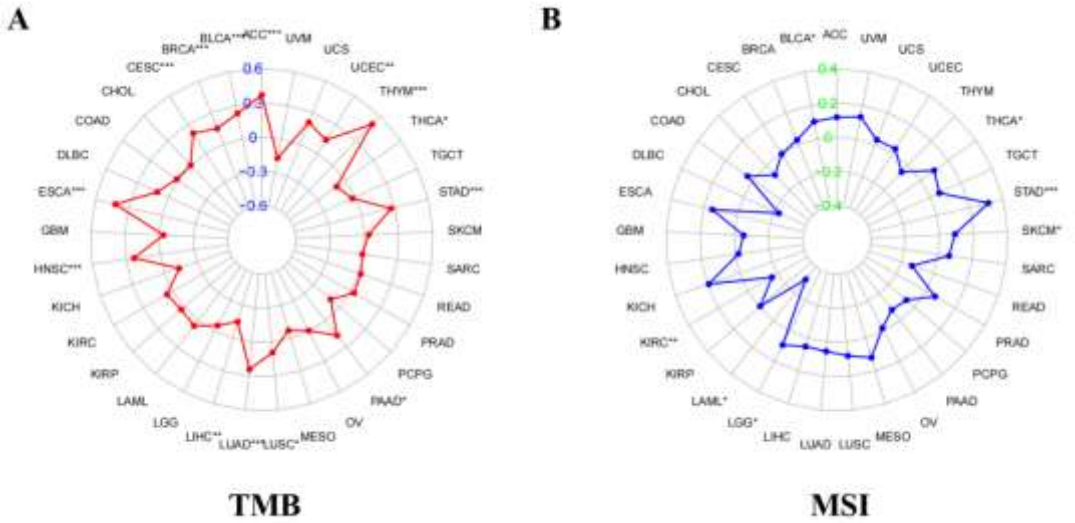


Figure 4 Associations between GRIN2D expression and TMB and MSI. (A) Clarify the relationship between GRIN2D expression and TMB. (B) Clarify the relationship between GRIN2D expression and MSI. *P < 0.05, **P < 0.01, and ***P < 0.001.

3.5 Correlation of GRIN2D expression with immunity

The tumor microenvironment (TME) plays a crucial role in the progression and treatment of cancer. To explore the potential correlation between GRIN2D expression and the tumor microenvironment, we utilized the ESTIMATE algorithm to assess immune scores and tumor purity in diverse samples from The Cancer

Genome Atlas (TCGA) cohort. Based on immune scores, we observed a positive correlation between GRIN2D expression and immune scores in BRCA, KICH, KIRC, KIRP, LIHC, PCPG, THCA, UCEC, and UVM. Conversely, in GBM, HNSC, LGG, STAD, and THYM, GRIN2D exhibited a negative correlation with immune scores (Fig. 5 and S3).

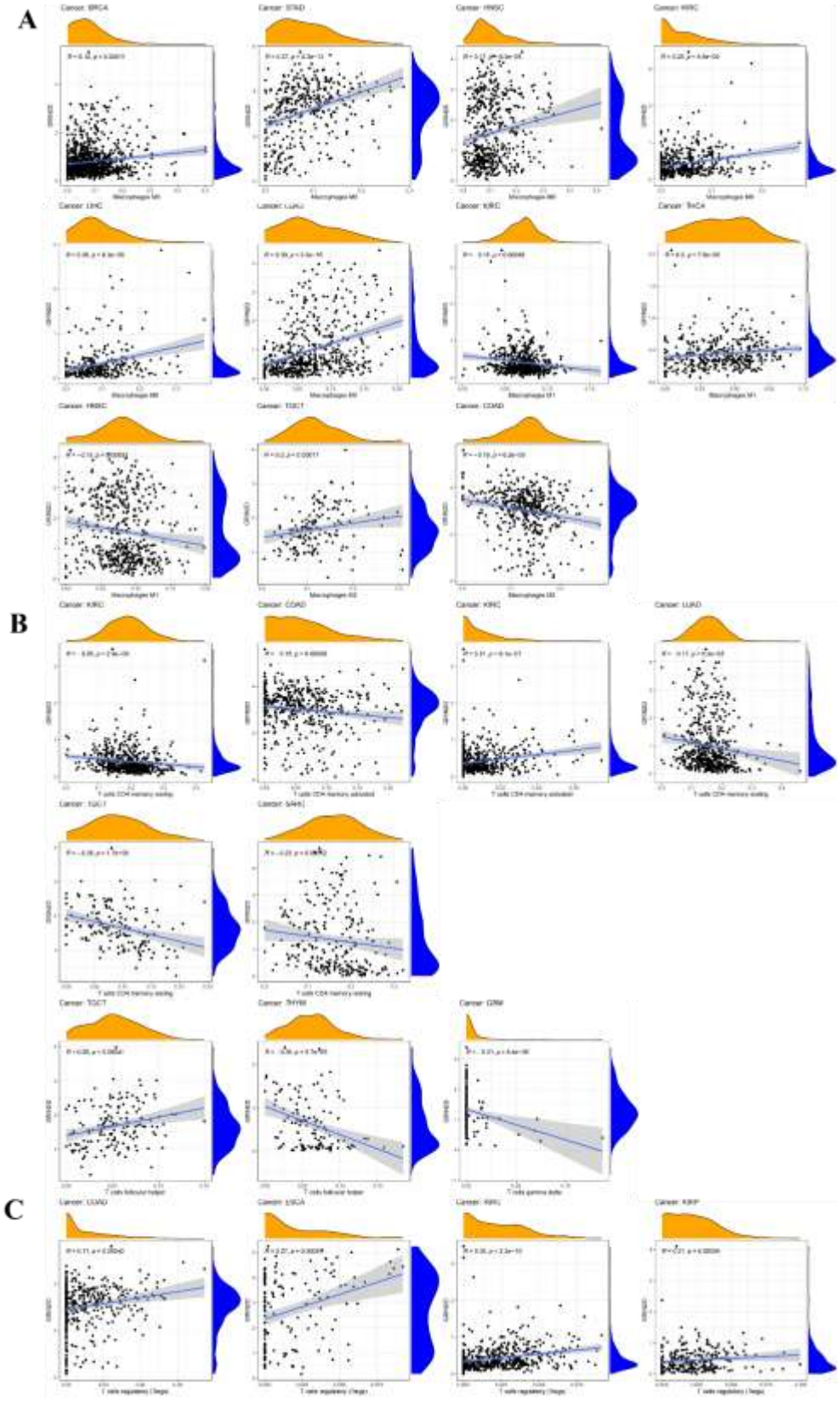
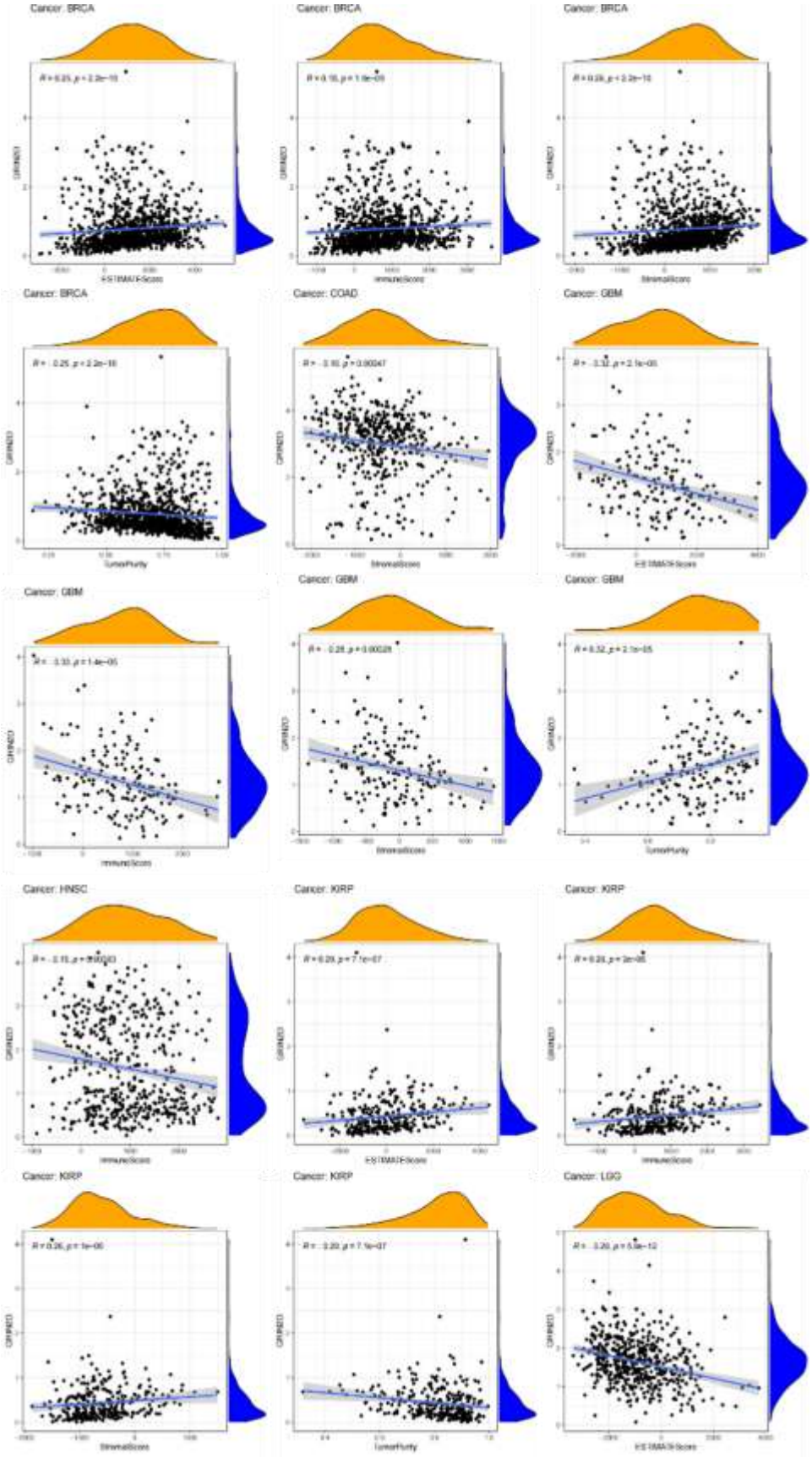
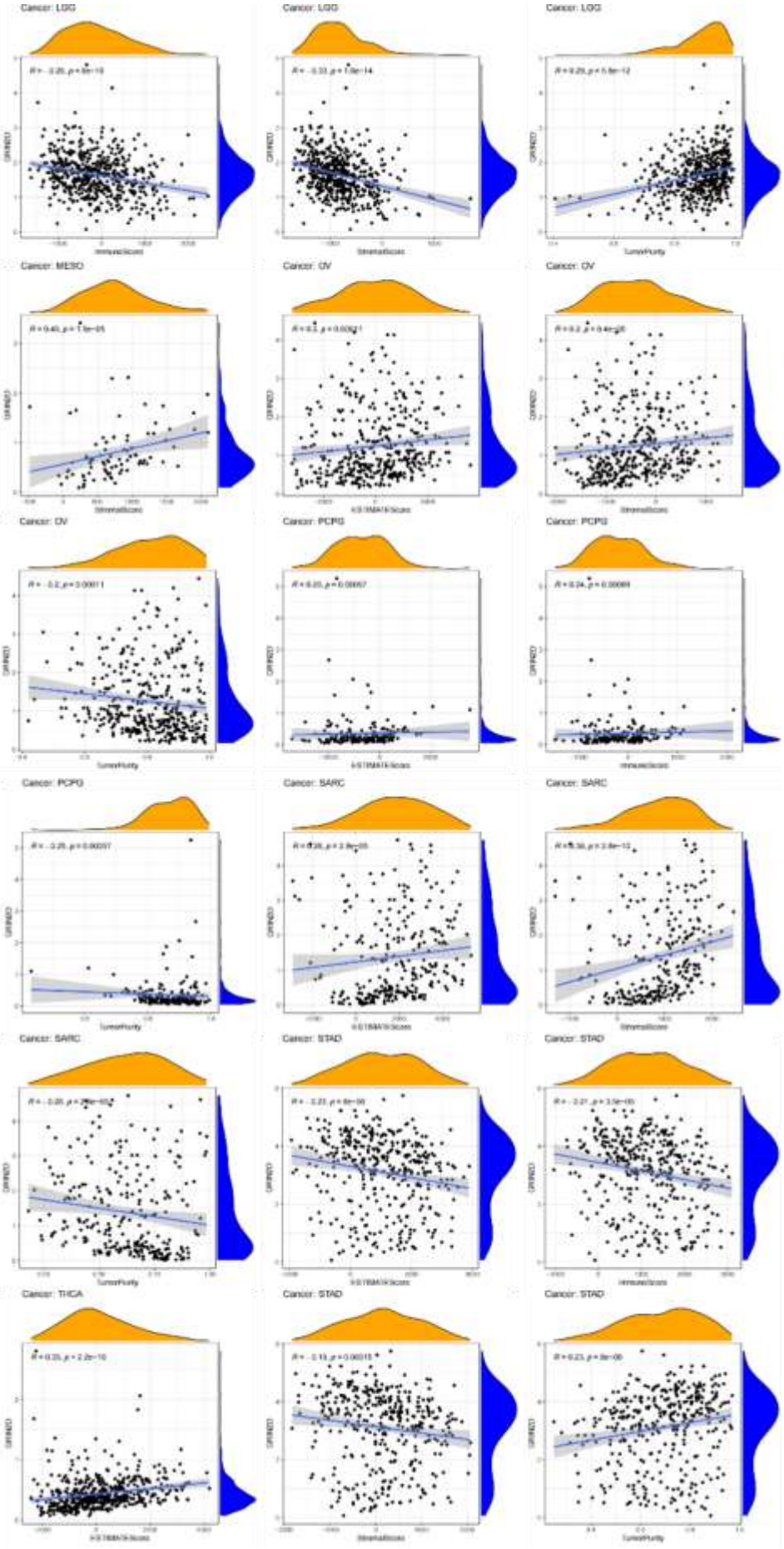


Figure 5 Correlation between GRIN2D gene expression and infiltration levels of different immune cells in pan-cancer. (A) Correlation between GRIN2D expression and tumor-associated macrophages

in BRCA, STAD, HNSC, KIRC, LIHC, LUAD, THCA, COAD, TGCT. (B) Correlation between GRIN2D expression and cytotoxic T cells and helper T cells in KIRC, COAD, LUAD, TGCT, SARC, THYM, GBM. (C) Correlation between GRIN2D expression and T cells regulatory (Tregs) in KIRC, COAD, ESCA, KIRP.




Cancer: LGG
Cancer: LGG
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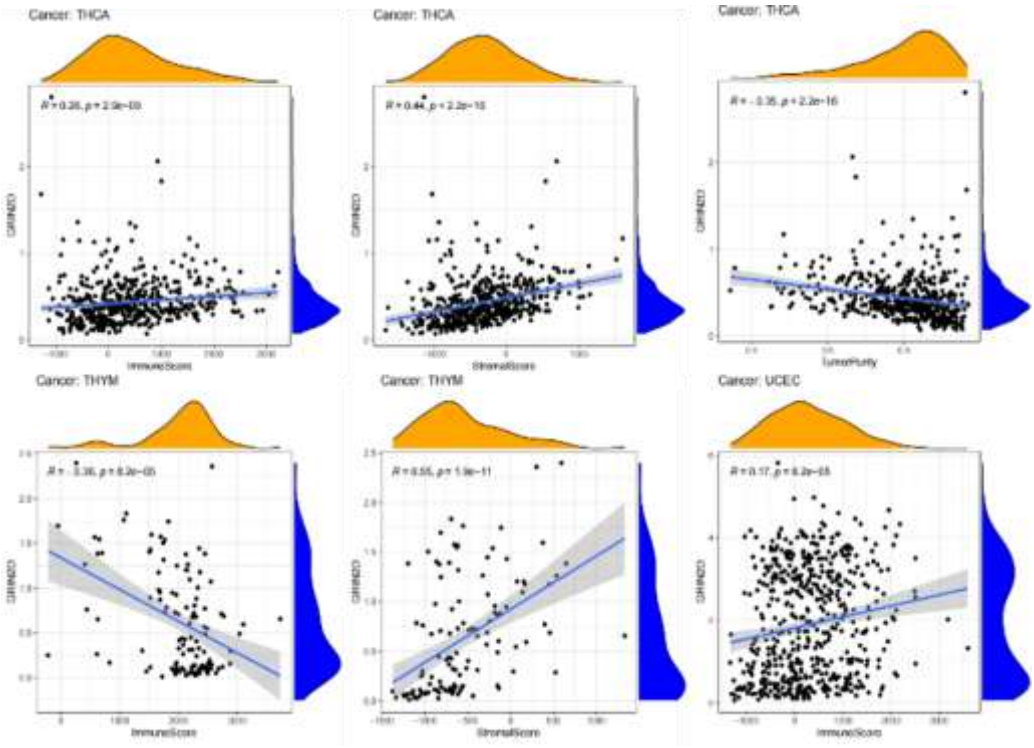


Figure S3. Correlation of GRIN2D expression with tumor microenvironment
Correlation between GRIN2D expression and immune score, stromal score, ESTIMATE score, tumor purity in a variety of tumors.

In different tumor types, specific immune cells may exert either promoting or inhibitory effects on cancer development. By analyzing the correlation between the abundance of various immune cells and GRIN2D expression across different tumors, we investigated the potential immunological impact of GRIN2D expression on tumor development. The expression levels of GRIN2D in LUAD, HNSC, thyroid carcinoma (THCA), THYM, KIRC, GBM, KIRP, COAD, LIHC, ovarian serous cystadenocarcinoma (OV), BRCA, ESCA, SARC, STAD, and TGCT were found to be closely associated with the infiltration of immune cells, playing a regulatory role in immune cell infiltration (Fig.6 and S2). Results indicated that GRIN2D expression was correlated with various tumor types and immune cells, including B cells, natural killer cells, tumor-associated macrophages, cytotoxic and helper T cells, regulatory T cells (Tregs), mast cells, and dendritic cells.

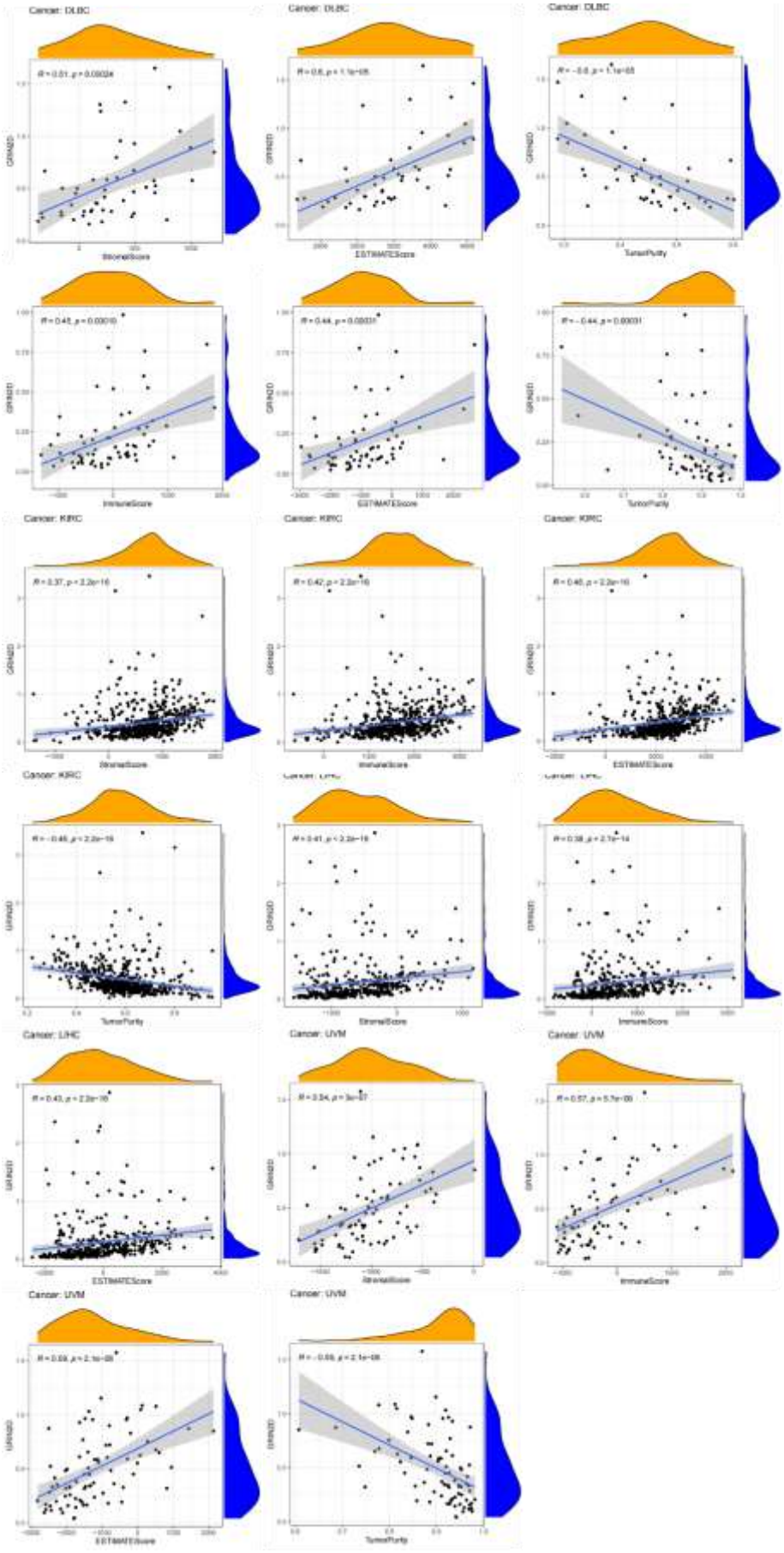


Figure 6 Correlation between GRIN2D expression and immune score, stromal score, ESTIMATE score, tumor purity in UVM, KIRC, KICH, LIHC and DLBC. These five tumors had the higher correlation coefficients.

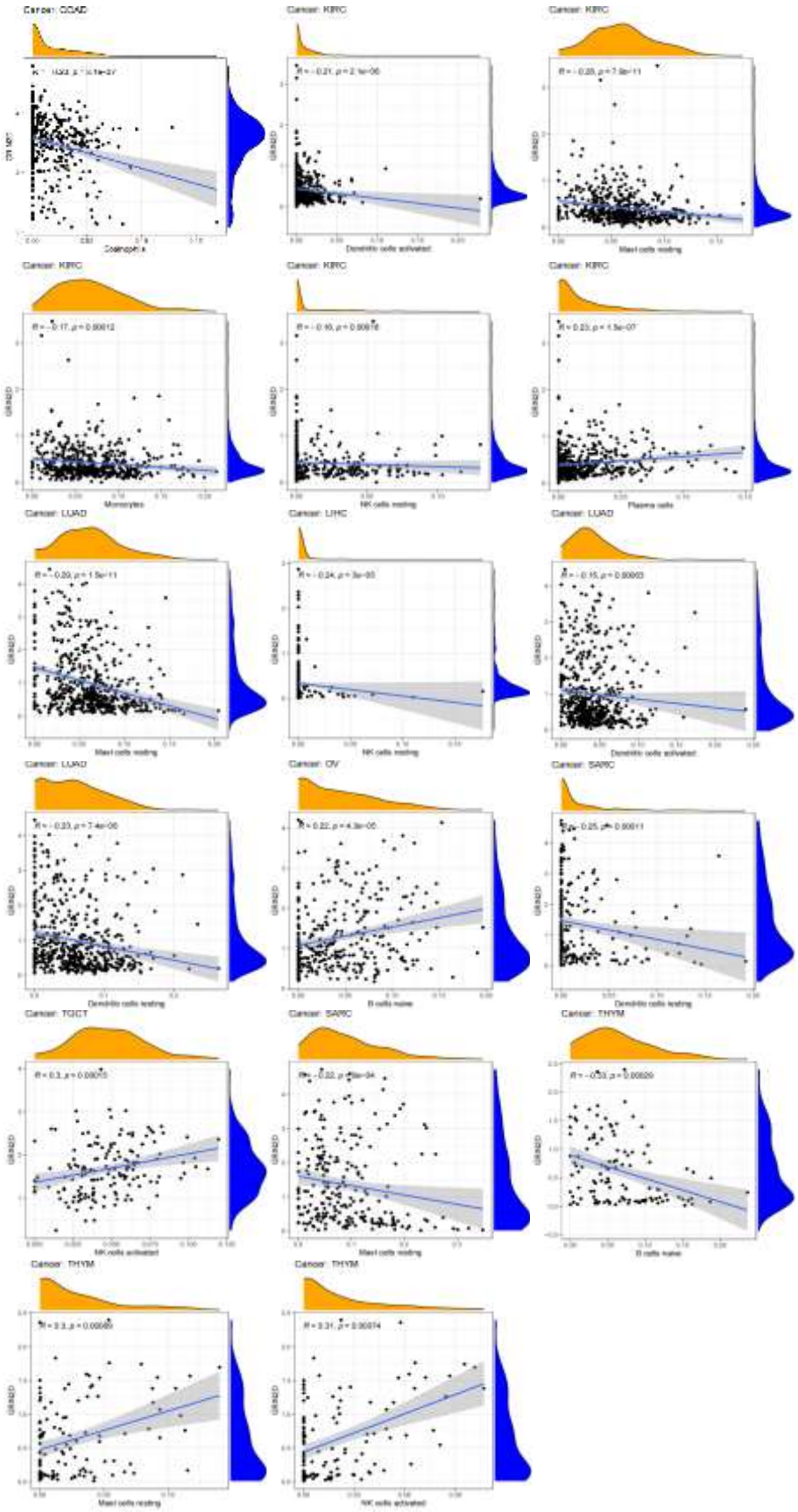


Figure S2. Correlation of GRIN2D expression with immunity cells
Correlation analysis between GRIN2D expression and multiple immune cells including B cells, dendritic cells, natural killer cells, mast cells, plasma cells and eosinophils in multiple cancer types.

In summary, GRIN2D may exert a regulatory role in cancer progression through its influence on the immune response across various tumor types. For instance, in THCA, GRIN2D expression positively correlated with ImmuneScore and was positively associated with Macrophages M1, suggesting a potential inhibitory effect on cancer progression. Conversely, in HNSC, the negative correlation between GRIN2D expression and ImmuneScore, as well as the negative association with the abundance of Macrophages M1, suggests a potential inhibitory role of GRIN2D in promoting tumor escape. In GBM, the negative correlation between GRIN2D expression and ImmuneScore, as well as the negative association with the abundance of T cells gamma delta, indicates a potential inhibitory effect on MT cells gamma delta, promoting tumor escape. Further investigations revealed associations between GRIN2D expression and various immune cell types in KIRP, ESCA, KIRC, LIHC, LUAD, and SARC, suggesting diverse regulatory roles in tumor development and escape.

This comprehensive analysis underscores the potential of GRIN2D to modulate cancer progression through its immunoregulatory effects in a variety of cancer types. The positive correlation of GRIN2D expression with ImmuneScore in THCA, and the positive association with Macrophages M1, suggests a potential inhibitory role of GRIN2D in cancer progression by enhancing Macrophages M1. Conversely, the negative correlation of GRIN2D expression with ImmuneScore in HNSC, and the negative association with the abundance of Macrophages M1, implies a potential role of GRIN2D in suppressing Macrophages M1 and promoting tumor escape. In GBM, the negative correlation of GRIN2D expression with ImmuneScore, and the negative association with T cells gamma delta abundance, suggests a potential inhibitory effect of GRIN2D on MT cells gamma delta, promoting tumor escape. In KIRP and ESCA, the positive correlation of GRIN2D expression with Tregs indicates a potential role of GRIN2D in inducing tumor progression by increasing Tregs abundance. In KIRC, the positive correlation of GRIN2D expression with ImmuneScore, and the positive association with Macrophages M0, Plasma cells, and Tregs, as well as the negative association with Dendritic cells activated, Macrophages M1, Mast cells resting, Monocytes, NK cells resting, T cells CD4 memory resting, suggests a potential role of GRIN2D in increasing Tregs levels, inhibiting the conversion of Macrophages M0 to Macrophages M1, and reducing the abundance of T cells CD4 memory resting and Dendritic cells activated, thereby promoting tumor progression. In LIHC, the positive correlation of GRIN2D expression with ImmuneScore, and the positive association with Macrophages M0, as well as the negative association with NK cells resting, suggests a potential role of GRIN2D in suppressing NK cells resting and promoting tumor escape. In LUAD and SARC, the negative correlation of GRIN2D expression with Dendritic cells resting, Mast cells resting, and T cells CD4 memory resting, suggests a potential role of GRIN2D in inhibiting Dendritic cells resting, Mast cells resting, and T cells CD4 memory resting, thereby inducing tumor progression.Since immune checkpoint related genes were closely related to tumor immune escape. Therefore, co-expression between GRIN2D and 44 immune checkpoint related genes were further explored and the results showed that GRIN2D is co-expressed with most genes in DLBC, KICH, LGG, LUAD, STAD, KIRC, KIRP, BRCA, COAD, LIHC, UVM, GBM, HNSC, THCA (Fig. 7). Among these genes, THFRSF14, CD276, LGALS9, TNFSF9, TNFRSF8, TNFRSF25 and TNFRSF18 showed stronger correlations.

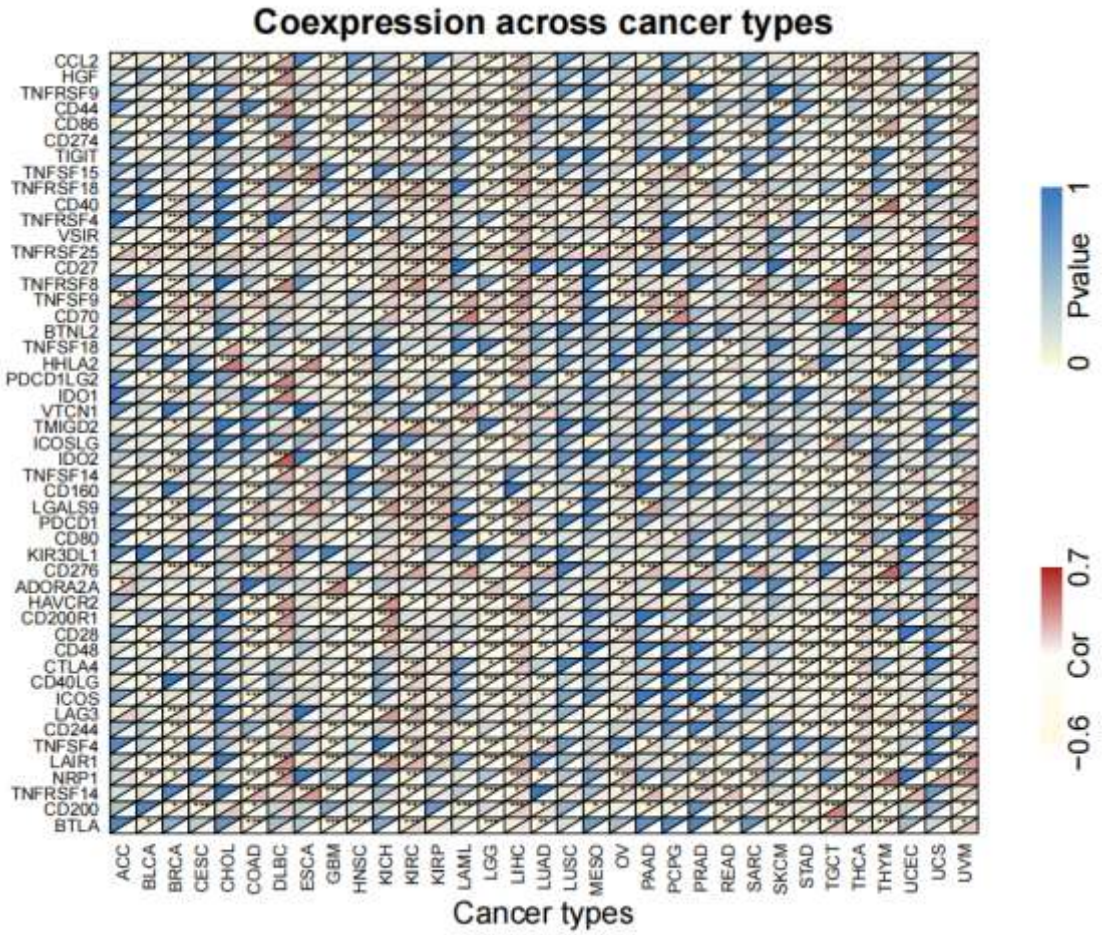
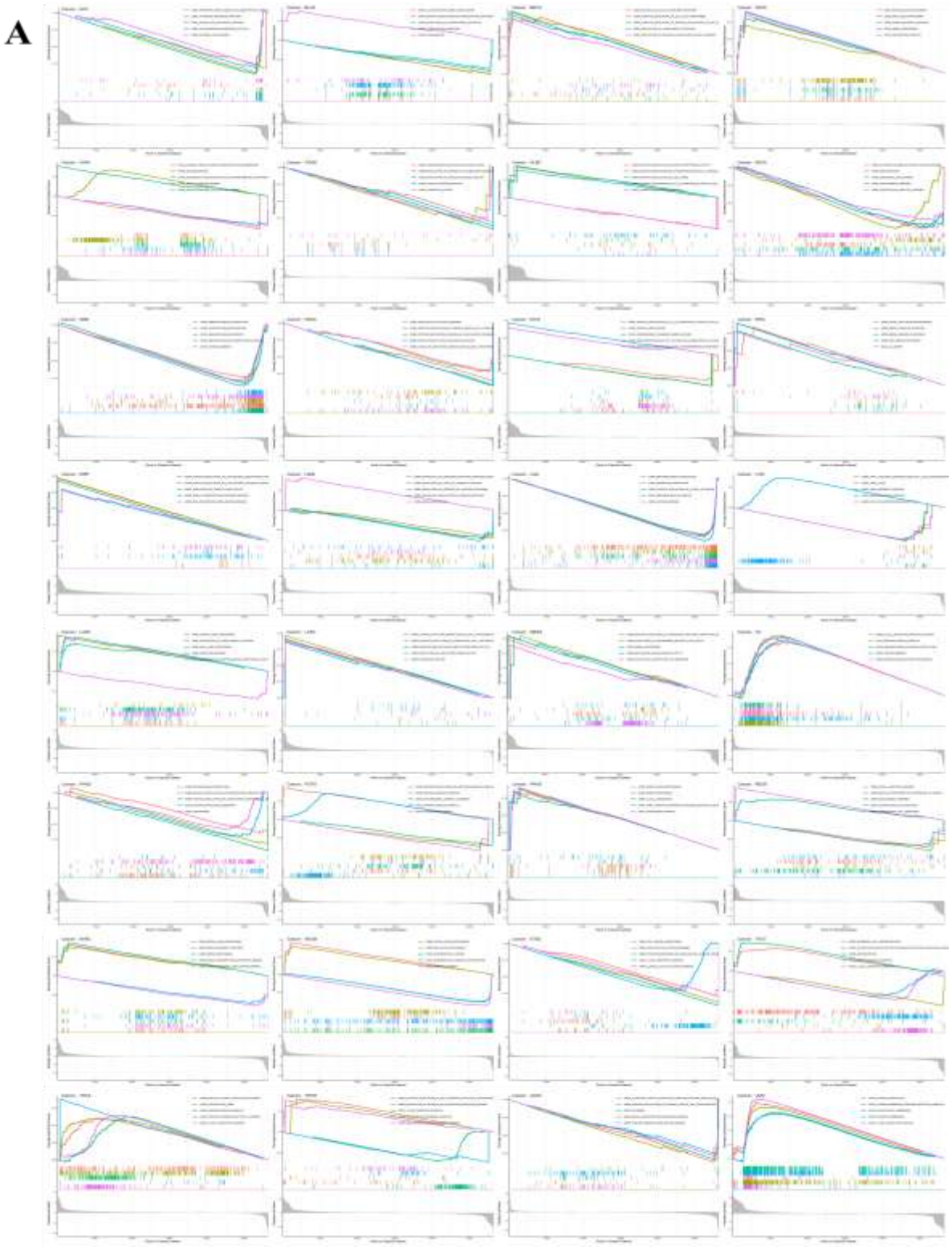


Figure 7 Heatmap showing the relationship between the immune checkpoint-related genes and GRIN2D expression. The correlation coefficient was shown in the bottom right triangle for each pair, and the p-value was shown in the top left triangle for each pair. *P < 0.05, **P < 0.01, ***P < 0.001.

3.6 GSEA for GRIN2D

To investigate the biological processes GRIN2D may be involved in tumor progression, we performed analysis using TCGA data and GO and KEGG data from the GSEA official website. The pathways with the top 5 enrichment scores in various tumors were shown in Figure 8. According to GO analysis results, immune-related biological processes were discovered to be highly correlated with GRIN2D expression, including complement activation, immune response, immunoglobulin complexing, antigen binding and phagocytosis recognition in BLCA, THCA, LGG, BRCA, KIRC, CHOL, UVM, GBM, HNSC, OV, PCPG, LIHC, Uterine Carcinosarcoma (UCS) and TGCT (Fig. 8A). Among them, the biological process of immunoglobulin complexing has been found in a variety of tumor types. KEGG enrichment analysis showed that several tumor-related and immune-related pathways such as antigen processing and presentation, autophagy regulation, and chemokine signaling pathways were activated in some tumors (Fig. 8B).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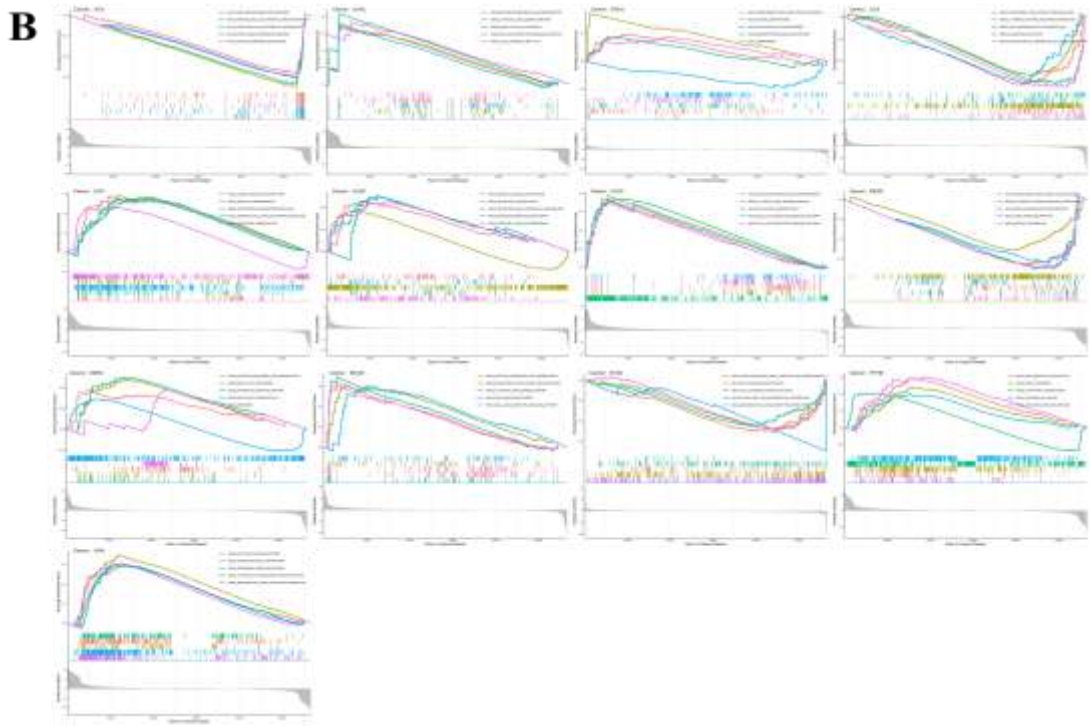

Figure 8 GSEA analysis of GRIN2D. (A) GO functional annotation of GRIN2D in various cancers. (B) KEGG pathway analysis of GRIN2D in multiple cancers. Each graph shows the 5 pathways or biological processes with the highest relative values of standard enrichment scores. Different colored curves represent various biological processes or regulatory pathways in various tumors.

3.7 Drug Sensitivity Analysis of GRIN2D

Since the above studies suggested that GRIN2D was related to the growth and progression of cancer, therefore, searching for potential drugs targeting GRIN2D offers a possibility for the treatment of pan-cancer. Correlation between GRIN2D expression and drug sensitivity in human cancer cell lines were further investigated using CellMiner database. The findings revealed a significant positive correlation between the expression of GRIN2D and the sensitivity of 34 drugs (Tab. S1). Notably, GRIN2D was more sensitive to 1,9-Pyrazoleanthrone, Foretinib, BMS-536924, Adavosertib, Astex FGF inhibitor, 8-Chloro-adenosine, ITRI-260, TAK-960 analog, Dovitinib, BML-277, AZD- 7762, PD-0325901, RO-4987655, LY-2606368, Lexibulin and Pimasertib (Fig. 9).

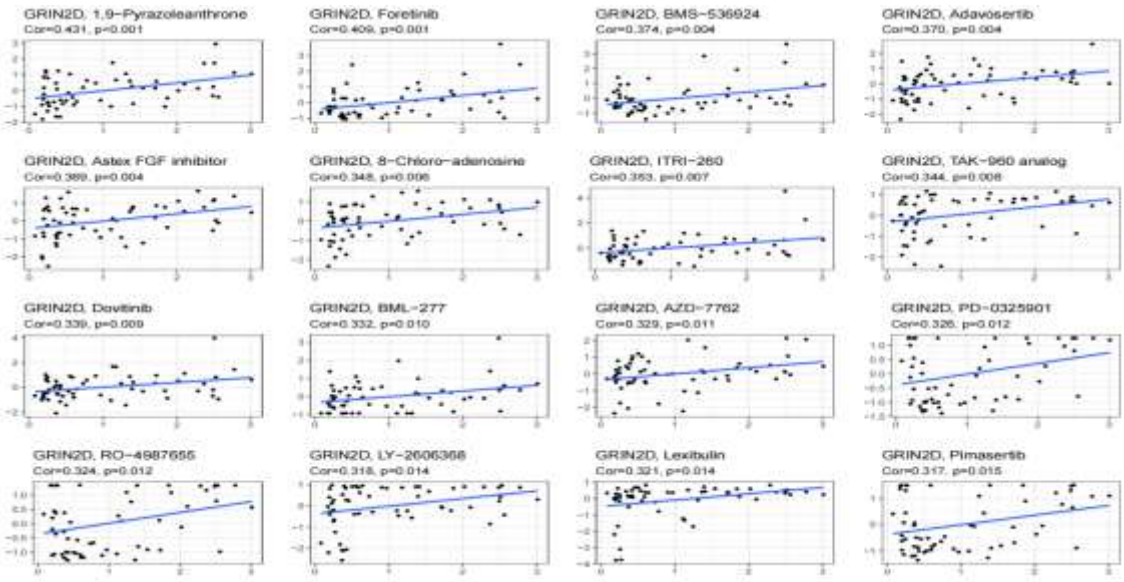

Figure 9 Drug sensitivity analysis of GRIN2D. The expression of GRIN2D was associated with the

sensitivity of 1,9-Pyrazoleanthrone, Foretinib, BMS-536924, Adavosertib, Astex FGF inhibitor, 8-Chloro-adenosine, ITRI-260, TAK-960 analog, Dovitinib, BML-277, AZD-7762, PD-0325901, RO-4987655, LY-2606368, Lexibulin and Pimasertib. A higher drug sensitivity score indicates that the cells was more sensitive to that drug treatment.

4. Discussion

NMDA receptors, one subtype of ionotropic glutamate receptors, are mainly distributed throughout the brain and closely related to neurological function [1, 2]. Studies have revealed that tumor cells contain numerous glutamate transport systems [25, 26], and glutamate synthesized and stored in tumor cells can be secreted outside of tumor cells through the glutamate transport system and subsequently bind to receptors to further exert regulatory effects on tumors [8, 9]. In gastric cancer, pancreatic cancer, small cell lung cancer, breast cancer, esophageal cancer and liver cancer, the increased expression of certain subunits of NMDA receptors can promote or inhibit tumor growth [15, 17, 18, 19, 27, 28, 29]. Relevant research reports on the role of a scaffold protein associated with NMDAR (GKAP), in modulating invasive growth and treatment response in pancreatic neuroendocrine tumors (PanNET), identify transcriptome signatures associated with low/inhibited NMDAR activity, showing favorable patient prognosis in various cancer types [30]. Pan-cancer analysis of NMDA receptors can reveal their similarities and distinctions across various tumors and provide possibility for the development of cancer medicines, diagnosis, and prevention [31]. We first analyzed the expression levels of 7 NMDA receptor subunits in different tumor tissues and normal tissues, and found that GRIN2D was higher expressed in various tumor tissues compared with normal tissues, while other genes showed less difference in expression levels between normal tissues and tumors. After that, the expression of GRIN2D was explored through the TCGA database and the TIMER2.0 database, and similar results were obtained, showing that the expression of GRIN2D in various tumors was significantly higher than that in normal tissues. Among the NMDAR subunits, in the already published data, the subunit more associated with tumor signalling is NMDAR2B expressed by GRIN2B gene. In this analysis we do not find high expression of this subunit in cancer subtypes and no so much difference of expression respect to normal tissue. The primary reason is that GRIN2B is predominantly expressed in brain tissue [3], exhibiting lower expression in other normal tissues and tumor tissues relative to the gene we studied, GRIN2D (Fig.1 and S4).

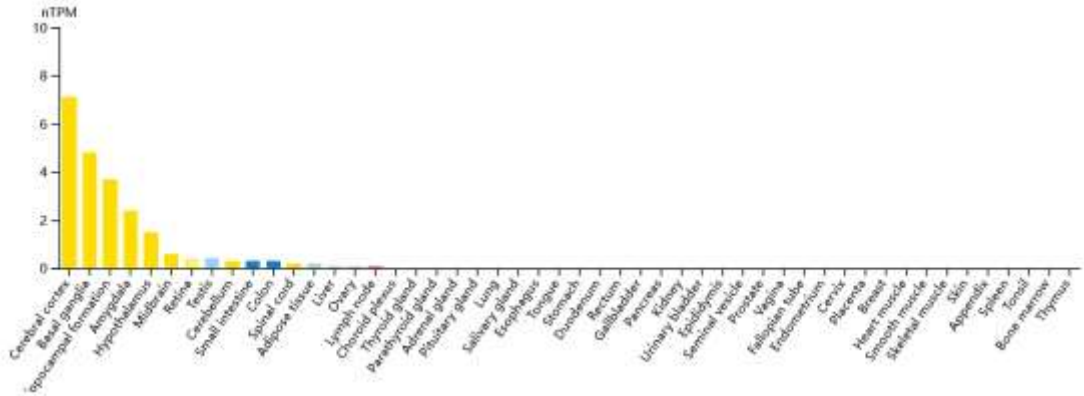

Figure S4. RNA expression of GRIN2B in normal tissues.
RNA expression levels of GRIN2B in normal tissues from HPA and GTEX databases. It can be seen that GRIN2B is mainly expressed in cerebral cortex, basal ganglia, hippocampal formation, amygdala and hypothalamus.

Secondly, although GRIN2B does not show differential expression in tumors and normal tissues, we cannot dismiss its potential role in tumor tissues [17, 14]. The role of GRIN2D in tumors is currently less explored in scientific research, and our preliminary investigation delves into the impact of GRIN2D on pan-cancer. A recent study has indicated high expression of

GRIN2D in pancreatic ductal adenocarcinoma (PDAC) cells, further promoting its oncogenic function [32], corroborating similar results from our bioinformatics analysis. Based on the significant differential expression characteristics of GRIN2D subunit in these seven subunits found in this study, further analysis and discussion on it was conducted.

Then, it was explored the significance of GRIN2D on tumor prognosis from different perspectives, including OS, DFS, DSS and PFS. The results showed that the expression of GRIN2D was associated with the prognosis of patients with several tumor types. Both OS and DSS analyses indicated that patients with high GRIN2D expression in ACC, KIRC, and LIHC had a lower survival rate, and conversely, high expression of LGG patients might lead to better prognosis. In the studies of differential expression analysis and clinical correlation analysis, the expression of GRIN2D in KIRC and LIHC was significantly higher than that in normal tissues, and the expression in late tumor was significantly higher than that in early tumor. Combined with prognostic analysis, GRIN2D had more significant significance for KIRC and LIHC, and the high expression of GRIN2D might promote the progression of these two tumors and the prognosis of patients is worse. These studies suggested that GRIN2D might serve as a prognostic and diagnostic biomarker in certain tumors, especially KIRC and LIHC.

TMB and MSI are promising pan-cancer predictive biomarkers with high predictive value for prognosis of tumor patients [21, 22], and can guide immunotherapy [33]. According to the study, in 14 tumor types, GRIN2D expression was discovered to be related to TMB, and in 7 tumor types, it was strongly related to MSI. It was suggested that the TMB and MSI of cancer were influenced by the expression level of GRIN2D, and the effect of immunotherapy and the prognosis of patients might also be affected. When exploring the relationship between the expression of checkpoint genes and GRIN2D, it was found that GRIN2D demonstrated a significant association with the expression of checkpoint genes, especially THFRSF14, CD276, LGALS9, TNFSF9, TNFRSF8, TNFRSF25, TNFRSF18. These genes were mainly involved in the immune response involving T cells. Therefore, the expression of GRIN2D might be related to the immunotherapy response of patients.

TME is made up of various elements, including stromal cells, immune cells, cancer cells, and others, and the tumor microenvironment affects not only the occurrence and progression of tumor metastasis, but also the primary tumor treatment efficacy [34]. Many studies have shown that the TME is critical for both tumor development and therapeutic response, and harnessing immune cells to fight tumors provides a new strategy for tumor therapy [35]. Various researches have found that anti-NMDA receptor encephalitis were associated with the autoimmunity [11], and tumor was the main cause of its occurrence. In the analysis of immune cells, it was revealed that the expression of GRIN2D was correlated with a variety of immune cells in tumors, among which cytotoxic T cells and helper T cells, Tregs and tumor-associated macrophages played more important role in a variety of tumor types. Tumor-associated macrophages (TAMs) exhibiting an M2-like phenotype are characterized by traits associated with angiogenesis, immune suppression, promotion of tumor growth, vascular invasion, metastasis, tumor stemness, and adverse prognosis. M2 macrophages produce anti-inflammatory cytokines such as IL-10 and inhibit the recruitment and activation of T cells. Conversely, TAMs with an M1-like phenotype (pro-inflammatory) are associated with tumor suppression [36]. Our investigation reveals that GRIN2D expression may positively correlate with Macrophages M0 (BRCA, HNSC, KIRC, LIHC, LUAD, and STAD), negatively correlate with Macrophages M1 (HNSC and KIRC), and positively correlate with Macrophages M2 (TGCT). GRIN2D might promote the differentiation of Macrophages M0 into Macrophages M2, inhibiting differentiation towards Macrophages M1 and fostering tumor progression. The activation of NMDA receptors is considered to be associated with the Foxo1/PPARγ signaling pathway, leading to the manifestation of a phenotype in macrophages that promotes tumor growth, specifically the M2 macrophage phenotype [37, 38]. Suppressing the expression of GRIN2D can reduce NMDA receptor activation, thereby diminishing the differentiation of M2 macrophages and exerting a certain anti-tumor effect. Simultaneously, we find evidence suggesting that GRIN2D may function

as a tumor suppressor gene in THCA by enhancing Macrophages M1 to inhibit cancer progression. The NMDAR is an ion channel receptor widely distributed in the nervous system, participating in the regulation of neurotransmitter release and communication between neurons. The NMDAR may be involved in modulating the activity of immune cells. Immune cells, such as T cells and macrophages, express NMDA receptors, and inhibiting the expression of GRIN2D may inhibit the activation of NMDAR and affect the function and immune response of immune cells. Tregs, acting as suppressors, shield tumor cells from the cytotoxic attacks of CD8+ T cells [39]. Our findings indicate a positive correlation between GRIN2D expression and Tregs in COAD, ESCA, KIRC, and KIRP. GRIN2D, acting as a tumor oncogene, may potentially promote Tregs generation. Cytotoxic T cells and helper T cells play pivotal roles in promoting tumor clearance through cellular lysis mechanisms or modulation of the TME. Their association with better prognoses is noted in melanoma, renal cell carcinoma, colorectal cancer, esophageal cancer, and squamous cell carcinoma. In COAD, GBM, KIRC, LUAD, SARC, TGCT, and THYM, GRIN2D expression is negatively correlated with cytotoxic T cells and helper T cells. GRIN2D may exert a pro-carcinogenic effect by suppressing the levels of these cells. The NMDAR may influence the immune system by regulating the release of neurotransmitters, such as glutamate. Experimental evidence demonstrates that glutamate binds to NMDAR on the surface of mast cells, significantly affecting the gene expression in mast cells, including the upregulation of a series of inflammation-related genes such as IL-6 and CCL2 [40]. Additionally, glutamate induces the upregulation of the transcription factor FosB, which may form an AP-1 complex by dimerizing with members of the Jun family, participating in various cellular processes, including cell proliferation and differentiation. The expression of GRIN2D is correlated with mast cells in various tumors (KIRC, LUAD, THYM, SARC). Inhibiting the expression of GRIN2D can suppress the binding of NMDAR to glutamate, thereby exerting an inhibitory effect on tumor proliferation. Notably, in KIRC, where poorer prognosis correlates with GRIN2D expression, a stronger association with various immune cells is observed. GRIN2D expression positively correlates with Macrophages M0, plasma cells, and Tregs, while negatively correlating with dendritic cells activated, Macrophages M1, resting mast cells, monocytes, resting NK cells, and resting memory CD4 T cells. In KIRC, GRIN2D may induce tumor progression by inhibiting Macrophages M0 to M1 differentiation, increasing Tregs levels, and reducing other immune cell levels that inhibit tumors. Further research is warranted to elucidate the intricate role of GRIN2D in this context.

Furthermore, the enrichment analysis results showed that several tumor-related and immune-related pathways such as autophagy regulation, complement activation, immune response, immunoglobulin complexing, and chemokine signaling pathways were activated in some tumors with low GRIN2D expression. These studies all revealed that GRIN2D may serve as a key immunoregulatory target in tumor progression. Membrane proteins, as potential mediators of the contact between tumor cells and the external environment, play a crucial role in the occurrence, development and metastasis of tumors, and are also the preferred choice for regulating tumor biological characteristics and potential therapeutic targets. The protein expression of GRIN2D is mainly on the cell membrane, which is more meaningful for tumor prognosis and as a potential therapeutic target.

At last, by using the CellMiner database, it was found that GRIN2D expression positively correlated with sensitivity to 34 drugs, including 1,9-Pyrazoleanthrone, Foretinib, BMS-536924 and Adavosertib. Among these drugs, Foretinib is an oral MET, RON and VEGFR2 multi-kinase inhibitor with antitumor activity. Adavosertib is a WEE1 inhibitor, mainly for the treatment of ovarian cancer and other solid tumors such as pancreatic cancer. Most of these drugs are targeted drugs in clinical trials for the treatment of various malignant tumors. As a non-competitive NMDA receptor antagonist, memantine (MK-801) has been FDA-approved for the treatment of Alzheimer's disease (AD) and is widely used in the field of the nervous system. Research has shown that memantine can inhibit tumor development in gastric cancer, SCLC, liver cancer, and pancreatic neuroendocrine tumors [15, 18, 19]. Concurrently, studies have revealed a

significant association between MK801 and pancreatic ductal adenocarcinoma (PDAC), with memantine significantly prolonging the survival time of PDAC genetically engineered mice [35]. MK-801 disrupts immunosuppressive activities in TAMs by inhibiting calcium influx and reactive oxygen species production [38]. Blocking NMDAR alters TAM phenotypes, enhancing their ability to promote anti-tumor immunity, and the combination of MK-801 with anti-PD-1 antibody eliminates established preclinical liver tumors, suggesting a potential therapeutic strategy for hepatocellular sarcoma. Therefore, memantine holds great potential as a future anticancer agent. In short, NMDA receptors might also be a potential target for these drugs to exert their antitumor activity.

5. Conclusion

In conclusion, the data in this study analyzed the close correlation and prognostic value of NMDA receptors in multiple human cancers. Within the genes encoding NMDA receptor subunits, GRIN2D exhibits heightened expression across various tumors, suggesting its potential influence on the prognosis of diverse cancers. The expression levels of GRIN2D show correlations with TMB, MSI, immune-related genes, and immune cell infiltration. Further analysis reveals that GRIN2D may primarily modulate tumor immune function by mediating T cells, regulatory T cells, and tumor-associated macrophages. The varied impact of GRIN2D on the immune functionality of the tumor microenvironment is evident across different cancer types. The study found two tumors more related to GRIN2D, KIRC and LIHC, which may provide reference for their diagnosis and prognosis, and to provide anti-cancer strategies from an immune perspective.

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Author Statements

Yijia Xu and Mingyi Zhao conceived and designed the experiments. Qingzheng Zhou, Yuying Lu, Yaru Liu, Yu Zhang and Jianfang Sun conducted the databases prediction. Qingzheng Zhou, Yuying Lu, Yu Zhang, Shubing Jia, Haochen Zhang and Long He analyzed the data. Qingzheng Zhou, Yuying Lu, Yu Zhang and Yijia Xu drafted the paper. All authors revised the paper and approved the final manuscript. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Conflict of Interest Statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Highlights

- The study found GRIN2D demonstrates distinct expression patterns compared to other genes within the NMDA receptor subunit-encoding family in the majority of tumors and normal tissues. GRIN2D was used as a potential tumor marker to evaluate the prognosis of tumor patients.
- It is first found that GRIN2D might become a key immunomodulatory target in tumor progression. GRIN2D may exert its influence on tumor immune escape by regulating macrophages, T cells, and regulatory T cells.
- The study found two tumors more associated with GRIN2D, kidney renal clear cell carcinoma (KIRC) and liver hepatocellular carcinoma (LIHC), for which NMDA receptors might be more significant for their diagnosis, prognosis and immunotherapy.

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Abbreviations

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
TCGA	The Cancer Genome Atlas
OS	overall survival
DSS	disease specific survival
DFS/DFI	disease free survival
PFI	progression free interval
RFS	relapse free survival
TMB	tumor mutational burden
MSI	microsatellite instability
TME	tumor microenvironment
AD	Alzheimer's disease
TAMs	Tumor-associated macrophages