International Journal of Immunopathology & Pharmacology

Original Research Article

Procollagen C-protease enhancer protein is a prognostic factor for glioma and promotes glioma development by regulating multiple tumor-related pathways and immune microenvironment

International Journal of Immunopathology and Pharmacology
Volume 36: 1–19

DOI: 10.1177/03946320221104548
journals.sagepub.com/home/iji
SAGE

Zijun Zhao[1,†], Jiahui Zhao[2,†], Zairan Wang[1,†], Yue Wu[3], Zhanzhan Zhang[1], Zihan Song[1], Jihao Miao[1], Boheng Liu[4], Shiyang Zhang[1], Boyu Sun[1] and Zongmao Zhao[1]

Abstract

Objectives: Glioma is a common type of brain tumor with high incidence and mortality rates. Procollagen C-protease enhancer protein (PCOLCE) has been shown to regulate tumor growth and metastasis in several cancers. However, the role of PCOLCE in glioma is unknown. This study aims to assess the association between PCOLCE and prognosis of glioma, and investigated the potential mechanisms.

Methods: The prognostic value of PCOLCE was determined using data from nine publicly available glioma cohorts. We also investigated the relationship between PCOLCE and glioma immune microenvironment and predicted response to immunotherapy based on the expression levels of PCOLCE. The potential roles of PCOLCE in glioma were also explored and validated in cell experiment.

Results: Survival analysis suggested that high-PCOLCE expression was associated with poor prognosis in glioma. Upregulation of PCOLCE enhanced an immune suppressive microenvironment in glioma by regulating immunocyte infiltration and Cancer-Immunity Cycle. Cox and ROC analysis revealed that PCOLCE was a prognostic factor for glioma and could be used to predict survival of the patients. Patients with low-PCOLCE expression were more likely to respond to Immunotherapy with ICI (immune checkpoint inhibitor) and survive longer. Enrichment analysis showed that PCOLCE was associated with multiple tumor-related pathways. Finally, we demonstrated that the knockdown of PCOLCE inhibited glioma development by regulating cell cycle and promoting apoptosis in in vitro experiments.

Conclusion: PCOLCE promotes glioma progression by regulating multiple tumor-related pathways and immune microenvironment and can be used as a prognostic factor for glioma.

[1]Department of Neurosurgery, The Second Hospital of Hebei Medical University, Shijiazhuang, China
[2]Department of Neurology, Beijing Tiantan Hospital, Capital Medical University, Beijing, China
[3]Department of Neurology, The Second Hospital of Hebei Medical University, Hebei, Shijiazhuang, China
[4]Department of Thoracic Surgery, The Fourth Hospital of Hebei Medical University, Hebei, Shijiazhuang, China

[†]These authors contributed equally to this work.

Corresponding author:
Zongmao Zhao, Department of Neurosurgery, The Second Hospital of Hebei Medical University, Heping West Road No. 215, Shijiazhuang 050000, China.
Email: zzm692017@sina.com

Keywords
Glioma, prognosis, procollagen C-protease enhancer protein, enrichment analysis, immunotherapy response, immune microenvironment

Background

Glioma is the most common type of tumor affecting the adult central nervous system (CNS).[1] Glioblastoma, a type of glioma, is the most invasive type of brain cancer, and is associated with poor prognosis as well as extremely low survival rates.[2–4] Despite great achievements in the field of basic research on gliomas in recent years, the results have not found clinical applications due to failures in clinical trials.

Procollagen C-protease enhancer protein (PCOLCE) facilitates the functions of procollagens and promotes the reestablishment of corneal and extracellular repair.[5,6] The dysregulation of PCOLCE expression has been shown to play critical roles in the development and occurrence of various diseases. Hassoun found that the level of PCOLCE was positively correlated with liver and muscle fibrosis,[7] while the absence of PCOLCE was associated with the impediment of corneal repair.[8] When PCOLCE interacts with mutated PABPN1, it is entrapped in the nuclear compartment, which may cause ophthalmopharyngeal muscular dystrophy.[9] A study showed that PCOLCE expression was significantly increased in osteosarcoma cancer tissues compared to their normal counterparts, and the upregulation of PCOLCE was associated with short survival time.[10] This study also demonstrated that the knockdown of PCOLCE inhibited the invasion, migration, and metastasis of tumor cells. Xiang and his team reported that PCOLCE was elevated in gastric cancer and was associated with poor prognosis of gastric cancer patients.[11] The same study also revealed that PCOLCE expression was positively correlated with the level of dendritic cells and macrophages in the tumor microenvironment.

Although several studies have investigated the role of PCOLCE in different cancers, there are few reports on the role of PCOLCE in glioma. In this study, we systematically analyzed the roles of PCOLCE using multiple glioma cohorts and investigated its relationship with prognosis and immune microenvironment of gliomas.

Method

Data acquisition

Our research was mainly based on bioinformatic analysis. All patient information including clinical and RNA sequencing data was downloaded from publically available databases. We selected nine glioma cohorts (TCGA: 667 samples, CGGA: 898 samples, Gravendeel: 276 samples, Rembrandt: 448 samples, LeeY: 191 samples, Kamoun: 152 samples, Freije: 85 samples, Murat: 80 samples, and Phillips: 77 samples) for bioinformatic analysis using the Gliovis platform (http://gliovis.bioinfo.cnio.es/).[12] And availability of clinical and RNA sequencing data for each patient. Glioma patients in each cohort were divided into the low-PCOLCE and high-PCOLCE expression groups according to the median PCOLCE expression level. The immunotherapy cohorts, GSE35640 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35640) and GSE78220 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE78220), used in our research were downloaded from GEO (Gene Expression Omnibus) database.

Survival and cox analysis

Kaplan–Meier curves were used to determine the OS (overall survival) between the two groups using R survival and survminer packages. Univariate Cox regression was used to explore potential prognostic indicators, while multivariate Cox regression analysis was used to determine if the gene signature was an independent risk factor for OS in glioma patients. SurvivorROC package was used to generate ROC (receiver operating characteristic) curves in R, and to further explore the OS estimation potential of the indicator in glioma patients.

Nomogram establishment

To better make evaluation of the survival for glioma patients, nomograms were developed using clinical features and PCOLCE expression based on data from TCGA and CGGA cohorts. Calibration curves were created for 1, 3, and 5-year OS to confirm the consistency between the actual and predicted OS. We subsequently drew the decision curves of 1, 3, and 5 years to compare the prediction values of nomogram and clinical features.

Analysis of immune microenvironment

We performed ssGSEA (single sample gene set enrichment analysis) to calculate the enrichment scores of 24 immune cell types through the R package "GSVA."[13] The enrichment scores represented the relative levels of 24 immune cell types, with the gene set signatures of each immune cell category being acquired from a previous study.[14]

The Cancer-Immunity Cycle refers to a series of processes entailing cancer eradication by immune system: (1) release of cancer cell antigen; (2) presentation of

cancer antigens; (3) priming and activation; (4) recruitments of immune cells; (5) infiltration of immune cells into the tumor; (6) recognition of cancer cells by T cells; and (7) killing of cancer cells.[15] In our study, the association between PCOLCE and Cancer-Immunity Cycle were explored. We also analyzed the association between PCOLCE expression and enrichment scores of 23 types of innate and adaptive immune responses in TCGA. The gene sets of the 23 types of immune responses for Gene Set Variation Analysis (GSVA) were obtained from a previous study.[16]

Prediction of immunotherapy response

To predict the efficacy of ICI (immune checkpoint inhibitor) for glioma patients with different levels of PCOLCE, we obtained the immunophenscore (IPS) of the TCGA glioma cohort from The Cancer Immunome Atlas (TCIA, https://tcia.at). Patients with high IPS scores are associated with good outcome after treatment with ICIs. We also performed TIDE[17] (Tumor immune dysfunction and exclusion) and ImmuCellAI[18] (Immune Cell Abundance Identifier) analysis to estimate the likelihood of patients with different PCOLCE expression levels benefiting from ICI therapy in the TCGA glioma cohort.

Identification of differently expressed genes (DEGs)

To establish the role of PCOLCE in glioma, the "limma" package in R was used to identify DEGs.[19] DEGs were subsequently analyzed in the Gene Ontology (GO) and KEGG (Kyoko Encyclopaedia of Genes and Genomes) database through the clusterProfiler R package.[20] GSEA (Gene set enrichment analysis) was also conducted to compare enriched pathways and biological process between low- and high-PCOLCE expression patients.

The gene sets of 18 tumor-related pathways were downloaded from the MSigDB (Molecular Signatures Database, http://www.gsea-msigdb.org/). GSVA analysis was conducted to compute the enrichment score of 18 tumor-related pathways.

Cell culture

A172 and U87 cells, purchased from the Chinese Academy of Sciences, were cultured in DMEM with 10% Gibico FBS+1% penicillin–streptomycin in a 5% CO2 incubator at 37°C.

The shRNA targeting PCOLCE, PCOLCE-sh#1 (s: 5'-CCGGTGAAGAAAGGAGTCAGT TATCCTCGAGGATAACTGACTCCTTTCTTCATTTTT-3'; as: 5'-AATTAAAAATGAAGAAA GGAGTCAGTTATCCTCGAGGATAACTGACTCCTTTCTTCA-3') and PCOLCE-sh#2 (s: 5'-CCGGCGCTGACCTTCGAGAAGTTTGCTCGAGCAAACTTCTCGAAGGTCAGCGTTTTT-3'; as: 5'-AATTAAAAACGCTGACCTTCGAGAAGTTTGCTCGAGCAAACT TCTCGAAGG TCAGCG-3'), were obtained from GenePharma (Shanghai, China).

Real-time PCR

Total RNA was isolated from cultured glioma cells using RNAiso (Takara, Japan). The cDNA The FastQuant RT Kit (Tiangen, Beijing, China) was performed through reverse transcription. The real-time PCR was performed by ViiA 7 Real-Time PCR detection system (DaanGene, Guangzhou, China). The primers for RT-PCR were as follows:PCOLCE (5′-GTGCGGAGGGGATGTGAAG-3′ and 5′-CGAAGACTCGGAATGA GAGGG-3′); GAPDH (5′-GGAGCGAGATCCCTCCA AAAT-3′ and 5′-GGCTGTTGTCATAC TTCTCATGG-3′).

In vitro experiment

CCK8 assay: the U87 and A172 cells at logarithmic growth phase were obtained and digested for cell counting kit-8 (CCK8) assay. 1×10^3 glioma cells were placed into 96-well plates and hatched for 1h under the condition of 37°C and 5% CO2.

Colony-forming assay: the U87 and A172 cells at logarithmic growth phase were obtained and digested, then plated into 6-well plates (300 cells per well) and cultured for 2 weeks at 5% CO_2 and 37°C. After that, the cells were fixed with 4% methanol (1 ml per well) and subsequently stained with crystal violet. Finally, cell discoloration was conducted using 10% acetic acid and the absorbance was measured at 450 nm.

Cell cycle assay: the U87 and A172 cells at logarithmic growth phase were obtained and digested after centrifuging, then washed three times with PBS. After resuspension, the cells were fixed with pre-cooled 100% ethanol overnight at 4°C. The fixed cells were subsequently washed with PBS and incubated with a buffer including PI and RNase for 1 h at 37°C. The percentage of each cell cycle was analyzed by the FlowJo software.

Cell apoptosis assay: the U87 and A172 cells were obtained and digested, and washed with PBS. After adding with 500-µl binding buffer, the glioma cells were resuspended into single existed cells. 5-µl PI solution and 5-µl Annexin V-APC were used for cell staining at room temperature for 10 min. We finally measured the apoptotic cells through the flow cytometer.

Results

Pan-cancer PCOLCE expression analysis

We performed a pan-cancer analysis to compare the expression of PCOLCE between normal and tumor tissues in

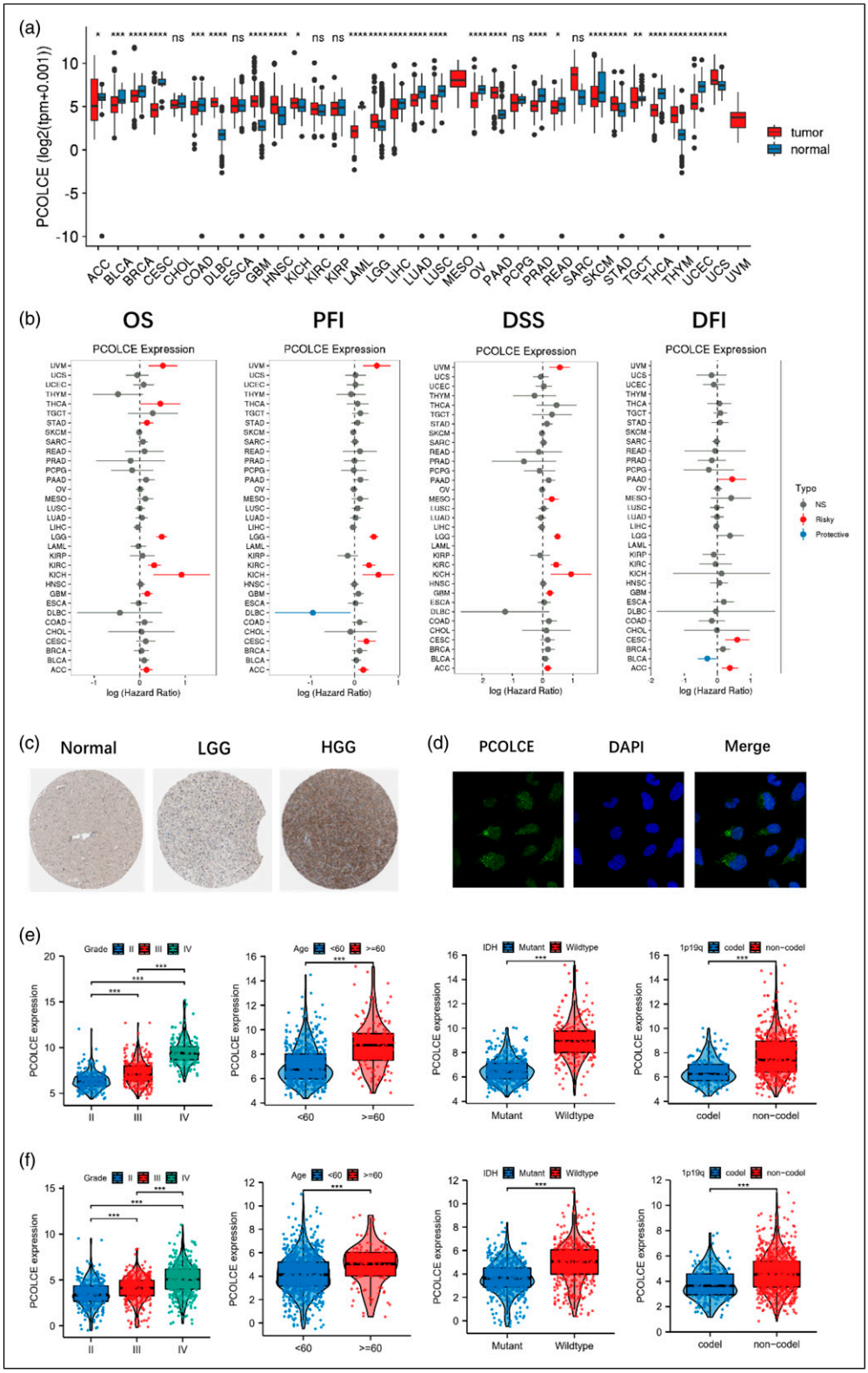

Figure 1. Pan-cancer POLCE expression analysis. (a) PCOLCE expression in 33 cancer types of TCGA database. (b) Cox analysis showed the relation between PCOLCE expression and overall survival (OS), progression-free interval (PFI), disease free interval (DFI), disease-specific survival (DSS). Risky: log(Hazard Ratio) > 0, $p<0.05$; Protective: log(Hazard Ratio) < 0, $p<0.05$; NS: no significance. (c) Expression of PCOLCE in normal brain tissue, LGG, and GBM obtained from HPA platform. (d) The immunofluorescence images of PCOLCE showed that PCOLCE protein mainly expressed in the Golgi apparatus and vesicles. (e–f) The association between PCOLCE expression and clinical features in TCGA (e) and CGGA (f). *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$; ns: no significance.

33 types of cancer found in TCGA using an online tool called UCSCXenaShiny (https://hiplot.com.cn/advance/ucsc-xena-shiny). In our research, low grade glioma (LGG) and glioblastoma multiforme (GBM) were both analyzed. The results showed that the expression of PCOLCE was higher in DLBC, GBM, KNSC, KICH, LGG, PAAD, SARC, STAD, THYM, and UCS, but lower in ACC, BLCA, BRCA, COAD, LAML, LIHC, LUAD, LUSC, OV, PRAD, READ, SKCM, TGCT, THCA, and UCEC compared to normal tissue (Figure 1(a)). Comparison of the protein expression levels of PCOLCE between normal and glioma tissues using the Human Protein Atlas[21] (HPA) revealed that PCOLCE expression was higher in LGG and HGG, compared with normal brain tissue (Figure 1(c)), which was contrasted with the results in pan-cancer analysis. Analysis of immunofluorescence images of tumor cells downloaded from the HPA platform showed that PCOLCE protein was mainly expressed in the Golgi apparatus and vesicles (Figure 1(d)). Cox analysis indicated that high expression of PCOLCE was significantly related to poor OS in both GBM and LGG (Figure 1(b)). Analysis of the relationship between PCOLCE expression and clinical characteristics such as age, grade, IDH, and 1p19q status based on TCGA and CGGA cohorts demonstrated that PCOLCE was expressed differently in different clinical subtypes (Figures 1(e)-(f)).

To identify the cell-type specific expression of PCOLCE in glioma tissue, we performed single cell sequencing analysis using Single Cell Expression Atlas (https://www.ebi.ac.uk/gxa/sc/home) and data from a previous study.[22] The results showed that the cells from 4 glioma samples could be divided into 7 clusters (Figure 2(a)), and PCOLCE was mainly expressed in neoplastic cells and vascular cells (Figure 2(b)).

Survival analysis of nine glioma cohorts based on PCOLCE expression

To estimate the prognostic value of PCOLCE in glioma, we performed survival analysis in nine glioma cohorts (CGGA, TCGA, Rembrandt, Gravendeel, LeeY, Kamoun, Freije, Murat, and Phillips). Glioma patients in each cohort were divided into low-expression and high-expression groups based on the median PCOLCE expression level. The Kaplan–Meier (KM) curves illustrated that glioma patients with high-PCOLCE expression in TCGA (HR: 4.78, 95% CI 3.56–3.44), CGGA (HR:2.37, 95% CI 2.01-2.80), Rembrandt (HR:2.38, 95% CI 1.89–2.99), and Gravendeel (HR:2.23, 95% CI 1.71–2.91), were associated with significantly worse prognosis than patients with low-PCOLCE expression (Figures 3(a)-(d)), while patients in the LeeY, Kamoun, Freije, Murat, and Phillips cohorts displayed similar trends with no statistical significance (Figures 3(e)-(i)). The results of survival analysis in the nine glioma cohorts were aggregated in a meta analysis and verified that patients with high-PCOLCE expression had poorer OS than patients with low-PCOLCE expression (HR = 2.18, 95% CI 1.98–2.40, Figure 3(j)).

We then explored the relationship between PCOLCE expression and survival in different glioma subtypes (GBM, LGG, IDH mutation, IDH wildtype, 1p19q codeletion, and 1p19q non-codeletion). Patients in each subtype were also divided into high-expression and low-expression groups based on the median PCOLCE expression level. The Kaplan–Meier curves (TCGA, CGGA, Rembrandt, and Gravendeel) showed that the high-expression group was associated with a shorter survival time than the low-expression group (TCGA: Supplemental Figure S1; CGGA: Supplemental Figure S2; Rembrandt: Supplemental Figure S3; Gravendeel: Supplemental Figure S4), particularly in the LGG cohort.

PCOLCE can serve as a predictive factor for OS in glioma patients

Cox analysis and ROC analysis were performed to further investigate the prognostic value of PCOLCE in glioma patients. In the TCGA cohort, the HR (hazard ratio) of univariate and multivariate Cox were 1.615 (95%CI = 1.519–1.718, $p<0.001$) and 1.126 (95%CI = 1.039–1.718, $p=0.046$), respectively (Figure 4(a)), and the AUC (area under the ROC curve) in 1, 3, and 5 years were 0.845, 0.874, and 0.791, respectively (Figure 5(a)). In the CGGA cohort, the HR of univariate and multivariate Cox were 1.286 ($p < 0.001$) and 1.068 ($p = 0.025$), respectively (Figure 4(b)), and the AUC in 1, 3, and 5 years were 0.655, 0.709, and 0.731, respectively (Figure 5(b)). In the Rembrandt cohort, the HR of univariate and multivariate Cox were 1.376 ($p < 0.001$) and 1.251 ($p = 0.011$), respectively (Figure 4(c)), and the AUC in 1, 3, and 5 years were 0.647, 0.752, and 0.747, respectively (Figure 5(c)). In the Gravendeel cohort, the HR of univariate and multivariate Cox were 1.273 ($p < 0.001$) and 1.071 ($p = 0.093$), respectively (Figure 4(d)), and the AUC in 1, 3, 5 years were 0.697, 0.748, 0.710, respectively (Figure 5(d)).

To better predict the prognosis of glioma patients, we developed a risk model (Supplemental Figure S5(c)) using the top 10 most relevant genes of PCOLCE (Supplemental Figure S5(a)-(b)) in TCGA using the LASSO (least absolute shrinkage and selection operator) regression algorithm and the R package glmnet. The formula used to calculate the risk score was: risk score= 0.174 * SERPINH1 + 0.2818 * TIMP1 + (−0.1187) *COL3A1 + 0.0953 * EVA1B + 0.2752 * GUSB +0.1345 * GPX8. Patients were then divided into high- and low-risk group based on the median risk score.

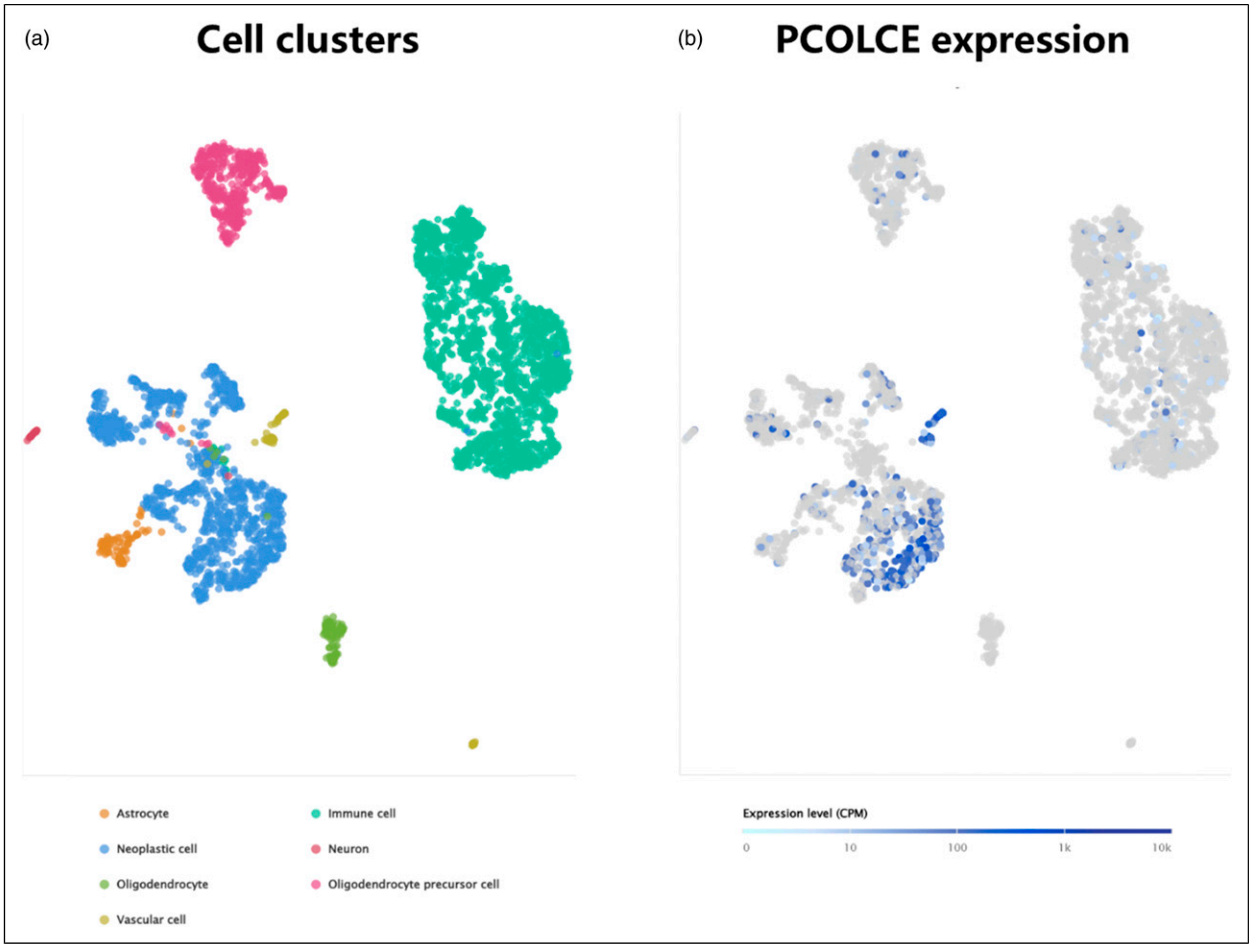

Figure 2. Single cell sequencing analysis. (a) Cells of four glioma samples were divided in to 7 clusters. (b) PCOLCE expression level in different cell clusters.

Survival analysis demonstrated that high-risk group had a worse prognosis than low-risk group (Supplemental Figure S5(d)). ROC curves showed good 1-, 3-, and 5-year predictive ability of the risk scores in gliomas (Supplemental Figure S5(e)). Univariate and multivariate Cox analysis indicated that the risk score was an independent prognostic factor for glioma (Supplemental Figure S5(f)-(g)).

Development and validation of the predictive nomogram

We established two nomograms in TCGA (Figure 6(a)) and CGGA (Figure 6(e)) based on PCOLCE expression and clinical and prognostic data. The calibration curves of the TCGA and CGGA nomograms were consistent with their standard curves (TCGA: Figure 6(b); CGGA: Figure 6(f)). We then investigated the predictive ability of the two nomograms using ROC curves (TCGA: Figure 6(c); CGGA: Figure 6(g)). Decision curves were also drawn to assess the performance of the two nomograms in clinical decision-making in 1, 3, and 5 years (TCGA: Figure 6(d); CGGA: Figure 6(h)).

Association between PCOLCE expression and glioma immune microenvironment

Dysregulation of the tumor immune microenvironment is necessary for the survival, metastasis, and immune escape of cancer cells. We investigated the association between infiltration of immune cells and PCOLCE expression in TCGA cohort. The results showed that multiple immuno-suppressive cells, such as macrophages, Th2 cells, and neutrophils, were significantly increased in patients with high-PCOLCE expression compared to patients with low-PCOLCE expression (Figure 7(a)), an indication that the expression of PCOLCE was positively correlated the level of immuno-suppressive cells (Figures 7(b)-(e)).

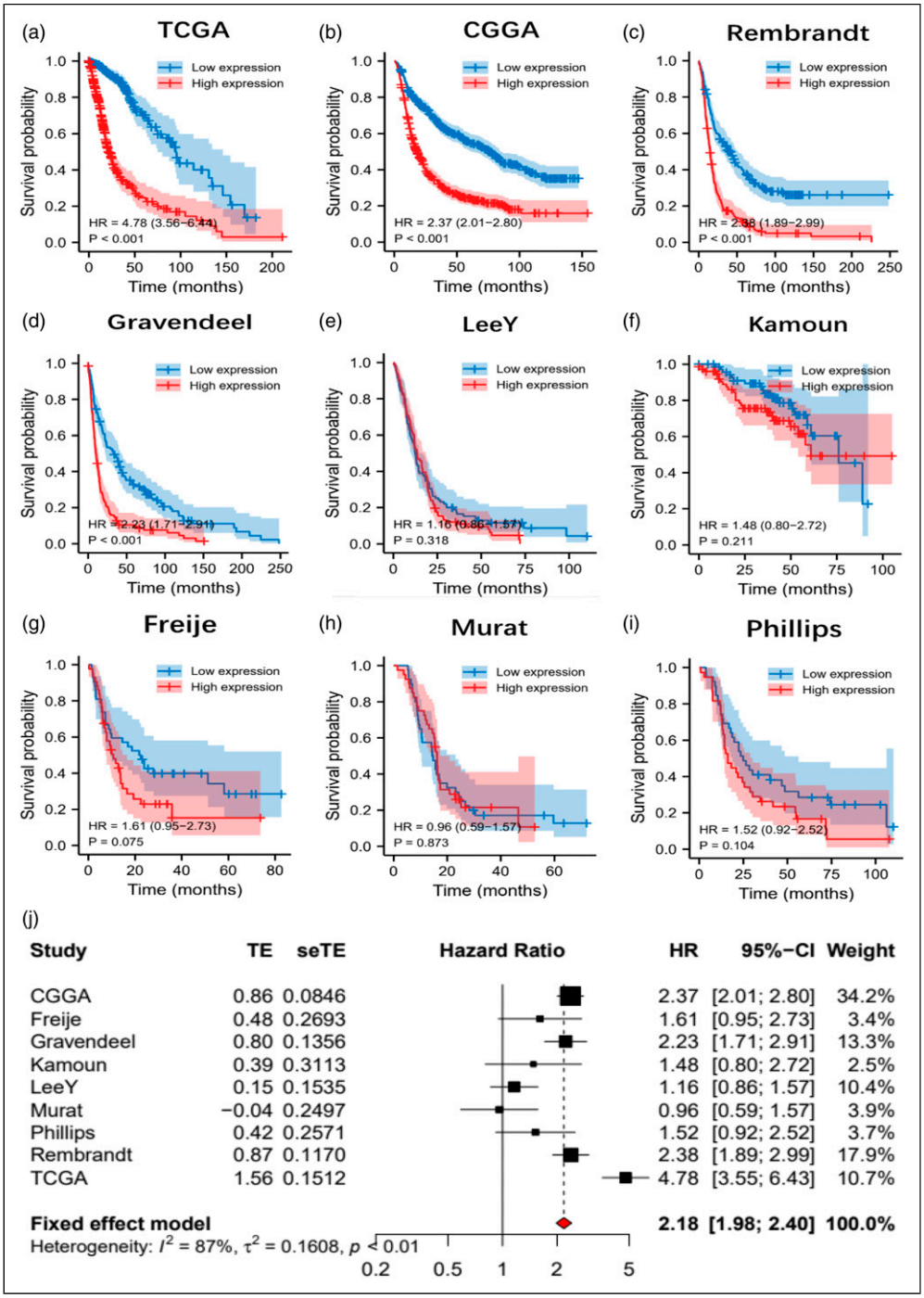

Figure 3. High-PCOLCE expression is associated with poor OS in glioma. (a–i) Kaplan–Meier curves of PCOLCE in (a) TCGA, (b) CGGA, (c) Rembrandt, (d) Gravendeel, (e) LeeY, (f) Kamoun, (g) Freije, (h) Murat, and (i) Phillips. (j) Meta analysis of the HRs for PCOLCE in nine glioma cohorts.

Anti-tumor immune activities consist of a range of stepwise events known as Cancer-Immunity Cycle. Immune phenotypes are tightly modulated by Cancer-immunity cycle in the tumor microenvironment. Our research demonstrated that PCOLCE were negatively correlated with the enrichment scores of Step2, Step3, Step5, and Step7 in Cancer-Immunity Cycle, and positively correlated with the enrichment scores of Step1 and

Step4 (Figure 7(g)). We then analyzed the association between PCOLCE expression and 23 gene sets containing innate and adaptive immunity to explore the presence of hot immunophenotypes in glioma. The results illustrated that high expression of PCOLCE was associated with the "hot" immunophenotypes (Figure 7(f)). Thus, our findings revealed that PCOLCE might participate in the development of immunosuppressive microenvironment by regulating immune cell infiltration and Cancer-Immunity Cycle.

Prediction of response to immunotherapy in patients with high and low-PCOLCE expression

The advent of immunotherapy and novel targeted therapy has tremendously prolonged the survival time of various cancers. Immune checkpoint inhibitors (ICI) are a type of immunotherapy associated with less toxicity and side effects compared with cytotoxic chemotherapy. We first compared the expression levels of 23 immune checkpoint-related genes between low- and high-expression PCOLCE groups in the TCGA cohort. The box plots showed that the expression of most immune checkpoint-related genes was higher in the patients with high-PCOLCE expression than patients with low-PCOLCE expression (Figures 8(a)-(b)). In addition, patients with low-PCOLCE expression had higher IPS scores than patients with high-PCOLCE expression, suggesting that patients with low expression of PCOLCE were more likely to benefit from ICI therapy (Figure 8(c)). Previous studies have revealed the potential of B7-H3 and CD73 in predicting response to ICI in glioma.[23,24] Our results showed that the expression levels of B7-H3 and CD73 were higher in glioma patients with high-PCOLCE expression than patients with low-PCOLCE expression (Figures 8(d)-(e)), indicating that patients with low-PCOLCE expression were more likely to respond to ICI. These findings were consistent with the TIDE scores of the two groups (Figure 8(f)). TIDE and ImmuCellAI platform were also utilized to predict response to ICI in high-PCOLCE and low-PCOLCE groups. Our results suggested that patients with low-PCOLCE expression had a higher response rate than patients with high-PCOLCE expression (Figures 8(g)-(h)).

To validate our findings on response to immunotherapy, we downloaded the data of two melanoma cohorts (GSE35640 and GSE78220) receiving immunotherapy from the GEO database. Melanoma patients in each cohort were divided into high- and low-expression groups based on the median PCOLCE expression level. The results showed that patients in low-PCOLCE expression group had a higher response rate to immunotherapy than patients in the high-PCOLCE expression group (Supplemental Figure S6(a)-(b)).

Determination and analysis of differently expressed genes (DEGs)

To explore the roles of PCOLCE in glioma, we identified the DEGs between patients with low- and high-expression of PCOLCE. We identified 5912 DEGs (p-value < 0.05, |log2FoldChange| > 1), including 4847 upregulated genes and 1065 downregulated genes (Figure 9(a)). KEGG and GO analysis suggested that the DEGs were mainly enriched in neutrophil migration, regulation of inflammatory response, PI3K-Akt signaling pathway, and chemokine signaling pathway (Figures 9(b)-(c)). GSEA analysis showed that epithelial mesenchymal transition, inflammatory response, apoptosis, JAK-STAT signaling pathway, cell cycle, and P53 signaling pathway were enhanced in patients with high expression of PCOLCE (Figures 9(d)-(e)). We also investigated the relationship between PCOLCE and 18 tumor-related pathways (Figures 9(f)-(g)), and found that PCOLCE expression was positively correlated with the enrichment scores of cell cycle (Figure 9(h)), apoptosis (Figure 9(i)), and P53 signaling pathway (Figure 9(j)).

Down-regulation of PCOLCE inhibited the development of glioma

The downregulation of PCOLCE expression has been shown to inhibit invasion, migration and metastasis of osteosarcoma cells. To explore the role of PCOLCE in glioma, CCK8, and colony-forming assay were performed after knockdown of PCOLCE expression. RT-PCR analysis demonstrated successful PCOLCE knockdown through PCOLCE-sh#1 and #2 (Figures 10(a)-(b)). Colony-forming assay and CCK8 assay showed that knockdown of PCOLCE inhibited cell growth (Figures 10(c)-(e)), and suppressed the proliferation of U87 and A172 cells (Figure 10(f)), respectively. The knockdown of PCOLCE expression also led to the arrest of U87 and A172 cell lines in the G2/M-phase (Figure 10(g)).

We finally investigated the function of PCOLCE in the apoptosis of glioma cells and found that knockdown of PCOLCE significantly promoted apoptosis in U87 cell line and A172 cells (Figures 11(a)-(b)).

Discussion

Glioma is the most common malignant tumor in central nervous system with poor prognosis.[25,26] The onset and progression of glioma is a complicated process because it includes the abnormality of cellular pathways and various genes. It is critical to find the key genes and fully understand their functions in the molecular mechanism of glioma in order to improve the treatment and diagnosis for patients. It was reported that PCOLCE could regulate

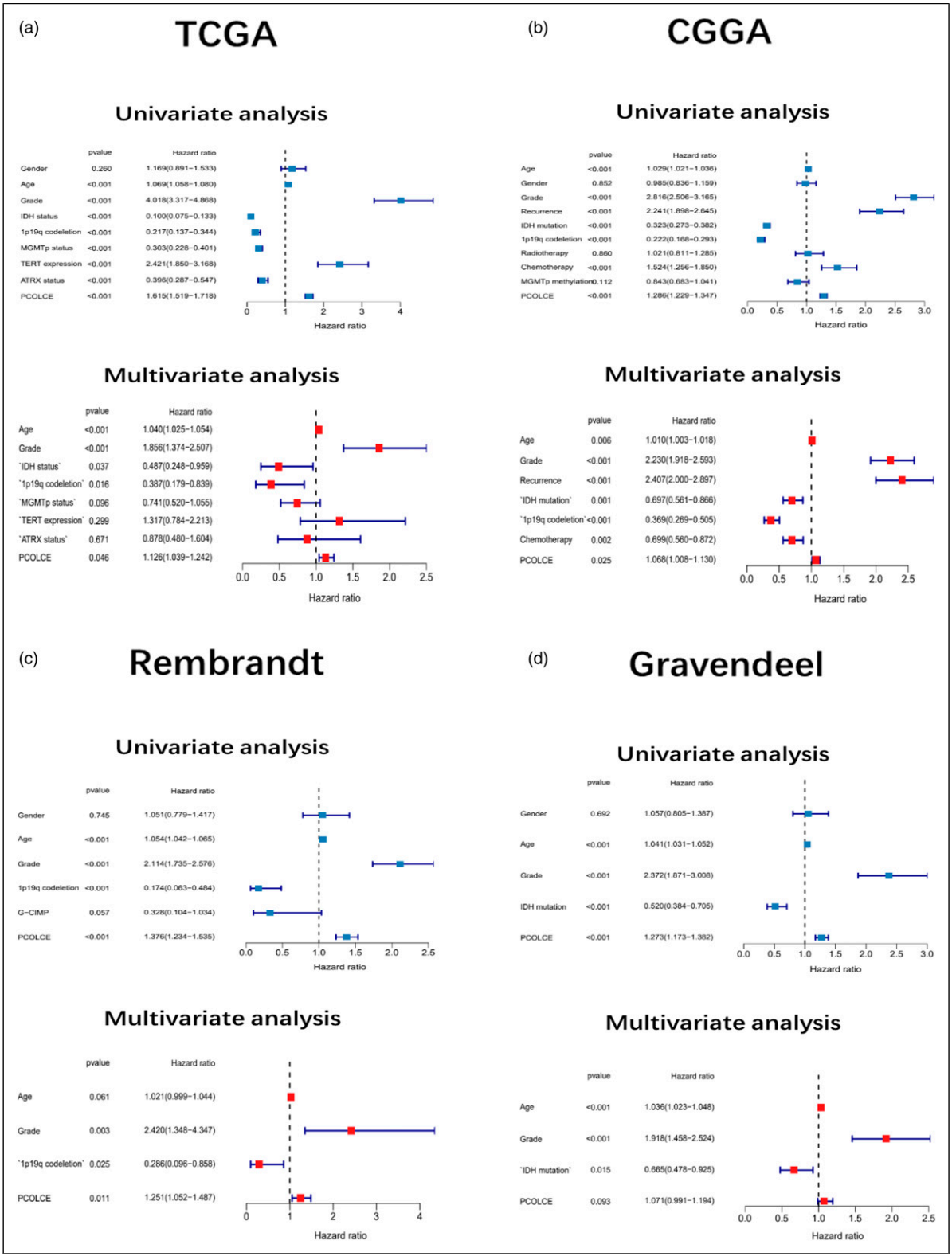

Figure 4. Cox analysis of PCOLCE and clinical features of glioma. (a) TCGA. (b) CGGA. (c) Rembrandt. (d) Gravendeel.

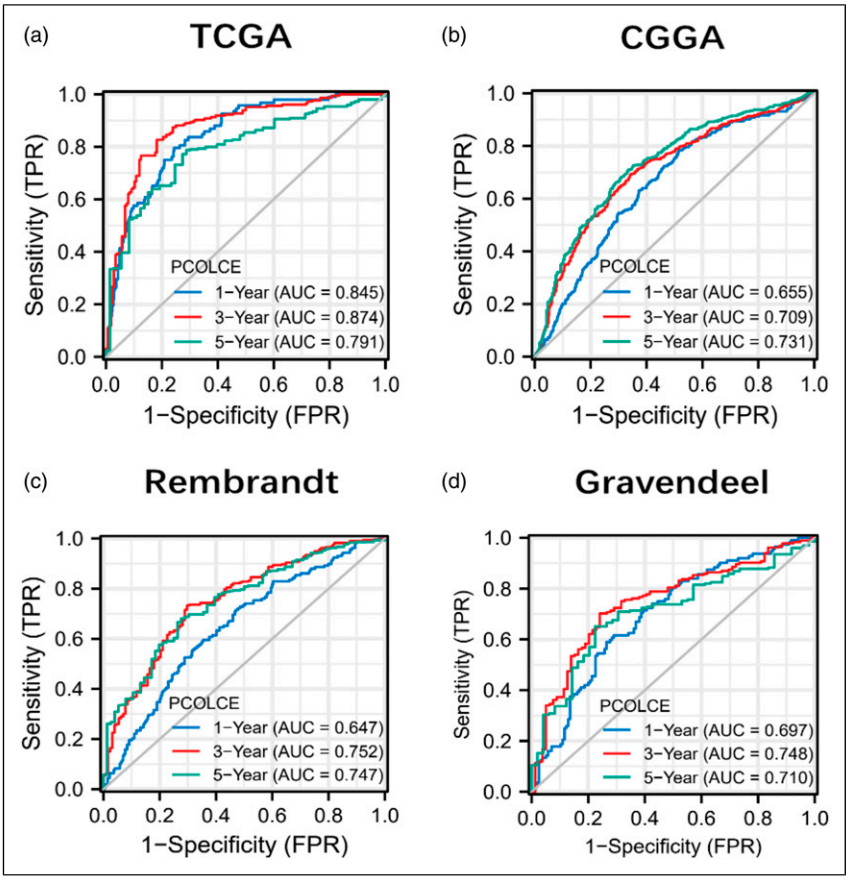

Figure 5. ROC curves of PCOLCE for predicting 1-year, 3-year, and 5-year survival. (a) TCGA. (b) CGGA. (c) Rembrandt. (d) Gravendeel.

tumor growth and metastasis in several cancers. However, the role of PCOLCE in glioma is still unclear. A recent study revealed that high expression of PCOLCE affected lymph node metastasis and was associated with poor prognosis in ovarian and gastric cancer.[11] Wang reported that PCOLCE expression was elevated by TWIST1 in osteosarcoma, and identified the N-glycosylation of PCOLCE as a critical promoter of metastasis of osteosarcoma.[10] This research is the first to systematically assess the roles and mechanisms of PCOLCE in glioma.

In this study, we used multiple glioma cohorts to demonstrate that PCOLCE is a potential prognostic factor for glioma. Analysis of the immune microenvironment showed that PCOLCE promoted an immune-suppressive microenvironment by regulating the immunophenotypes, infiltration of immune cells, and the processes associated with the Cancer-Immunity Cycle. We also found that patients with low-PCOLCE expression were more likely to benefit from ICI therapy than patients with high-PCOLCE expression. Moreover, we demonstrated that the knockdown of PCOLCE inhibited the growth of glioma cells by regulating cell cycle and promoting cell apoptosis.

Infiltrating immune cells and stromal cells play a key role in tumor evasion of detection and attack by the immune system. Various immune cells, such as bone marrow-derived suppressor cells (MDSC), cancer-associated fibroblasts (CAFs), macrophages, neutrophils, Th2 cells, and Tregs play essential roles in establishing an immunosuppressive microenvironment. Neutrophils are reported to promote cancer cell growth, increase metastasis, and enhance angiogenesis through multiple means.[27] CAFs promote cancer by releasing multiple chemokines and cytokines (CXCL2 and IL-6) for the recruitment and accumulation of Tregs in tumor microenvironment.[28] Th2 cells are reported to inhibit anti-cancer-immunity effects by secreting multiple cytokines.[29] Our research revealed that the high level of PCOLCE were significantly associated with increased infiltration of immunosuppressive cells (macrophages, Th2 cells, and neutrophils). The dysfunction of several steps in Cancer-Immunity Cycle can also cause immunosuppression and tumor progression. Our

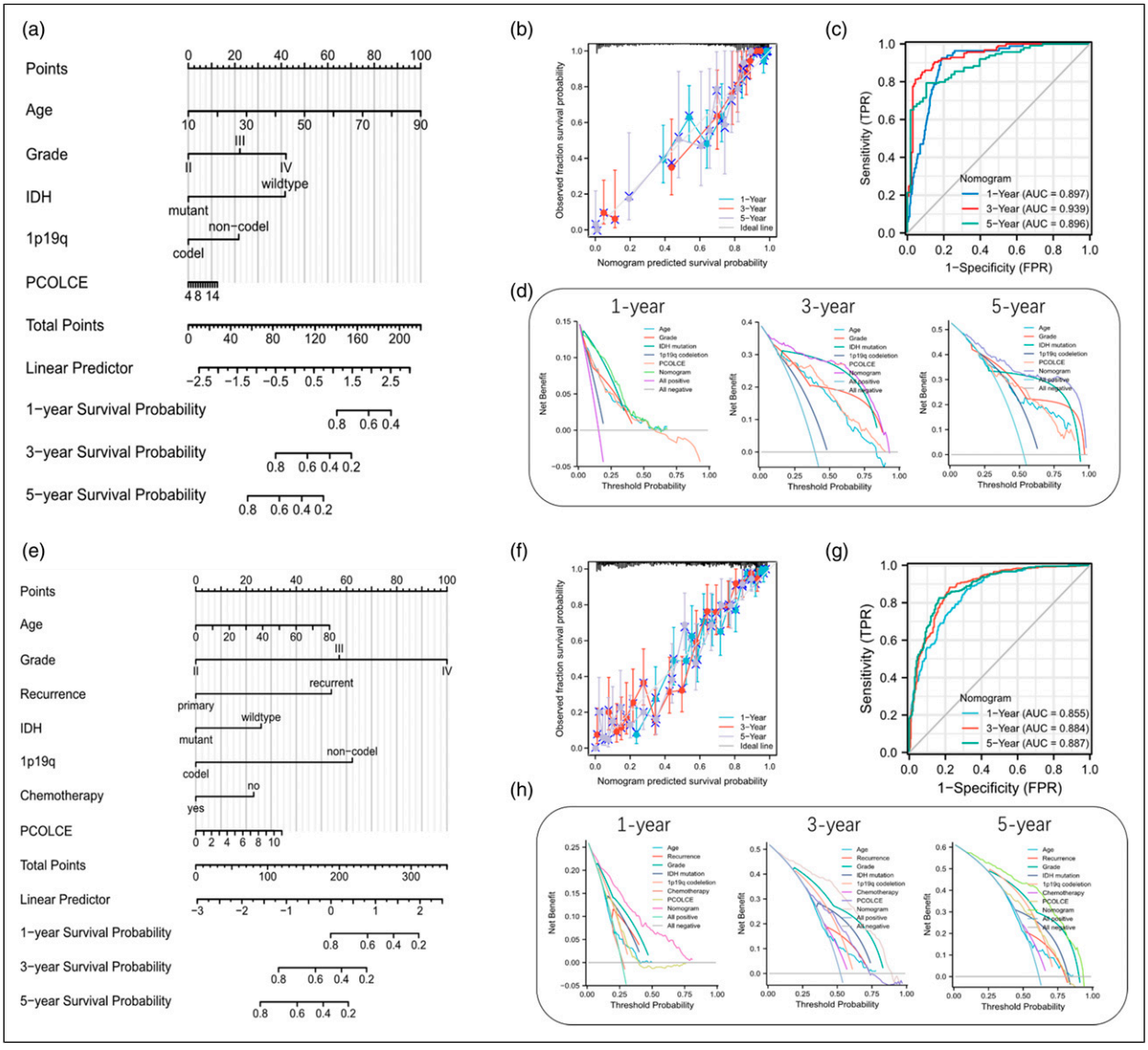

Figure 6. Development and validation of the predictive nomogram. (a) Nomogram development in TCGA cohort. (b) Calibration curves showed the prediction accuracy of TCGA nomogram. (c) ROC curves showed the prediction ability of TCGA nomogram in 1, 3 and 5 years. (d) Decision curves showed the performance of TCGA nomogram in clinical decision-making. (e) Nomogram development in CGGA cohort. (f) Calibration curves showed the prediction accuracy of CGGA nomogram. (g) ROC curves showed the prediction ability of CGGA nomogram in 1, 3, and 5 years. (h) Decision curves showed the performance of CGGA nomogram in clinical decision-making.

study indicated that the high level of PCOLCE was significantly related to the inhibition of Step2, Step3, Step5, and Step7 in Cancer-Immunity Cycle. These findings suggest that PCOLCE promote pro-tumor effects by regulating Cancer-Immunity Cycle and infiltration of immune cells.

In recent years, great achievements and breakthroughs have been acquired in the fields of cancer immunotherapy, which remarkably changed our understandings about anti-tumor treatment modality. ICIs (immune checkpoint inhibitor) are the most common type of immunotherapy for cancer, which activate anti-cancer immunity by promoting infiltration and accumulation of T cells in the tumor.[30,31] Nevertheless, glioma patients do not benefit from immunotherapy due to the immunosuppressive state and T-cell deficiency in the tumor microenvironment.[32,33] CD73 was recently identified as a predictor of response to ICI in GBM. Deletion of CD73 enhanced the anti-tumor effect of ICIs by increasing T-cell infiltration and macrophage polarization to generate an immunostimulatory phenotype in a mouse model.[23] B7-H3 is another predictor of therapeutic efficacy of immunotherapy in GBM,

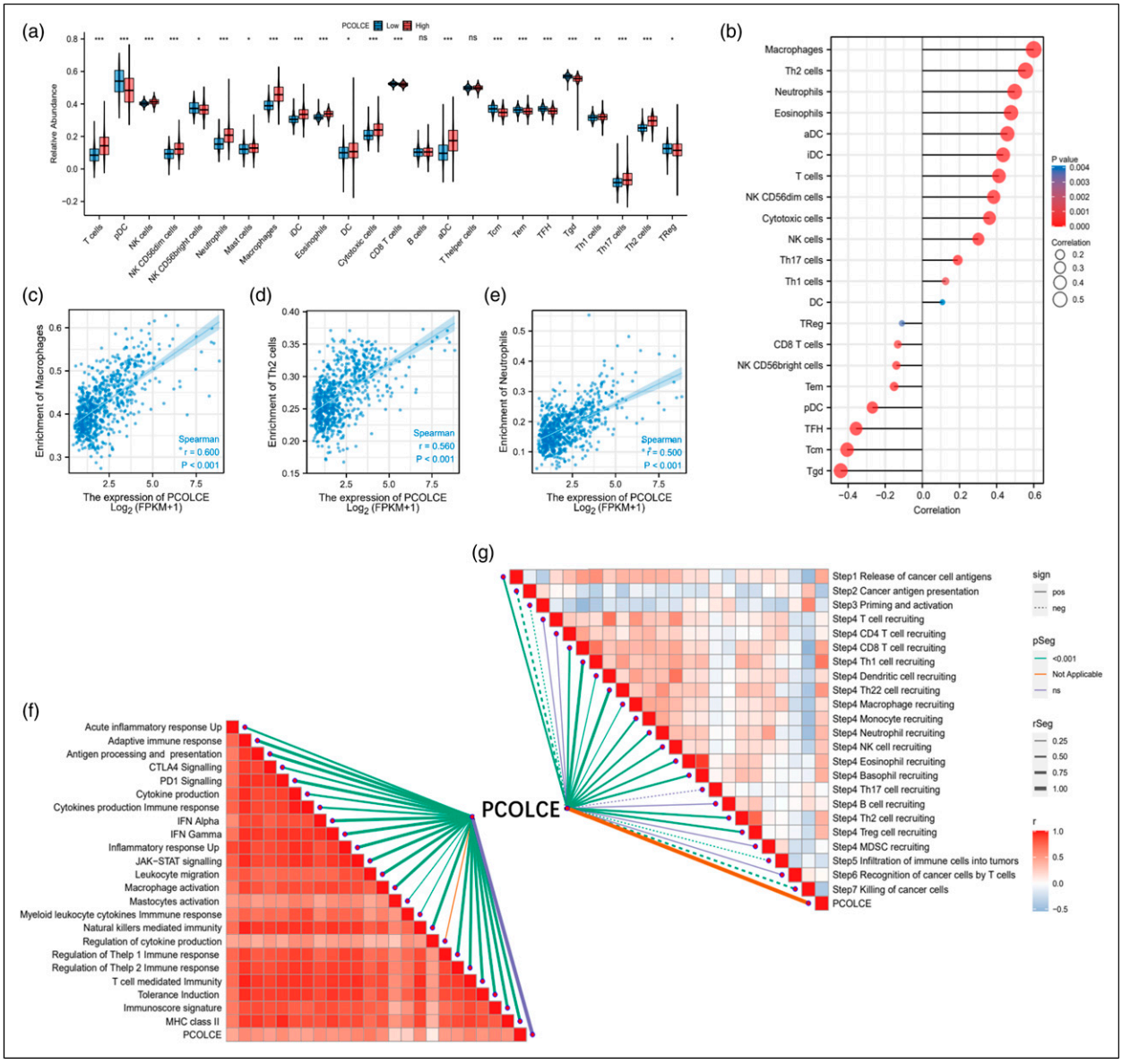

Figure 7. Relationship between PCOLCE expression and glioma immune microenvironment. (a) Relative levels of 24 immune cell types in high and low-PCOLCE expression groups. (b) Lollipop plot showed the correlation between PCOLCE expression and multiple immune cells. (c–e) Correlation between PCOLCE expression and (c) macrophages, (d) Th2 cells and (e) neutrophils. (f) Relationship between PCOLCE expression and 23 gene sets containing innate and adaptive immunity. (g) Relationship between PCOLCE expression and Cancer-Immunity Cycle.

and GBM patients with low expression of B7-H3 are associated with prolonged survival.[24] In our study, PCOLCE expression was positively correlated to CD73 and B7-H3 expression, suggesting that the low-PCOLCE expression group might have better response to ICI therapies than the high-PCOLCE expression group. Similar results were observed in our prediction using ImmuCellAI and TIDE, and validated using data from two public datasets (GSE35640 and GSE78220), showing that patients with lower levels of PCOLCE had higher response to immunotherapy.

Enrichment analysis indicated that pro-tumor biological processes and pathways, such as DNA repair, P53 signaling pathway, PI3K-AKT-MTOR signaling pathway, IL6-JAK-STAT3 signaling pathway, hypoxia, and glycolysis were enhanced in patients with high-PCOLCE expression.

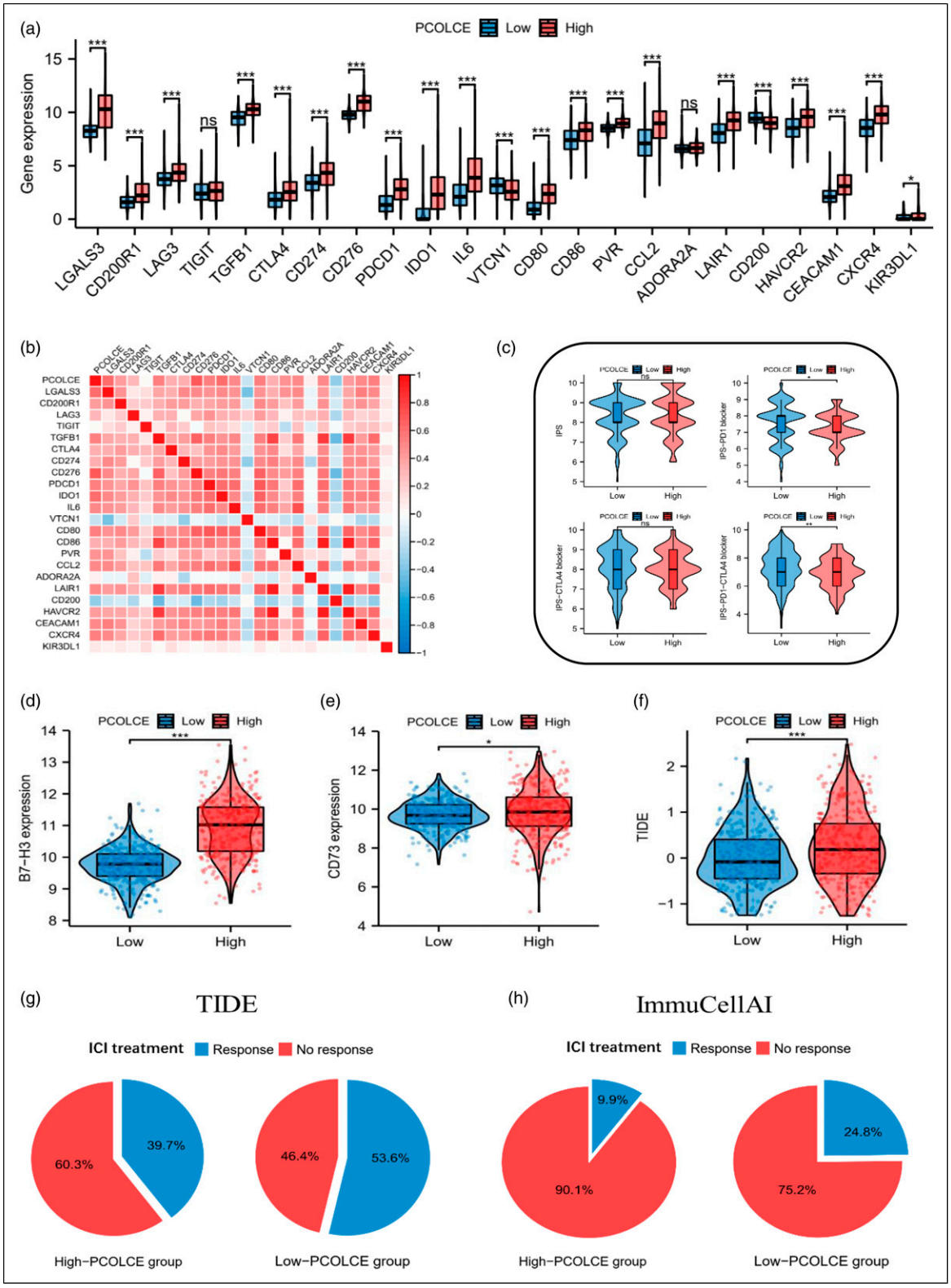

Figure 8. Prediction of response to immunotherapy in the low- and high-PCOLCE expression groups. (a) Level of multiple immune checkpoint-related genes in low-and high-PCOLCE expression groups. (b) Heatmap showing the correlation between PCOLCE and the immune checkpoint-related genes. (c) IPS scores in low-and high-PCOLCE expression groups. (d) B7-H3 expression in low- and high-PCOLCE expression groups. (e) CD73 expression in low- and high-PCOLCE expression groups. (f) TIDE scores in low- and high-PCOLCE expression groups. (g–h) Prediction of ICI response using TIDE and ImmuCellAI platform.

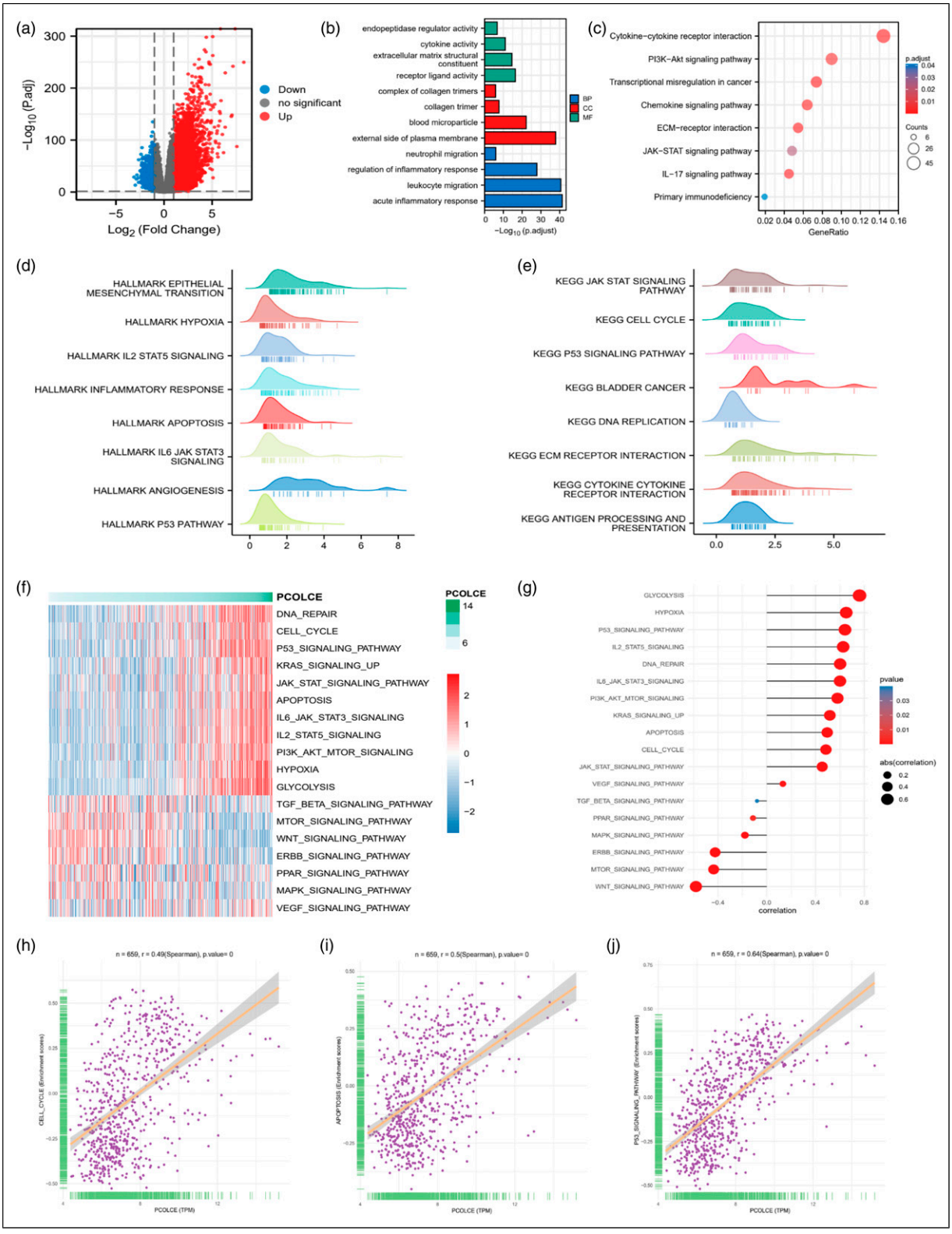

Figure 9. Determination of DEGs and enrichment analysis. (a) Volcano plot showing the DEGs in low- and high-PCOLCE expression groups. (b) GO analysis of DEGs. (c) KEGG analysis of EDGs. (d–e) GSEA analysis showing the activated processes and pathways in high-PCOLCE group. (f–g) The association between PCOLCE and 18-cancer related pathways. (h) Correlation between PCOLCE and cell cycle. (I) Correlation between PCOLCE and apoptosis. (j) Correlation between PCOLCE and P53 signaling pathway.

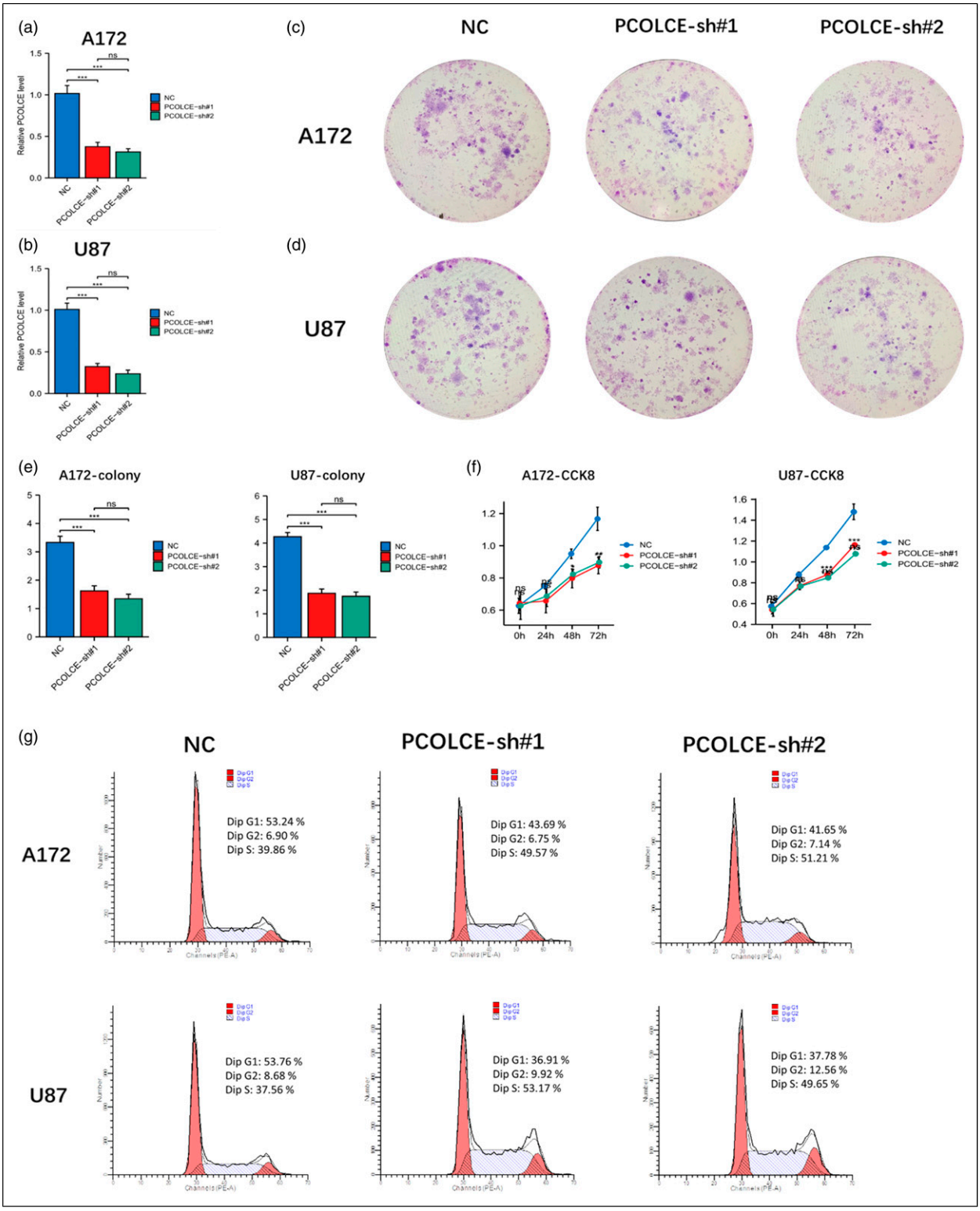

Figure 10. Experiment validation. (a) Knockdown of PCOLCE in A172 cells. (b) Knockdown of PCOLCE in U87 cell. (c) The colony-forming assay showed that the proliferative capacity of A172 cells was inhibited after knocking down PCOLCE. NC, normal control. (d) The colony-forming assay showed that the proliferative capacity of U87 cells was inhibited after knocking down PCOLCE. (e) Statistical analysis for colony-forming assay; (f) Statistical analysis for CCK8 assay. (g) Cell cycle assay showed that inhibition of PCOLCE expression caused the arrest of A172 and U87 in the G2/M-phase. *$p < 0.05$; ***$p < 0.001$; ns, not statistically significant.

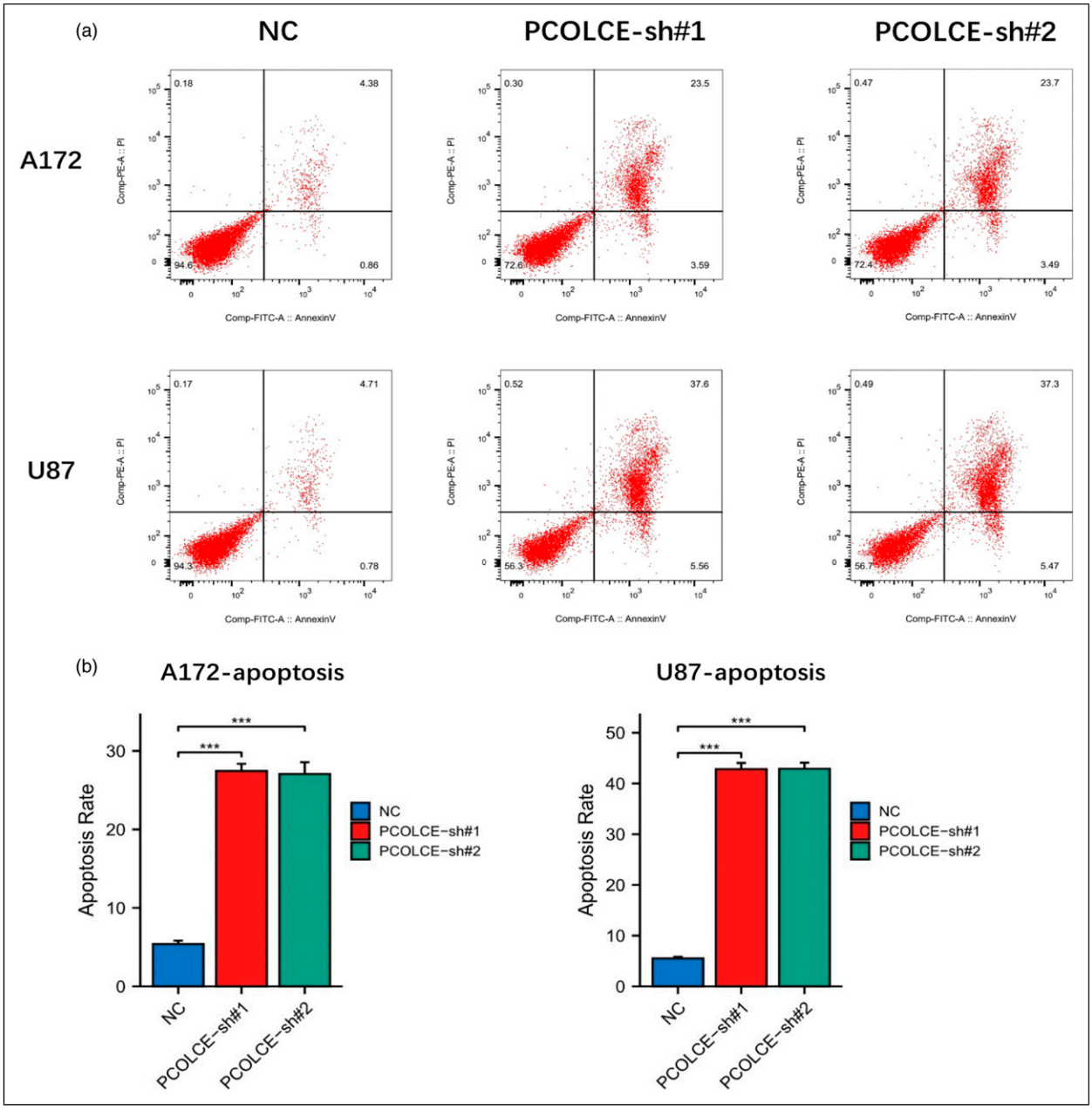

Figure 11. Cell apoptosis assay. (a) apoptosis analysis of A172 and U87 cell lines transfected with PCOLCE-sh#1, PCOLCE-sh#2 and NC; (b) Statistical analysis of cell apoptosis in A172 and U87 cells. ***$p < 0.001$.

Results obtained through bioinformatics analysis were validated in cell experiments, where downregulation of PCOLCE was found to inhibit glioma cell growth by regulating cell cycle and promoting cell apoptosis.

In summary, our research demonstrated that PCOLCE is a prognostic factor for glioma. Using bioinformatics analysis, we investigated the difference in immune phenotypes and biological processes between patients with low and high PCOLCE expression. We also predicted response to immunotherapy in patients with different levels of PCOLCE and found that patient in the low-PCOLCE expression group responded better to ICI treatment. Moreover, the results of cell experiments indicated that PCOLCE promoted glioma progression by regulating cell cycle and apoptosis.

This study had some limitations. First, we did not conduct a priori sample size calculation, which prevents us from determining the power of our sample size. Second, the patient data used in our research was downloaded from openly available website, and our conclusions are mainly

based on bioinformatics analysis. There is need to validate the roles of PCOLCE in glioma through in vivo and in vitro experiments. Although our conclusion on response to immunotherapy was verified in two melanoma cohorts, there is need to validate the findings in glioma immunotherapy cohorts. Large multicenter and prospective studies are required to enhance the clinical applications of our findings.

Conclusions

Our findings revealed that PCOLCE was associated with immune phenotypes, multiple pathways, and therapy response in glioma. These findings provide insight into the molecular mechanisms underlying glioma and possible therapeutic strategies.

Acknowledgments

We are very grateful to the authors of the databases used in this article, and their data facilitated our research.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was supported by National Natural Science Foundation of China (81870984); National Key R & D Program Intergovernmental Cooperation on International Scientific and Technological Innovation of the Ministry of Science and Technology of China (2017YFE0110400); Hebei Natural Science Foundation General Project— Beijing-Tianjin-Hebei Basic Research Cooperation Project (H2018206675); Special Project for the Construction of Hebei Province International Science and Technology Cooperation Base (193977143D); Government funded Project on Training of outstanding Clinical Medical Personnel and Basic Research Projects of Hebei Province in the Year of 2017; Government funded Project on Training of outstanding Clinical Medical Personnel and Basic Research Projects of Hebei Province in the Year of 2019.

Data availability

The data set is available from Gliovis platform.

ORCID iD

Zongmao Zhao https://orcid.org/0000-0002-3753-3949

Supplemental Material

Supplemental material for this article is available online.

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Appendix

Abbreviations

PCOLCE:	procollagen C-protease enhancer protein
ICI:	immune checkpoint inhibitor
CNS:	central nervous system
OS:	overall survival
ROC:	receiver operating characteristic
ssGSEA:	single sample gene set enrichment analysis
GSVA:	Gene Set Variation Analysis
TCIA:	The Cancer Immunome Atlas

ImmuCellAI: Immune Cell Abundance Identifier
TIDE: Tumor immune dysfunction and exclusion
DEG: differently expressed genes
GO: Gene Ontology
KEGG: Kyoko Encyclopaedia of Genes and Genomes
GSEA: Gene set enrichment analysis
CCK8: cell counting kit-8
LGG: low grade glioma
HGG: high grade glioma
HPA: Human Protein Atlas
MDSC: marrow-derived suppressor cell