Pan-cancer Analysis of the Prognostic and Immunological Effects of PIK3C3

Qianxin Xie
Zhejiang Chinese Medical University
Jie Zhou
Zhejiang Chinese Medical University
Junfeng Guan
Zhejiang Chinese Medical University
Siqi She
Zhejiang Chinese Medical University
Junfeng Li
Zhejiang Chinese Medical University
Wenhong Liu
Zhejiang Chinese Medical University
Ye Xu
Zhejiang Chinese Medical University
Mengjiao Hu
Zhejiang Chinese Medical University
Fangfang Tao (taoff@zcmu.edu.cn)
Zhejiang Chinese Medical University

Research Article

Keywords: PIK3C3, pan-cancer analysis, tumor, immunotherapy, prognosis

Posted Date: September 13th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3268034/v1

Additional Declarations: No competing interests reported.

Abstract

Background

Autophagy-related protein plays a pivotal role in cancer development, progression, and prognosis. Among these proteins, PIK3C3 holds significant importance as it is involved in canonical autophagy, endocytosis, and vesicle trafficking, thereby exerting influential effects on various types of cancer progression. However, the diverse biological significance of PIK3C3 in pan-cancer has not been systematically and comprehensively studied.

Methods: Data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) were utilized, and bioinformatics approaches were also employed to explore the potential mechanisms of PIK3C3 in diverse cancers.

Results: PIK3C3 exhibited upregulation in several tumors and showed prognostic associations. Low expression of PIK3C3 predicted poorer overall survival (OS) in kidney renal clear cell carcinoma patients, while high expression of PIK3C3 predicted poorer OS in adrenocortical carcinoma, bladder urothelial carcinoma, brain lower grade glioma, and liver hepatocellular carcinoma. Additionally, PIK3C3 expression significantly correlated with immune infiltrating cells and tumor mutational burden, microsatellite instability and neoantigens in several cancer types. Furthermore, knockdown of PIK3C3 in colorectal cancer cells lines significantly suppressed cell proliferation and metastasis.

Conclusion: PIK3C3 can be used as an auxiliary indicator for early tumor diagnosis and a prognostic marker for many types of tumors.

1. Introduction

Cancer is a leading cause of death worldwide and remains a significant challenge despite the availability of various treatment methods, including surgery, radiotherapy, chemotherapy, targeted therapy and immunotherapy[1]. Tumor gene mutations and drug resistance make it increasingly difficult to effectively treat certain types of cancer[2]. Therefore, identifying more sensitive tumor markers and alternative drug targets for early cancer diagnosis and treatment is crucial.

Phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3), also known as yeast vesicular protein sorting 34 (Vps34)[3], is a member of the phosphatidylinositol 3-kinase (PI3K) family[4] that encodes 887 amino acids[5]. PIK3C3 catalyzes the phosphorylation of phosphatidylinositol (PtdIns) to produce phosphatidylinositol-3-phosphate (PI3P), which is a critical phospholipid in the process of autophagy[4]. After PI3P generation, PIK3C3 forms a complex with other proteins to nucleate autophagosomes and initiate autophagy.

Autophagy is a catabolic process that maintains cellular homeostasis by breaking down dysfunctional or unnecessary proteins and organelles[6], and also plays a dual role in cancers, inhibiting the growth of

benign tumors while promoting the growth of advanced cancers[7]. An increasing number of pan-cancer studies have shown that autophagy-related genes are closely associated with a variety of cancers[8, 9]. The autophagy-associated molecule ATG5 is intricately linked to various cancers, and its expression level has demonstrated associations with tumor immune infiltration and the tumor microenvironment, particularly in breast invasive carcinoma (BRCA), kidney renal clear cell carcinoma (KIRC) and liver hepatocellular carcinoma (LIHC)[10]. It has been mentioned that VPS13A, another important autophagy molecule, the absence of VPS13A causes a defective autophagy flux. VPS13A has been shown to be associated with rhabdomyosarcoma, gastric cancer and ovarian cancer[11]. Since PIK3C3 is an essential component of the autophagy process, PIK3C3 has emerged as a key player in oncogenesis and development. In colorectal cancer, inhibitors of PIK3C3 have been shown to inhibit colorectal cancer stem cells and improve the sensitivity of chemotherapy[12]. Similarly, PIK3C3 inhibitors can inhibit hepatocellular carcinoma stem cell activity[13]. Numerous studies have targeted PIK3C3 and found that PIK3C3 inhibitors can effectively inhibit cancer development[14–18]. A comprehensive understanding of the role of the autophagy molecule PIK3C3 in malignancies could be gained through a pan-cancer study on PIK3C3.

Tumor development encompasses a multifaceted process characterized by cancer cell proliferation, anti-apoptosis, neoangiogenesis, invasion, metastasis and immune evasion[19]. The tumor microenvironment (TME) plays a crucial role in this process, comprising diverse cellular components including cancer-associated fibroblasts, neuroendocrine cells, immune cells, inflammatory cells, blood and lymphatic vessels and extracellular matrix (ECM)[20]. Immune and inflammatory cells comprise a significant proportion of the tumor microenvironment[21], and tumor-infiltrating lymphocytes have been shown to play a crucial role in various cancers, serving as biomarkers of cancer and predicting patient prognosis[22–24]. It has been shown that the impact of autophagy on tumor immunity and the modulation of tumor-infiltrating immune cells. This range of effects encompasses the recognition and presentation of tumor antigens by antigen presenting cells (APCs) as well as the activation and development of T cell receptor (TCR)-specific lymphocyte activation and development. The emerging evidence highlighting the synergistic interplay between autophagy and immunity in shaing tumor progreesion implies that autophagy may be a potential target for cancer immunotherapy[25–28].

In this study, we present a comprehensive analysis of PIK3C3 in pan-cancer. We integrated data from various databases and performed a thorough investigation of PIK3C3 expression in different common cancer types. We examined the association between PIK3C3 expression and several biomarkers, including TMB, MSI, NEO, DNA methylation, and immune infiltration levels, as well as various immune cells in different cancer types. We observed a significant correlation between high PIK3C3 expression and poor prognosis in multiple cancers. Moreover, high PIK3C3 expression was closely associated with increased cellular infiltration. We employed several online tools to investigate the underlying mechanisms of PIK3C3 in different cancers, using data from TCGA and GTEx databases.

2. Materials and methods

2.1. Expression analysis of PIK3C3 in different cancers

The differences in PIK3C3 gene expression between different tumor tissues and normal tissues were explored in SangerBox (http://sangerbox.com/Tool), while TCGA was matched with GTEx data using the GEPIA2 (http://gepia2.cancer-pku.cn/)[29]. The "stage plots" in GEPIA2 can present PIK3C3 gene expression at different stages in several tumor types, and Log2 (TPM + 1) on a logarithmic scale is used for the violin plot. UALCAN (http://ualcan.path.uab.edu/index.html) data analysis portal was used to analyze promoter methylation[30].

2.2. Association of PIK3C3 with different tumor survival rates

In GEPIA2, the "survival analysis" module was used to reveal the overall survival (OS) and disease-free survival (DFS) curves of high and low PIK3C3 expression groups in different cancer types using the Kaplan-Meier method. The association between PIK3C3 expression in cancer and patient prognosis, including OS, disease-specific survival (DSS), disease-free interval (DFI) and progression-free interval (PFI) were also investigated.

2.3. Analysis of genetic alterations and immune infiltration of PIK3C3

The cBioPortal (https://www.cbioportal.org/) has the capability to analyze the frequency of genetic alterations, types of alterations (mutations, amplifications, multiple alterations) and mutation sites. The expression of PIK3C3 in tumor infiltrating immune cells (TIICs) was evaluated on the SangerBox website, and the relationship of PIK3C3 expression with immune checkpoint genes (ICPGs) and TME biomarkers (including TMB, MSI, NEO) was also explored. In addition, the correlation between PIK3C3 expression and immune subtypes of tumor types and molecular subtypes of tumor types was analyzed using the TISIDB database (http://cis.hku.hk/TISIDB/index.php).

2.4. Cell culture

Colorectal cancer cells HCT8, HCT116 and RKO were purchased from Zhejiang Meisen Cell Technology Co. HCT8 cells were cultured in 1640 medium (Cat. C11875500BT; Gibco) supplemented with 10% fetal bovine serum (FBS, Cat. A6903FBS-500; Invigentech), 1% Penicillin-Streptomycin Solution (Cat. H8611; HAKATA), HCT116 and RKO cells were cultured in high-sugar DMEM medium (Cat. CTCC-002-008; MeisenCTCC) supplemented with 10% fetal bovine serum, 1% Penicillin-Streptomycin solution, and the cells were cultured in a 37°C, 5% CO_2 incubator.

2.5. PIK3C3 siRNA and transfection

HCT8, HCT116 and RKO cells were inoculated onto 6-well plates (without Penicillin-Streptomycin solution) at a density of 3×10^5 cells/well and incubated at 37°C for 24 hours. The siRNA and Lipofectamine 2000 were diluted with DMEM medium, left for 5 minutes at room temperature, and the

two were mixed well and left for 20 minutes at room temperature. Then, the mixture was transferred to a 6-well plate and incubated at 37°C. After transfection for 6–8 hours, replace the cell culture medium.

2.6 Real-time quantitative fluorescence PCR

Total RNA was extracted from colorectal cancer cells using Trizol reagent (Cat. R701-01; Vazyme). The PCR conditions were set as 95°C for 5 minutes, followed by 45 cycles of 95°C for 10s, 60°C for 10s and 72°C for 10s. mRNA expression was performed by SYBR Premix Ex Taq II (Cat. RR820A; TaKaRa).

The primer sequences were

PIK3C3, FORWARD: TGGAAGCCGATGGATGTAGAGGAC REVERSE: ACAGCATAACGCCTCACAGTTGG

GAPDH, FORWARD: GGTGGTCTCCTCTGACTTCAACA REVERSE: GTTGCTGTAGCCAAATTCGTTGT

CDH1, FORWARD : ATCCTGACCAGCAGTTCGTTGTTG REVERSE : GTTCCTCGTTCTCCACTCTCACATG

CDH2, FORWARD: CGATAAGGATCAACCCCATACA REVERSE : TTCAAAGTCGATTGGTTTGACC

Vimentin, FORWARD : ACTAGCCGCAGCCTCTATTCCTC REVERSE : GAAGTCCACCGAGTCTTGAAGCAG

2.7. Wetern blot and Antibodies

For cells transfected for 48 hours, cells were lysed with ice-cold RIPA buffer containing protease inhibitor and phosphatase inhibitor. Cell lysates were collected from the culture plates and total protein was collected by centrifugation. Protein concentration was quantified using the BCA Protein Assay Kit (Cat. 23225; Thermo), then add protein loading buffer and boil for 5 minutes. Proteins were separated by SDS-PAGE and transferred to PVDF membranes, then the membranes were closed with milk for 2 hours and incubated with anti-PIK3C3 (1:1000, 4263S; CST) and β-actin (1:1000, AF2811; Beyotime) overnight at 4°C, followed by HRP-conjugated secondary protein (1:1000, RS0002; ImmunoWay) for 2 hours at room temperature. Chemiluminescence signal of proteins was generated by ECL reagent and quantified by ImageJ software.

2.8. CCK8 assay

For CCK8 assay, 3×10^3 cells transfected for 48h were inoculated onto 96-well plates. After 24 hours of incubation, the used medium was replaced with 100 μL of fresh medium containing 10 μL CCK8 (Cat. BS350B; Biosharp) and the cells were incubated for 1 hour at 37°C. Cell proliferation was determined by measuring the OD at 450 nm using a microplate spectrophotometer.

2.9. Statistical analysis

GraphPad Prism was applied for statistical analyses and illustrated data, each of the experiment were conducted for three times. One-way ANOVA or two-tailed Student's t test were utilized for statistically analyzing the experimental data and the data were described as mean ± SD. The difference of $p < 0.05$ was viewed statistically significant.

3. Results

3.1. Differential Expression of PIK3C3 in Cancers

The result of PIK3C3 expression in different cancers by the Sangerbox was shown in Fig. 1A, PIK3C3 was upregulated in certain cancers, including glioblastoma (GBM), Glioma (GBMLGG), LGG, BRCA, stomach adenocarcinoma (STAD), lung squamous cell carcinoma (LUSC), LIHC, pancreatic adenocarcinoma (PAAD), acute myeloid leukemia (LAML), cholangiocarcinoma (CHOL) (p 0.0001), head and neck squamous cell carcinoma (HNSC), skin cutaneous melanoma (SKCM), uterine carcinosarcoma (UCS) (p 0.01), uterine corpus endometrial carcinoma (UCEC) and colon and rectal adenocarcinoma (COADREAD) (p 0.05). Whereas the expression of PIK3C3 in lung adenocarcinoma (LUAD), kidney renal papillary cell carcinoma (KIRP), thyroid carcinoma (THCA), ovarian serous cystadeno carcinoma (OV) and testicular germ cell tumors (TGCT) (p 0.0001), rectum adenocarcinoma (READ), kidney chromophobe (KICH) (p 0.01) and ACC (p 0.05) was lower than normal tissues (Fig. 1A).

To further explored the expression of PIK3C3 in various cancer types, we compared tumor tissues to control tissues in the GTEx database using GEPIA2, and generated box line plots to visualize the expression differences. As shown in Fig. 1B, PIK3C3 expression was upregulated in CHOL, lymphoid neoplasm diffffuse large B cell lymphoma (DLBC), GBM, LGG, PAAD and thymoma (THYM). We further probed the difference in PIK3C3 expression in CHOL, GBM, PAAD, pheochromocytoma and paraganglioma (PCPG) and THYM using UALCA. The results showed that the total expression of PIK3C3 in CHOL, GBM, and THYM was higher than normal tissues, while the expression in PAAD and PCPG was lower than normal tissues, as depicted in Fig. 1C. Additionally, we investigated the correlation between PIK3C3 expression levels and pathological staging in several cancers, including DLBC, KIRC, LIHC, OV, and STAD, the results are shown in Fig. 1D. The correlation between pathological stages and PIK3C3 expression levels in other cancer types with no statistical significance were shown in Supplemental Fig. 1.

3.2. Prognostic Index of PIK3C3 in Different Cancers

In the analysis of the TCGA database, it was found that low expression of PIK3C3 was associated with poorer OS and DFS in KIRC patients, while high expression of PIK3C3 was associated with poorer OS and DFS in ACC, LGG patients ($p < 0.05$) (Fig. 2A and 2B), high expression of PIK3C3 was associated with poorer OS in and DFS in SKCM ($p < 0.05$) patients. Notably, PIK3C3 expression in Colon adenocarcinoma (COAD) patients did not correlated with patient OS, however, its promoter methylation was significantly associated with DSS, DFI and PFI, as shown in Fig. 2C. DSS, DFI, PFI of others cancer types were shown in Supplemental Fig. 2, 3, 4.

3.3. Alteration of PIK3C3 Gene Analysis Data

According to Fig. 3A, the analysis indicates that the gene alteration of PIK3C3 occurs most frequently, with mutation being the predominant type of genetic change. Specifically, PIK3C3 exhibits a high

prevalence of mutation in various cancer types, including UCS, UCEC, STAD, BLCA, SKCM, COAD, LUAD, cervical squamous cell carcinoma (CESC), ACC, LAML, THYM, BRCA, KIRP and GBM. Similarly, amplification occurs in OV, HNSC, sarcoma (SARC), LGG and LIHC is the main types of PIK3C3 gene alterations. Additionally, Fig. 3B contains more detailed information regarding the mutation type, location and the number of cases with "Missense" as the main type of mutation. Moreover, a diagram of PIK3C3 mutation sites revealed a somatic mutation frequency of 1.5%. The differentinal alterations at the P94Q/S site included 161 missense, 18 truncating, and 14 splice and 2 Fusion alterations. Notably, P94Q mutations were observed in two patients with skin cutaneous melanoma and one with lung squamous cell carcinoma, while P94S was detected in one patient with lung squamous cell carcinoma.

3.4. PIK3C3 Promoter Methylation Expression in Different Cancers

Based on analysis of the TCGA database, the promoter methylation level of PIK3C3 was founded to be significantly higher in COAD, esophageal carcinoma (ESCA), LUSC and SARC than in normal tissues ($p < 0.05$), while it was lower in rostate adenocarcinoma (PRAD) and TGCT (Fig. 4). These findings suggest that aberrant promoter methylation of PIK3C3 may play a role in the development and progression of certain types of cancer.

3.5. Expression of PIK3C3 in Immune and Molecular Subtypes of Different Cancers

Cancer is a heterogeneous disease, and molecular subtyping of cancers can aid in the identification of the pathways and processes underlying specific cancer subsets. The role of PIK3C3 expression on immune and molecular subtypes among human cancers was explored with the TISIDB website. PIK3C3 expression was different in various immune subtypes including C1 (wound healing), C2 (IFN-gamma dominant), C3 (inflammatory), C4 (lymphocyte depleted), C5 (immunologically quiet) and C6 (TGF-b dominant) in ACC, BLCA, CESC, COAD, ESCA, KICH, KIRC, LGG, PAAD, PRAD, SARC, SKCM, STAD and UCEC (Fig. 5A).

In addition, PIK3C3 in ACC, BRCA, COAD, ESCA, HNSC SKCM, LUSC, STAD and UCEC was differentially expressed in multiple molecular isoforms ($p < 0.01$). For COAD, ESCA and STAD, PIK3C3 was identified to express more in the molecular subtype of CIN than other molecular subtypes. For adrenocortical carcinoma, PIK3C3 was expressed the highest in the molecular subtype of CIMP_high. For breast invasive carcinoma and lung squamous cell carcinoma, PIK3C3 was expressed the highest in the molecular subtype of Basal. For head and neck squamous cell carcinoma, PIK3C3 was expressed the highest in the molecular subtype of Atypical. For skin cutaneous melanoma, PIK3C3 was expressed the highest in the molecular subtype of hotspot_Mutants. For uterine corpus endometrial carcinoma, PIK3C3 was expressed the highest in the molecular subtype of CN_HIGH.

3.6. PIK3C3 Expression is Associated with ICPGs Expression in Different Cancers

Immune surveillance affects the prognosis of cancer patients, and malignancies evade immune system recognition by employing immune checkpoints such as PD-1, PD-L1 and CTLA-4[31, 32]. To further assess the correlation between PIK3C3 expression and TME in a pan-cancer dataset, we investigated the relationship between PIK3C3 expression and ICPGs expression. Notably, we observed that PIK3C3 expression was positively correlated with most ICPGs expression in the vast majority of cancers including UVW, OV, READ, COAD, CHOL, STAD, LAML, TGCT, HNSC, LGG, KIRC, PCPG, PRAD, KICH, THCA, UCEC, ESCA, LUSC, BRCA, LUAD, UCS, ACC and SKCM (Fig. 6).

3.7. Correlation between PIK3C3 Expression and Immune Cell Infiltration in Different Cancers

The correlation between PIKC3 expression and the majority of immune cells in various cancers was displayed in Fig. 7A, PIK3C3 gene expression linked positively with T_cells_CD4_memory_resting and macrophage M1 and M2 infiltration levels, but negatively with the infiltration of most immune cells. A pan-cancer analysis of the association between PIK3C3 expression and the level of immune infiltration based on the TIMER database was performed, as shown in Fig. 7B, the expression of PIK3C3 was significantly correlated with the abundance of infiltrating immune cells, including $CD8^+$T cells in 22 cancers, $CD4^+$T cells in 15 cancers, neutrophils in 22 cancers, dendritic cells in 22 cancers, macrophages in 25 cancers and B cells in 16 cancers. The relationship between PIK3C3 expression and infiltration of diverse immune cell subtypes was also explored, T cell CD4 memory resting and Macrophages M2 were significantly and positively correlated with PIK3C3 in variety of cancers, as shown in Fig. 7C.

3.8. Pan-cancer Analysis of the Correlation between PIK3C3 and Biomarkers of Immunotherapeutic response

Tumor mutational bureden (TMB), microsatellite instability (MSI) and neoantigens (NEO) are emerging biomarkers associated with immunotherapeutic response. Therefore, an investigation about the relationship between PIK3C3 expression and TMB, MSI and NEO was arranged, in terms of TMB, PIK3C3 expression was positively correlated in CESC, COAD, COADREA, Stomach and Esophageal carcinoma(STES) and STAD, and was negatively correlated in LAML(Fig. 8A). In terms of MSI, PIK3C3 expression was positively associated with 7 cancers, including GBM, COAD, COADREAD, STES, STAD, KIRC and READ, and negatively associated with GBMLGG, PRAD, THCA and DLBC (Fig. 8B). Additionally, PIK3C3 expression was positively correlated with NEO in GBM, CESC, COAD, COAD and READ, as shown in Fig. 8C.

3.9. PIK3C3 Knockdown Inhibits Cell Proliferation and Metastasis

From the preceding bioinformatics findings, the expression of PIK3C3 in COAD patients was notably higher than in normal tissues. Interestingly, despite the lack of correlation between PIK3C3 expression and overall survival, its promoter methylation was significantly association with DSS, DFI and PFI. In order to delve deeper into the connection between PIK3C3 mutation and colon cancer development, three colorectal cancer cell lines (HCT8, HCT116, RKO) were employed. By using siRNA to knockdown PIK3C3, the knockdown rate was exceeded 50% in all three cells (Fig. 9A) and western blot experiments also confirmed the decrease in PIK3C3 protein expression in all three cells (Fig. 9B). The average knockdown rate of PIK3C3 protein was shown in Supplemental Fig. 7. As shown in Fig. 9C, after the knockdown of PIK3C3, the proliferation of tumor cells was inhibited and the proliferation of the three colorectal cancer cells was reduced by roughly 40%.

During cancer progression, epithelial tumor cells may undergo epithelial-to-mesenchymal transition (EMT), a morphological and functional remodeling, that deeply alters tumor cell features, leading to loss of epithelial markers, such as E-cadherin(CDH1), changes in cell polarity and intercellular junctions and increase of mesenchymal markers such as N-cadherin (CDH2) and vimentin. Therefore, using RT-PCR experiments to detect the expression of EMT-related mRNAs, it was found that after knockdown of PIK3C3, the expression of CDH1 was elevated and the expression of both CDH2 and Vimentin was decreased. The results of these experiments suggest that PIK3C3 is a key gene that promotes proliferation and migration in colorectal cancer cells.

4. Discussion

Pan-cancer analysis can reveal the similarities and differences between different cancers and provide insights into the design of cancer prevention and personalized treatment strategies[33]. In this study, we comprehensively examined the expression of PIK3C3 in a pan-cancer datasets.

Our results demonstrate the upregulation of PIK3C3 in several cancer types including CHOL, DLBC, GBM, LGG, PAAD and THYM, indicating its potential as a prognostic marker. Previous studies have highlighted the efficacy of PIK3C3 inhibitors in suppressing the growth and migration of various cancer types, such as non-small cell lung cancer, pancreatic cancer, colorectal cancer, breast cancer, head and neck squamous cell carcinoma, and melanoma[14, 17, 18, 34–37]. Combining PIK3C3 inhibitors with conventional anticancer drugs or immunotherapies has been a common clinical practice, leading to synergistic anticancer effects and improved therapeutic outcomes.

DNA methylation, a crucial epigenetic modification, often results in the silencing or inactivation of tumor suppressor genes when hypermethylation occurs within the promoter region [38, 39]. Notably, the elevated levels of promoter methylation of PIK3C3 was founded in COAD, ESCA, LUSC, and SARC when compared to the normal tissues. VPS13A has high methylation levels in cells from tumor patients[11].

The prognosis of tumor patients is closely linked to immune infiltration[40]. Tumor-infiltrating immune cells as integral component of the tumor microenvironment are associated with tumor progress, prognosis and responses to immunotherapy[41], and our findings reveal a strong correlation between PIK3C3 expression and immune cell populations across various cancer types. Moreover, PIK3C3 expression demonstrates associations with distinct immune subtypes and molecular subtypes within different cancer types. The oncogenesis and progression of cancers are closely associated with genomic mutations. In COAD and COADREAD, PIK3C3 expression positively correlates with TMB, MSI and NEO, indicating enhanced antitumor immunity and potential predictability of immunotherapy response based on PIK3C3 expression. Notably, immune checkpoint inhibitors therapy has shown promising results in improving the survival rates of patients with metastatic colorectal cancer [42].

Intriguingly, although the expression of PIK3C3 did not correlate with patient overall survival in COAD, the promoter methylation of PIK3C3 exhibited significant associations with DSS, DFI and PFI. It is well known that metastasis and recurrence are important mortality factors for patients. Notably, optimal treatment has led to colorectal cancer having one of the best long-term prognoses, with curative resection being possible in 98% of patients presenting with resectable tumors and no distant metastases[43]. Therefore, this may explain the lack of association between PIK3C3 expression and OS in COAD. DSS, DFI, PFI, the above three indicators were more reflective of cancer-induced death. In addition, colorectal cancer patients with mismatch repair (MMR) deficiency had a better prognosis, and metastatic colorectal cancer with MMR deficiency accounted for about 5% of poor prognosis, indicating that gene mutations play a very important role in the progression and treatment of colorectal cancer[44], and in COAD, PIK3C3 mutation is the main type of gene alteration, further suggesting that high expression of PIK3C3 may cause poor prognosis in COAD patients.

Several preclinical and clinical studies have been conducted to develop pharmacological inhibitors targeting autophagy. Targeting PIK3C3/VPS34 genetically or using pharmacological inhibitors SB02024 or SAR405 in tumor cells significantly reduce tumor growth, decrease tumor weight, and improved survival in a mouse model of CT26 colorectal cancer[45]. Based on the results of our raw letter analysis and the feasibility of autophagy and immunotherapy, we selected colorectal cancer for phenotypic experiments to further validate the role of PIK3C3 in colorectal cancer cells. Notably, our findings align with the study by Kobylarz MJ et al., confirming that PIK3C3 promotes tumor cell proliferation and metastasis[36].

5. Conclusion

In summary, our findings provide a comprehensive understanding of the function of PIK3C3 in the prognosis and immunotherapy of different types of cancers. PIK3C3 has the potential to be used as a target for cancer immunotherapy and deserves more attention.

Supplementary Materails

Figure S1: PIK3C3 gene expression level in 19 tumor tissues and normal tissues in GEO database. Figure S2: Correlation between PIK3C3 promoter methylation and DSS in several patients. Figure S3: Correlation between PIK3C3 promoter methylation and DFI in several patients. Figure S4: Correlation between PIK3C3 promoter methylation and PFI in several patients. Figure S5: The average knockdown rate of PIK3C3 protein. $^{**}p < 0.01$, and $^{***}p < 0.001$.

Declarations

Supplementary Materails

Figure S1: PIK3C3 gene expression level in 19 tumor tissues and normal tissues in GEO database. Figure S2: Correlation between PIK3C3 promoter methylation and DSS in several patients. Figure S3: Correlation between PIK3C3 promoter methylation and DFI in several patients. Figure S4: Correlation between PIK3C3 promoter methylation and PFI in several patients. Figure S5: The average knockdown rate of PIK3C3 protein. $^{**}p < 0.01$, and $^{***}p < 0.001$.

Funding Statement: This work was supported by the National Natural Science Foundation of China [grant numbers 82003753, 82204875, 82074391]; Natural Science Foundation of Zhejiang Province [grant numbers LQ21H080002, LY21H270004]; and Zhejiang Chinese Medical University (grant numbers 2021RCZXZK07).

Author Contributions: Q.X.: conceptualization, writing—original draft preparation. J.Z.: methodology, formal analysis. J.G.: formal analysis. J.L.: software, validation. S.S.: data curation. W.L.: writing—reviewing and editing. Y.X., M.H. and T.F.: conceptualization, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sung, H.; Ferlay, J.; Siegel, R. L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F., Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* **2021,** *71* (3), 209-249.

2. Zugazagoitia, J.; Guedes, C.; Ponce, S.; Ferrer, I.; Molina-Pinelo, S.; Paz-Ares, L., Current Challenges in Cancer Treatment. *Clin Ther* **2016,** *38* (7), 1551-66.
3. Lindmo, K.; Stenmark, H., Regulation of membrane traffic by phosphoinositide 3-kinases. *J Cell Sci* **2006,** *119* (Pt 4), 605-14.
4. Chu, C. A.; Wang, Y. W.; Chen, Y. L.; Chen, H. W.; Chuang, J. J.; Chang, H. Y.; Ho, C. L.; Chang, C.; Chow, N. H.; Lee, C. T., The Role of Phosphatidylinositol 3-Kinase Catalytic Subunit Type 3 in the Pathogenesis of Human Cancer. *Int J Mol Sci* **2021,** *22* (20).
5. Zhou, X.; Takatoh, J.; Wang, F., The mammalian class 3 PI3K (PIK3C3) is required for early embryogenesis and cell proliferation. *PLoS One* **2011,** *6* (1), e16358.
6. Huang, T.; Song, X.; Yang, Y.; Wan, X.; Alvarez, A. A.; Sastry, N.; Feng, H.; Hu, B.; Cheng, S. Y., Autophagy and Hallmarks of Cancer. *Crit Rev Oncog* **2018,** *23* (5-6), 247-267.
7. Onorati, A. V.; Dyczynski, M.; Ojha, R.; Amaravadi, R. K., Targeting autophagy in cancer. *Cancer* **2018,** *124* (16), 3307-3318.
8. Wu, G.; Xu, Y.; Zhang, H.; Ruan, Z.; Zhang, P.; Wang, Z.; Gao, H.; Che, X.; Xia, Q.; Chen, F., A new prognostic risk model based on autophagy-related genes in kidney renal clear cell carcinoma. *Bioengineered* **2021,** *12* (1), 7805-7819.
9. Kondapuram, S. K.; Coumar, M. S., Pan-cancer gene expression analysis: Identification of deregulated autophagy genes and drugs to target them. *Gene* **2022,** *844*, 146821.
10. White, E., Deconvoluting the context-dependent role for autophagy in cancer. *Nat Rev Cancer* **2012,** *12* (6), 401-10.
11. Zhang, X. Q.; Li, L., Biological Function and Clinical Value of VPS13A in Pan-Cancer Based on Bioinformatics Analysis. *Int J Gen Med* **2021,** *14*, 6825-6838.
12. Kumar, B.; Ahmad, R.; Sharma, S.; Gowrikumar, S.; Primeaux, M.; Rana, S.; Natarajan, A.; Oupicky, D.; Hopkins, C. R.; Dhawan, P.; Singh, A. B., PIK3C3 Inhibition Promotes Sensitivity to Colon Cancer Therapy by Inhibiting Cancer Stem Cells. *Cancers (Basel)* **2021,** *13* (9).
13. Liu, F.; Wu, X.; Qian, Y.; Jiang, X.; Wang, Y.; Gao, J., PIK3C3 regulates the expansion of liver CSCs and PIK3C3 inhibition counteracts liver cancer stem cell activity induced by PI3K inhibitor. *Cell Death Dis* **2020,** *11* (6), 427.
14. Chen, C. H.; Hsieh, T. H.; Lin, Y. C.; Liu, Y. R.; Liou, J. P.; Yen, Y., Targeting Autophagy by MPT0L145, a Highly Potent PIK3C3 Inhibitor, Provides Synergistic Interaction to Targeted or Chemotherapeutic Agents in Cancer Cells. *Cancers (Basel)* **2019,** *11* (9).
15. Chen, C. H.; Changou, C. A.; Hsieh, T. H.; Lee, Y. C.; Chu, C. Y.; Hsu, K. C.; Wang, H. C.; Lin, Y. C.; Lo, Y. N.; Liu, Y. R.; Liou, J. P.; Yen, Y., Dual Inhibition of PIK3C3 and FGFR as a New Therapeutic Approach to Treat Bladder Cancer. *Clin Cancer Res* **2018,** *24* (5), 1176-1189.
16. Meunier, G.; Birsen, R.; Cazelles, C.; Belhadj, M.; Cantero-Aguilar, L.; Kosmider, O.; Fontenay, M.; Azar, N.; Mayeux, P.; Chapuis, N.; Tamburini, J.; Bouscary, D., Antileukemic activity of the VPS34-IN1 inhibitor in acute myeloid leukemia. *Oncogenesis* **2020,** *9* (10), 94.

17. Young, C. D.; Arteaga, C. L.; Cook, R. S., Dual inhibition of Type I and Type III PI3 kinases increases tumor cell apoptosis in HER2+ breast cancers. *Breast Cancer Res* **2015,** *17*, 148.
18. Huang, S.; Sinicrope, F. A., Celecoxib-induced apoptosis is enhanced by ABT-737 and by inhibition of autophagy in human colorectal cancer cells. *Autophagy* **2010,** *6* (2), 256-69.
19. Hanahan, D.; Weinberg, R. A., Hallmarks of cancer: the next generation. *Cell* **2011,** *144* (5), 646-74.
20. Chen, F.; Zhuang, X.; Lin, L.; Yu, P.; Wang, Y.; Shi, Y.; Hu, G.; Sun, Y., New horizons in tumor microenvironment biology: challenges and opportunities. *BMC Med* **2015,** *13*, 45.
21. Bindea, G.; Mlecnik, B.; Tosolini, M.; Kirilovsky, A.; Waldner, M.; Obenauf, A. C.; Angell, H.; Fredriksen, T.; Lafontaine, L.; Berger, A.; Bruneval, P.; Fridman, W. H.; Becker, C.; Pagès, F.; Speicher, M. R.; Trajanoski, Z.; Galon, J., Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* **2013,** *39* (4), 782-95.
22. Orhan, A.; Khesrawi, F.; Tvilling Madsen, M.; Peuliche Vogelsang, R.; Dohrn, N.; Kanstrup Fiehn, A. M.; Gögenur, I., Tumor-Infiltrating Lymphocytes as Biomarkers of Treatment Response and Long-Term Survival in Patients with Rectal Cancer: A Systematic Review and Meta-Analysis. *Cancers (Basel)* **2022,** *14* (3).
23. Mohammed, A. A.; Mostafa Elsayed, F.; Algazar, M.; Rashed, H. E., Predictive and Prognostic Value of Tumor- Infiltrating Lymphocytes for Pathological Response to Neoadjuvant Chemotherapy in Triple Negative Breast Cancer. *Gulf J Oncolog* **2022,** *1* (38), 53-60.
24. Cai, Z.; Jiang, J.; Huang, L.; Yuan, Y.; Zheng, R.; Zhang, J.; Qiu, W., The Prognostic Impact of Combined Tumor-Infiltrating Lymphocytes and Pretreatment Blood Lymphocyte Percentage in Locally Advanced Nasopharyngeal Carcinoma. *Front Oncol* **2021,** *11*, 788497.
25. Nedjic, J.; Aichinger, M.; Emmerich, J.; Mizushima, N.; Klein, L., Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. *Nature* **2008,** *455* (7211), 396-400.
26. Sensi, M.; Anichini, A., Unique tumor antigens: evidence for immune control of genome integrity and immunogenic targets for T cell-mediated patient-specific immunotherapy. *Clin Cancer Res* **2006,** *12* (17), 5023-32.
27. Zhou, S.; Sun, X.; Jin, Z.; Yang, H.; Ye, W., The role of autophagy in initiation, progression, TME modification, diagnosis, and treatment of esophageal cancers. *Crit Rev Oncol Hematol* **2022,** *175*, 103702.
28. Jin, Z.; Sun, X.; Wang, Y.; Zhou, C.; Yang, H.; Zhou, S., Regulation of autophagy fires up the cold tumor microenvironment to improve cancer immunotherapy. *Front Immunol* **2022,** *13*, 1018903.
29. Tang, Z.; Kang, B.; Li, C.; Chen, T.; Zhang, Z., GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis. *Nucleic Acids Res* **2019,** *47* (W1), W556-w560.
30. Chandrashekar, D. S.; Karthikeyan, S. K.; Korla, P. K.; Patel, H.; Shovon, A. R.; Athar, M.; Netto, G. J.; Qin, Z. S.; Kumar, S.; Manne, U.; Creighton, C. J.; Varambally, S., UALCAN: An update to the integrated cancer data analysis platform. *Neoplasia* **2022,** *25*, 18-27.
31. Mlecnik, B.; Bindea, G.; Pagès, F.; Galon, J., Tumor immunosurveillance in human cancers. *Cancer Metastasis Rev* **2011,** *30* (1), 5-12.

32. Galon, J.; Angell, H. K.; Bedognetti, D.; Marincola, F. M., The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. *Immunity* **2013,** *39* (1), 11-26.
33. Chen, F.; Fan, Y.; Cao, P.; Liu, B.; Hou, J.; Zhang, B.; Tan, K., Pan-Cancer Analysis of the Prognostic and Immunological Role of HSF1: A Potential Target for Survival and Immunotherapy. *Oxid Med Cell Longev* **2021,** *2021*, 5551036.
34. Noman, M. Z.; Parpal, S.; Van Moer, K.; Xiao, M.; Yu, Y.; Viklund, J.; De Milito, A.; Hasmim, M.; Andersson, M.; Amaravadi, R. K.; Martinsson, J.; Berchem, G.; Janji, B., Inhibition of Vps34 reprograms cold into hot inflamed tumors and improves anti-PD-1/PD-L1 immunotherapy. *Sci Adv* **2020,** *6* (18), eaax7881.
35. New, J.; Arnold, L.; Ananth, M.; Alvi, S.; Thornton, M.; Werner, L.; Tawfik, O.; Dai, H.; Shnayder, Y.; Kakarala, K.; Tsue, T. T.; Girod, D. A.; Ding, W. X.; Anant, S.; Thomas, S. M., Secretory Autophagy in Cancer-Associated Fibroblasts Promotes Head and Neck Cancer Progression and Offers a Novel Therapeutic Target. *Cancer Res* **2017,** *77* (23), 6679-6691.
36. Kobylarz, M. J.; Goodwin, J. M.; Kang, Z. B.; Annand, J. W.; Hevi, S.; O'Mahony, E.; McAllister, G.; Reece-Hoyes, J.; Wang, Q.; Alford, J.; Russ, C.; Lindeman, A.; Beibel, M.; Roma, G.; Carbone, W.; Knehr, J.; Loureiro, J.; Antczak, C.; Wiederschain, D.; Murphy, L. O.; Menon, S.; Nyfeler, B., An iron-dependent metabolic vulnerability underlies VPS34-dependence in RKO cancer cells. *PLoS One* **2020,** *15* (8), e0235551.
37. Verykiou, S.; Alexander, M.; Edwards, N.; Plummer, R.; Chaudhry, B.; Lovat, P. E.; Hill, D. S., Harnessing autophagy to overcome mitogen-activated protein kinase kinase inhibitor-induced resistance in metastatic melanoma. *Br J Dermatol* **2019,** *180* (2), 346-356.
38. Mehdi, A.; Rabbani, S. A., Role of Methylation in Pro- and Anti-Cancer Immunity. *Cancers (Basel)* **2021,** *13* (3).
39. Wang, M.; Ngo, V.; Wang, W., Deciphering the genetic code of DNA methylation. *Brief Bioinform* **2021,** *22* (5).
40. Bi, K. W.; Wei, X. G.; Qin, X. X.; Li, B., BTK Has Potential to Be a Prognostic Factor for Lung Adenocarcinoma and an Indicator for Tumor Microenvironment Remodeling: A Study Based on TCGA Data Mining. *Front Oncol* **2020,** *10*, 424.
41. Tamborero, D.; Rubio-Perez, C.; Muiños, F.; Sabarinathan, R.; Piulats, J. M.; Muntasell, A.; Dienstmann, R.; Lopez-Bigas, N.; Gonzalez-Perez, A., A Pan-cancer Landscape of Interactions between Solid Tumors and Infiltrating Immune Cell Populations. *Clin Cancer Res* **2018,** *24* (15), 3717-3728.
42. Ganesh, K.; Stadler, Z. K.; Cercek, A.; Mendelsohn, R. B.; Shia, J.; Segal, N. H.; Diaz, L. A., Jr., Immunotherapy in colorectal cancer: rationale, challenges and potential. *Nat Rev Gastroenterol Hepatol* **2019,** *16* (6), 361-375.
43. Hohenberger, W.; Bittorf, B.; Papadopoulos, T.; Merkel, S., Survival after surgical treatment of cancer of the rectum. *Langenbecks Arch Surg* **2005,** *390* (5), 363-72.
44. Battaglin, F.; Naseem, M.; Lenz, H. J.; Salem, M. E., Microsatellite instability in colorectal cancer: overview of its clinical significance and novel perspectives. *Clin Adv Hematol Oncol* **2018,** *16* (11),

735-745.

45. Janji, B.; Hasmim, M.; Parpal, S.; De Milito, A.; Berchem, G.; Noman, M. Z., Lighting up the fire in cold tumors to improve cancer immunotherapy by blocking the activity of the autophagy-related protein PIK3C3/VPS34. *Autophagy* **2020,** *16* (11), 2110-2111.

Figures

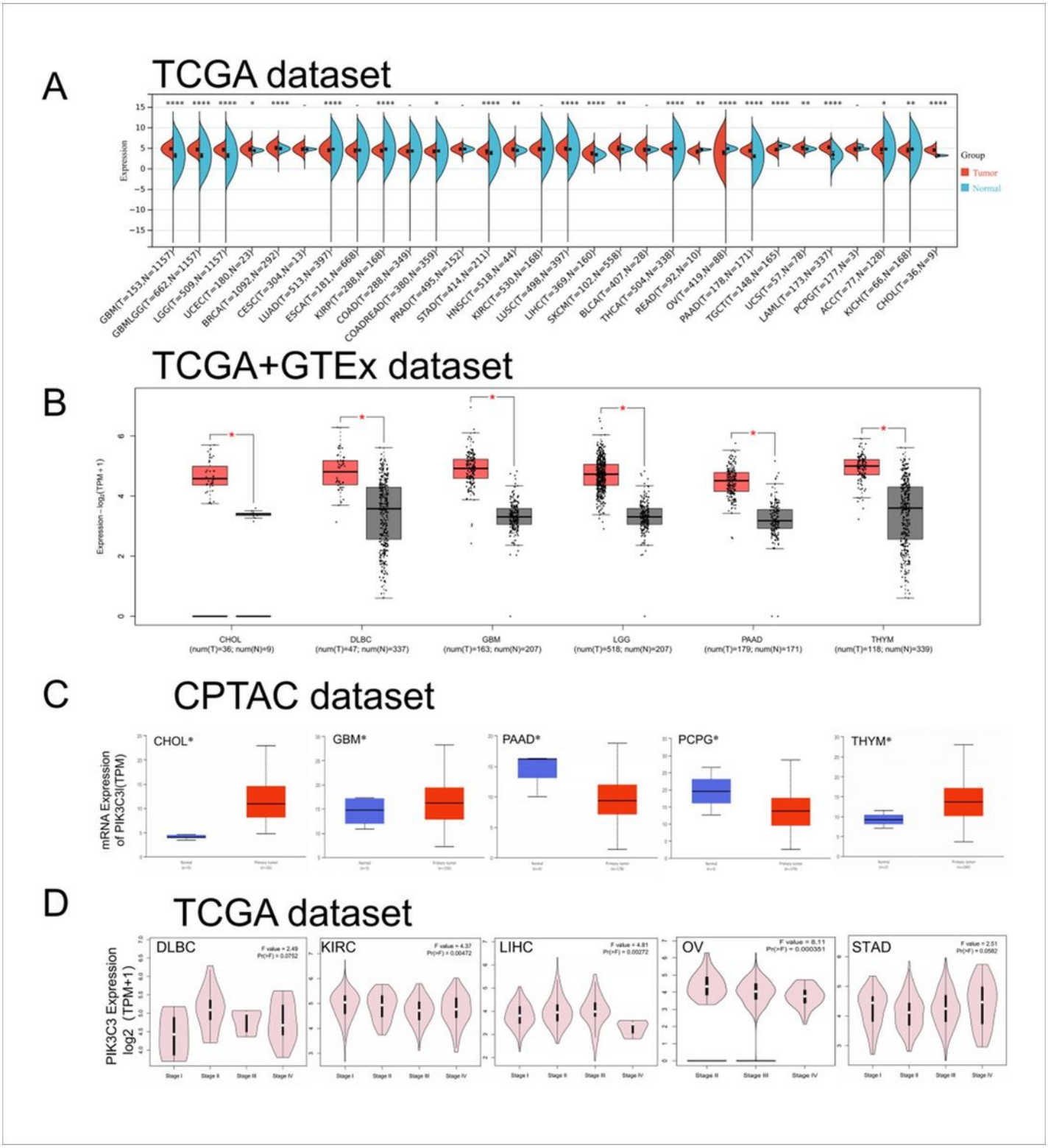

Figure 1

PIK3C3 gene expression in tumors and pathological stages of tumors. **(A)**PIK3C3 gene expression level in tumor tissues and normal tissues,(Blue represents normal tissues, red represents tumor tissues). *** $p <$ 0.001; ** $p < 0.01$; * $p < 0.05$. **(B)** PIK3C3 gene expression in TCGA+GTEx, including CHOL, DLBC, GBM, LGG, PAAD and THYM. * $p< 0.05$. **(C)** Total mRNA expression level of PIK3C3 in tumor tissues and normal tissues of CHOL, GBM, PAAD, PCPG and THYM. * $p < 0.05$. **(D)**PIK3C3 expression level in different pathological stages of DLBC, KIRC, LIHC, OV and STAD.

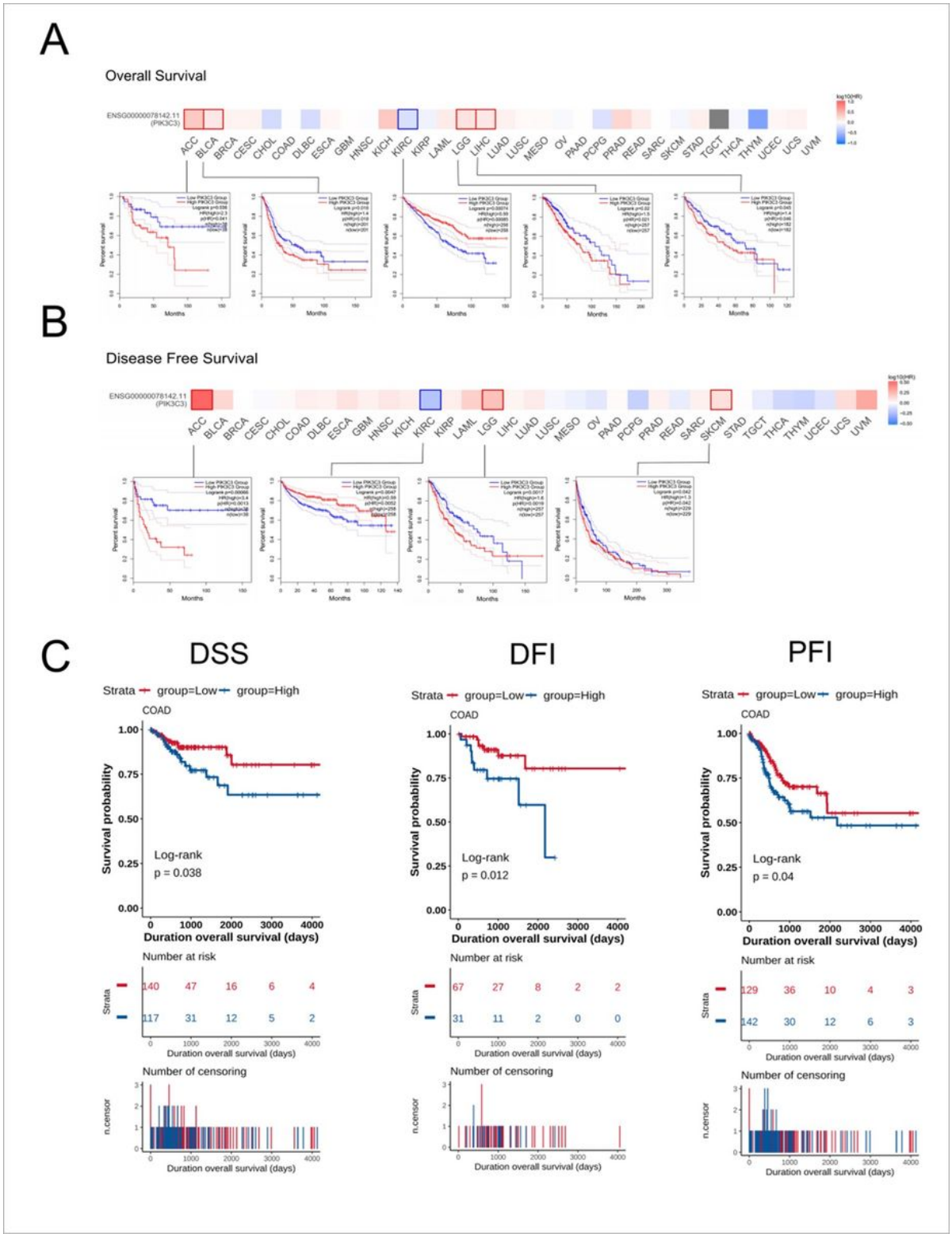

Figure 2

Correlation between PIK3C3 expression in TCGA and survival of cancer patients. **(A)**Correlation between PIK3C3 expression and OS in patients with KIRP, ACC, BLCA, LGG, and LIHC. **(B)** Correlation between PIK3C3 expression and DFS in KIRC, ACC, LGG and SKCM patients. **(C)** Correlation between PIK3C3 promoter methylation and DSS, DFI and PFI in COAD patients.

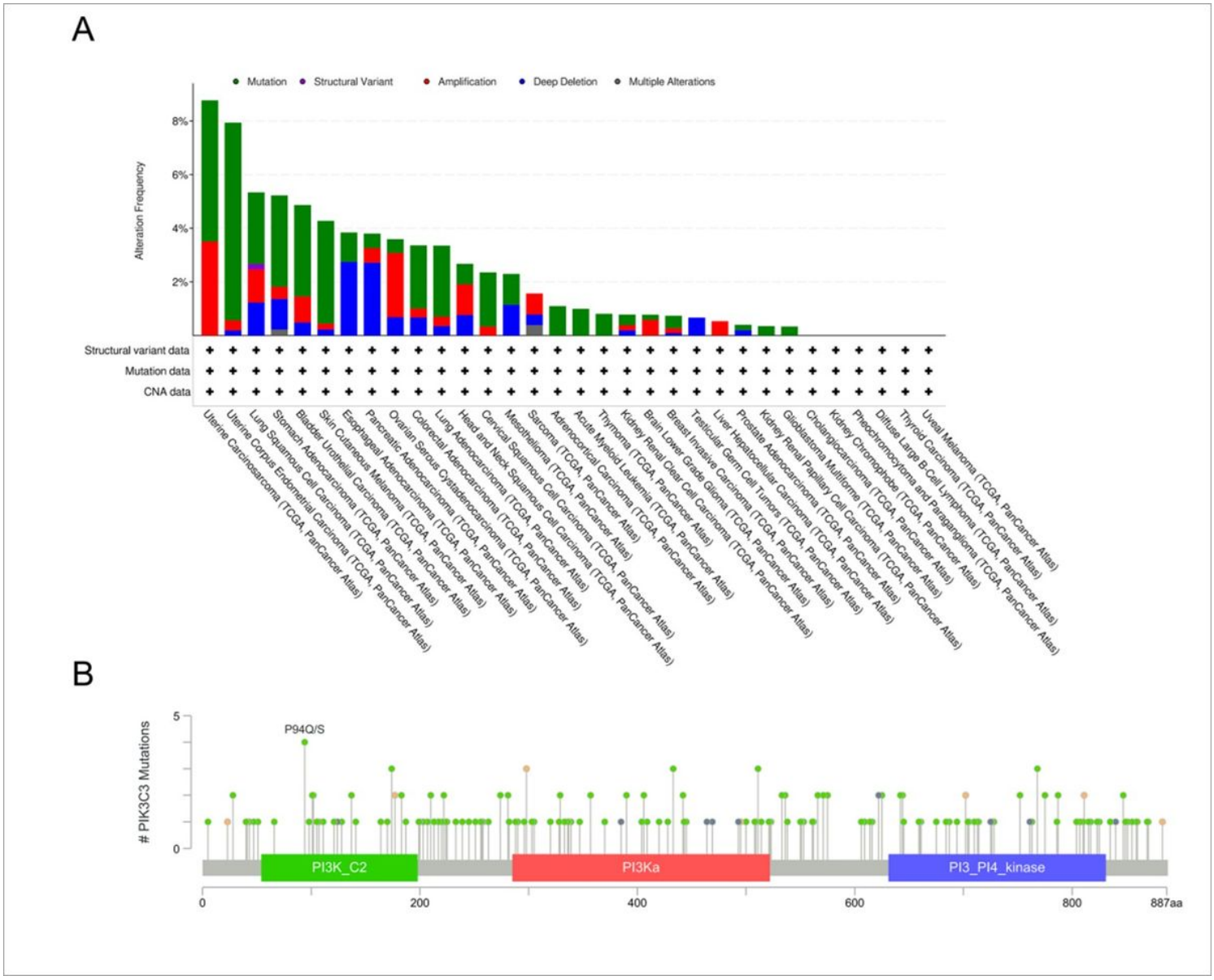

Figure 3

PIK3C3 gene alteration frequency, types, sites and cases. **(A)** PIK3C3 alteration types and frequency. **(B)** PIK3C3 mutation types, sites and cases. “+” represents that data are available.

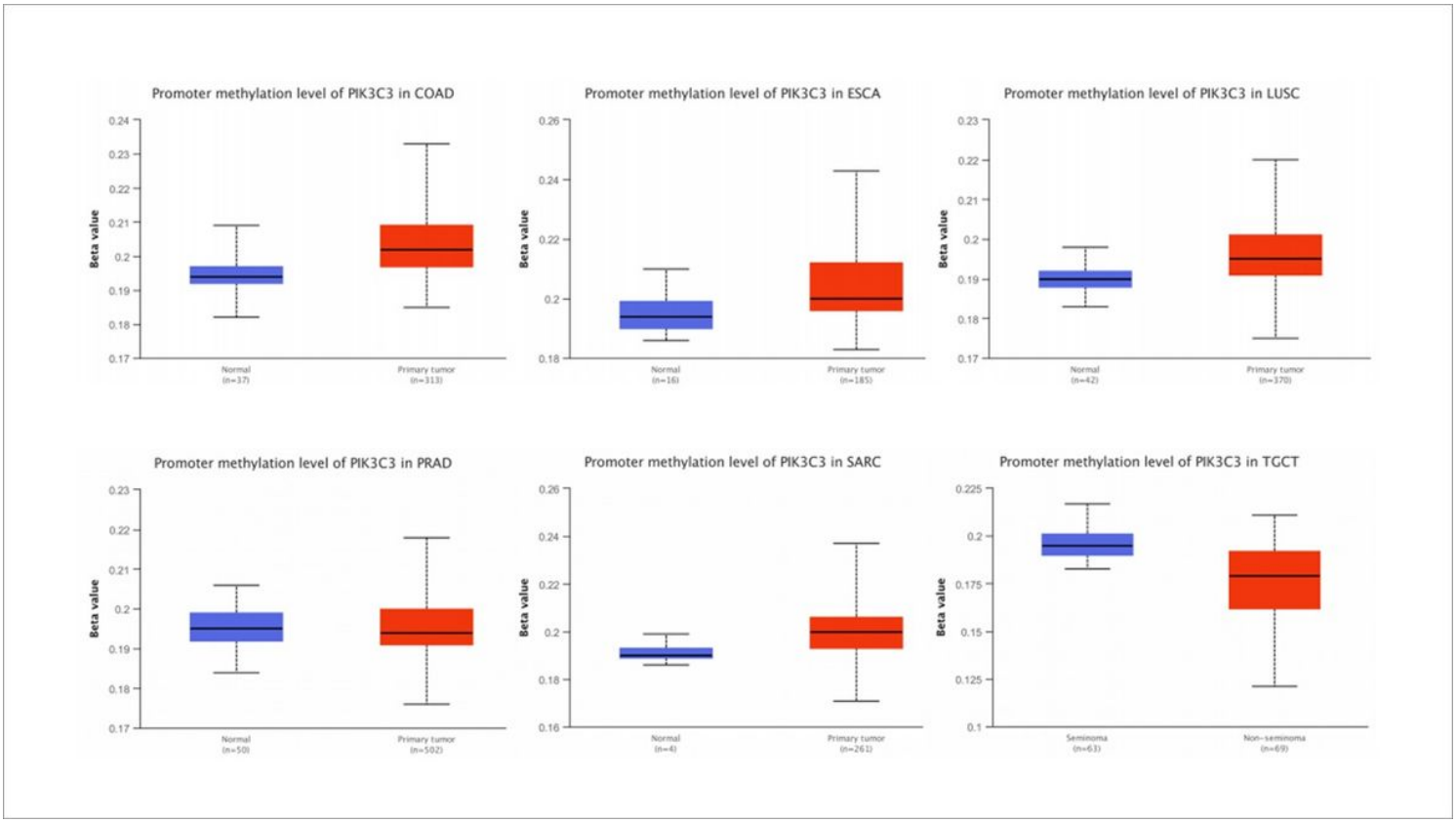

Figure 4

PIK3C3 protein promoter methylation analysis in different tumors. *$p < 0.05$.

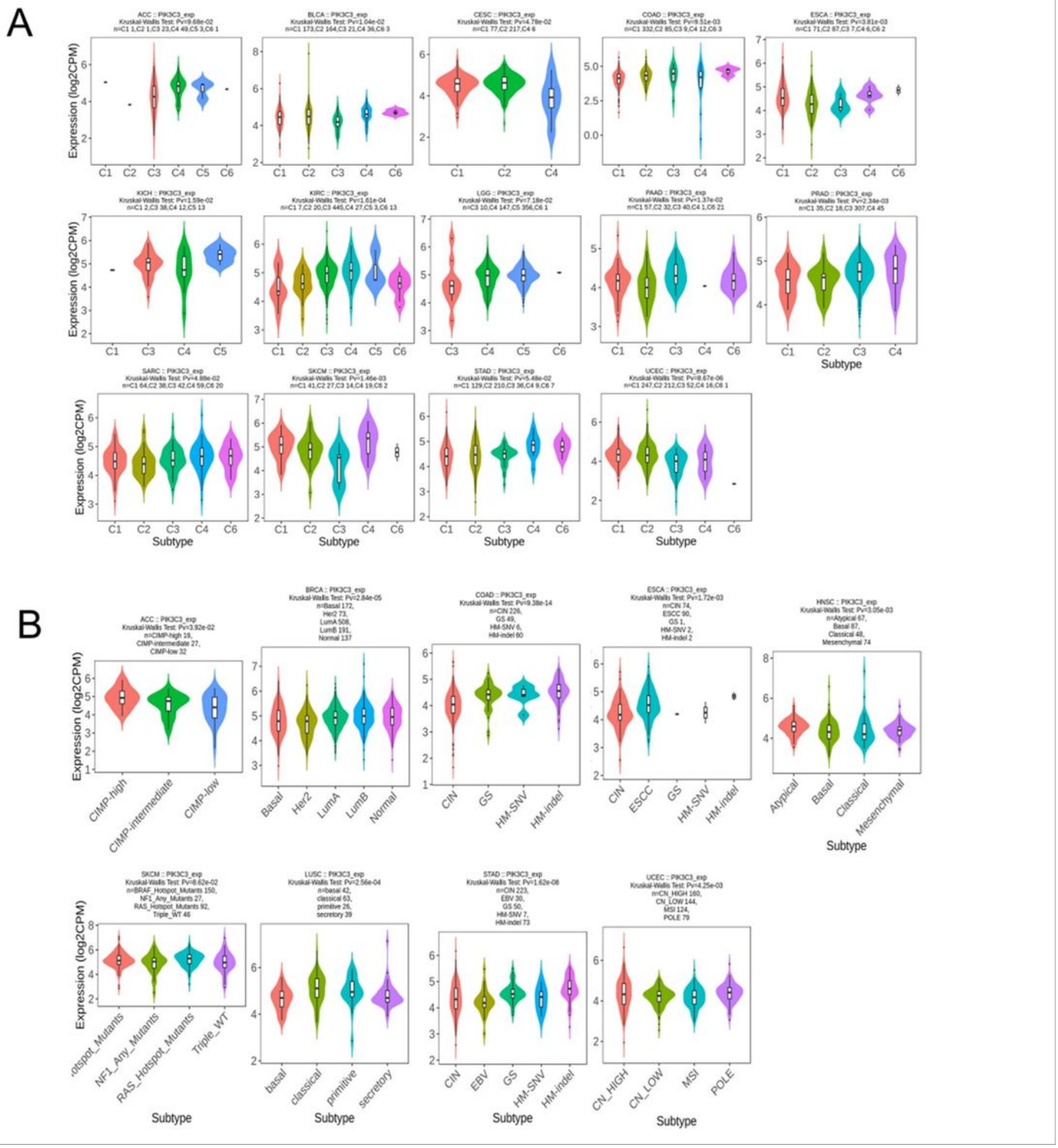

Figure 5

PIK3C3 expression in cancer immune and molecular subtypes. **(A)** Relationship between PIK3C3 expression and cancer immune subtypes. **(B)** Relationship between PIK3C3 expression and molecular subtypes of cancer.

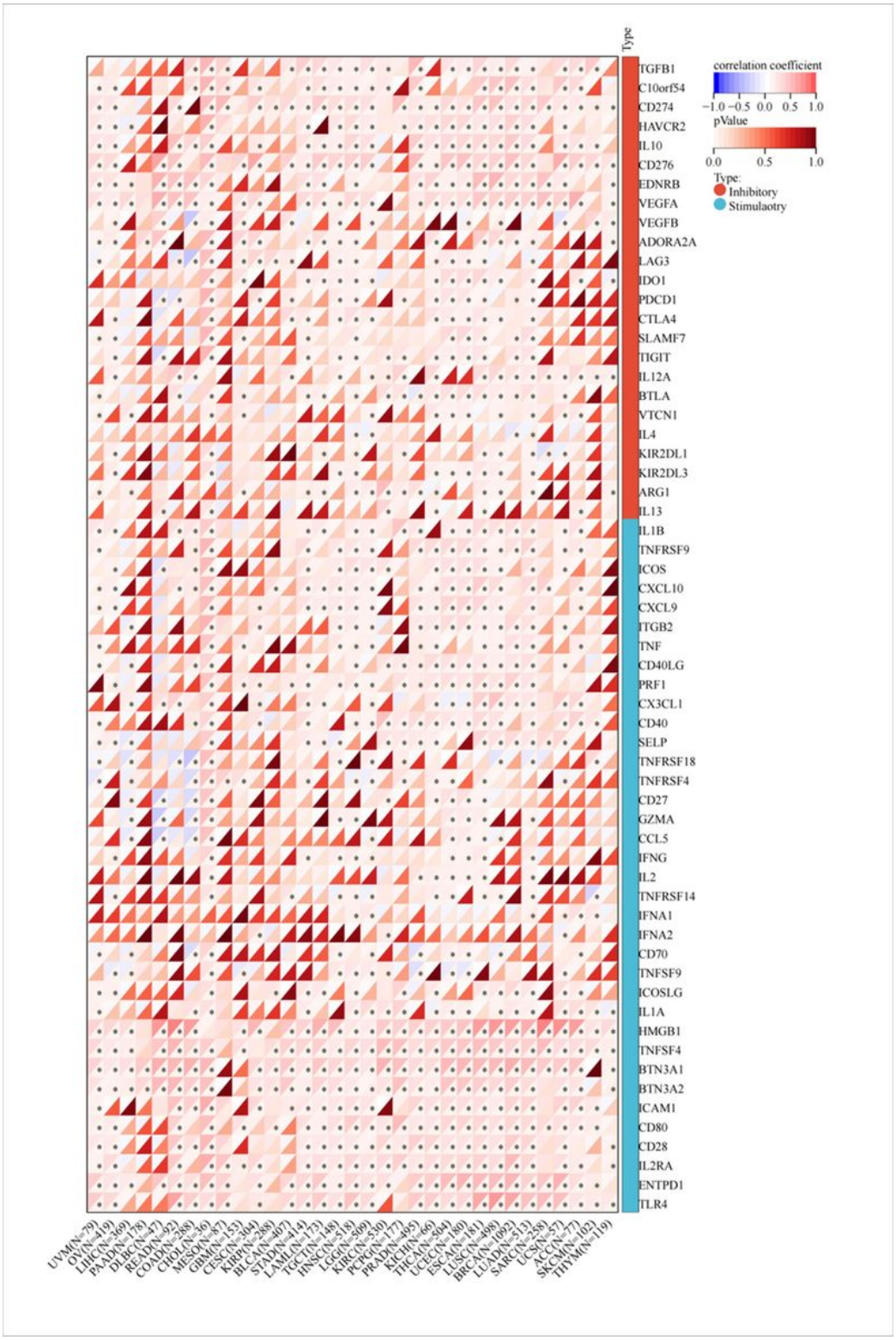

Figure 6

Correlation matrix between PIK3C3 expression and ICPGs expression. * p<0.05.

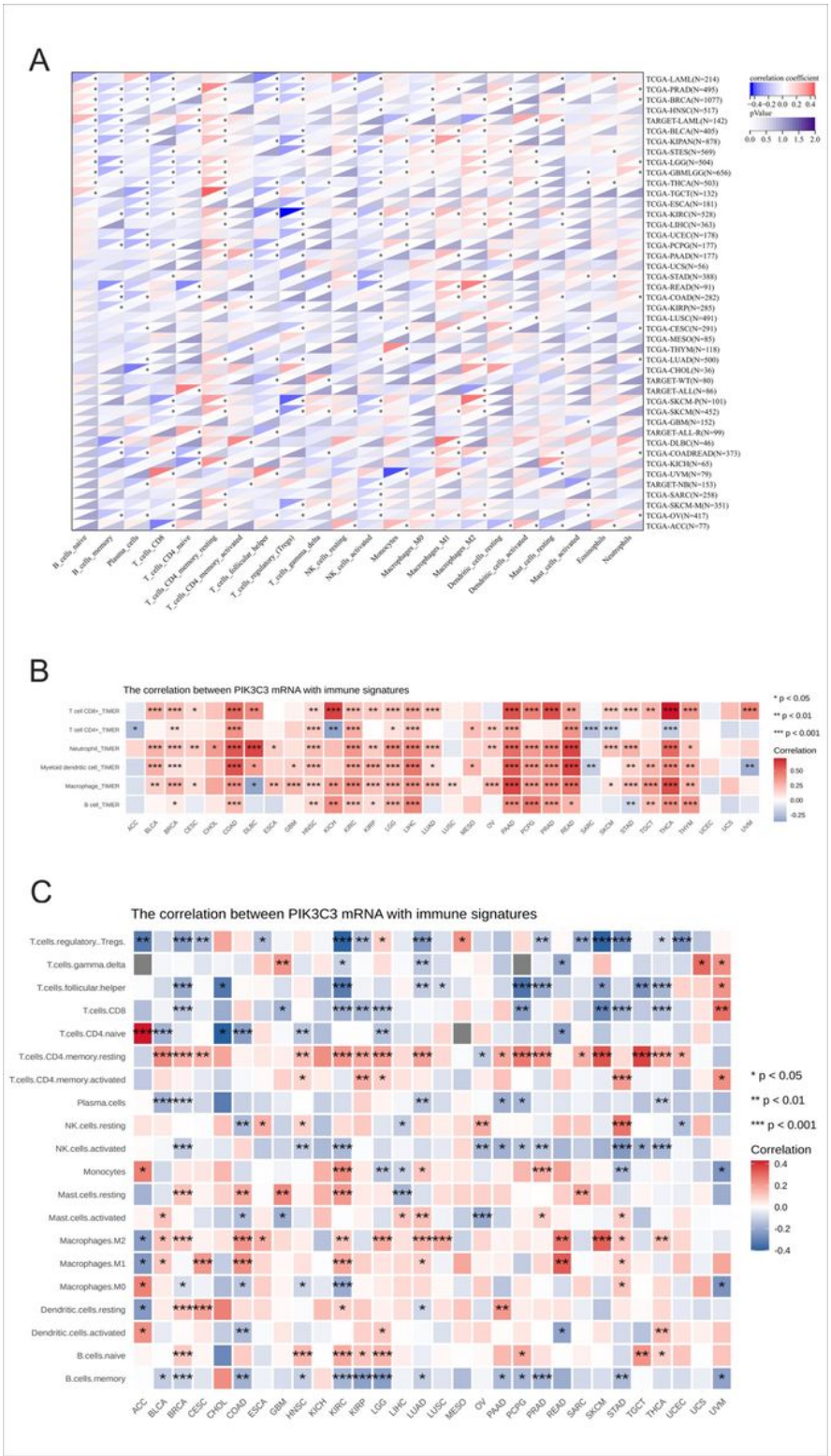

Figure 7

PIK3C3 expression correlated with immune infiltration. **(A)** Correlation matrix between PIK3C3 expression and immune cell content. *p < 0.05 **(B)** PIK3C3 expression was significantly correlated with the level of infiltration of various immune cells in the TIMER database. **(C)** PIK3C3 expression was significantly correlated with the level of infiltration of various immune cell subtypes. *p< 0.05, **p < 0.01, and ****p < 0.001.

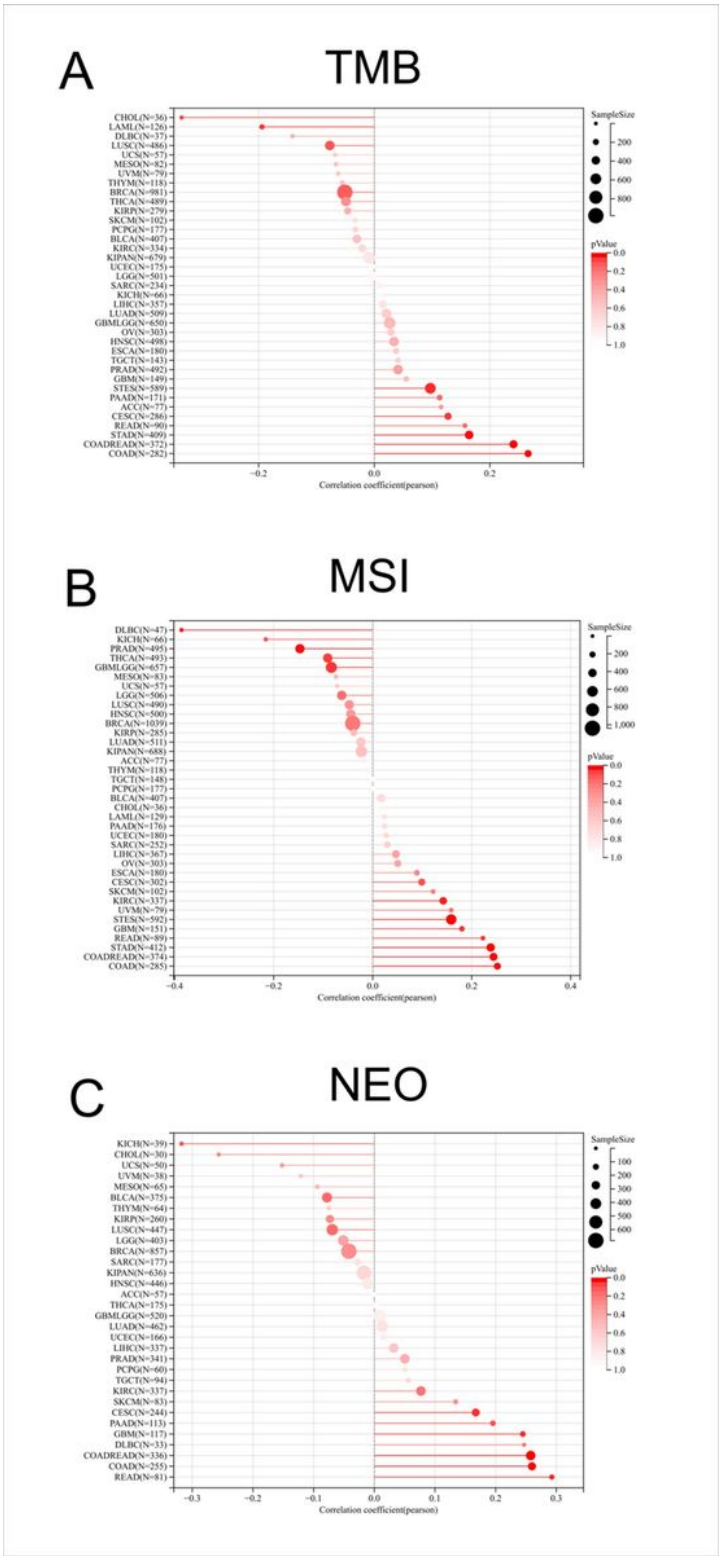

Figure 8

Relationship between PIK3C3 expression and anti-immune indexes. **(A)** Relationship between PIK3C3 expression and TMB. **(B)** Relationship between PIK3C3 expression and MSI. **(C)** Relationship between PIK3C3 expression and NEO.

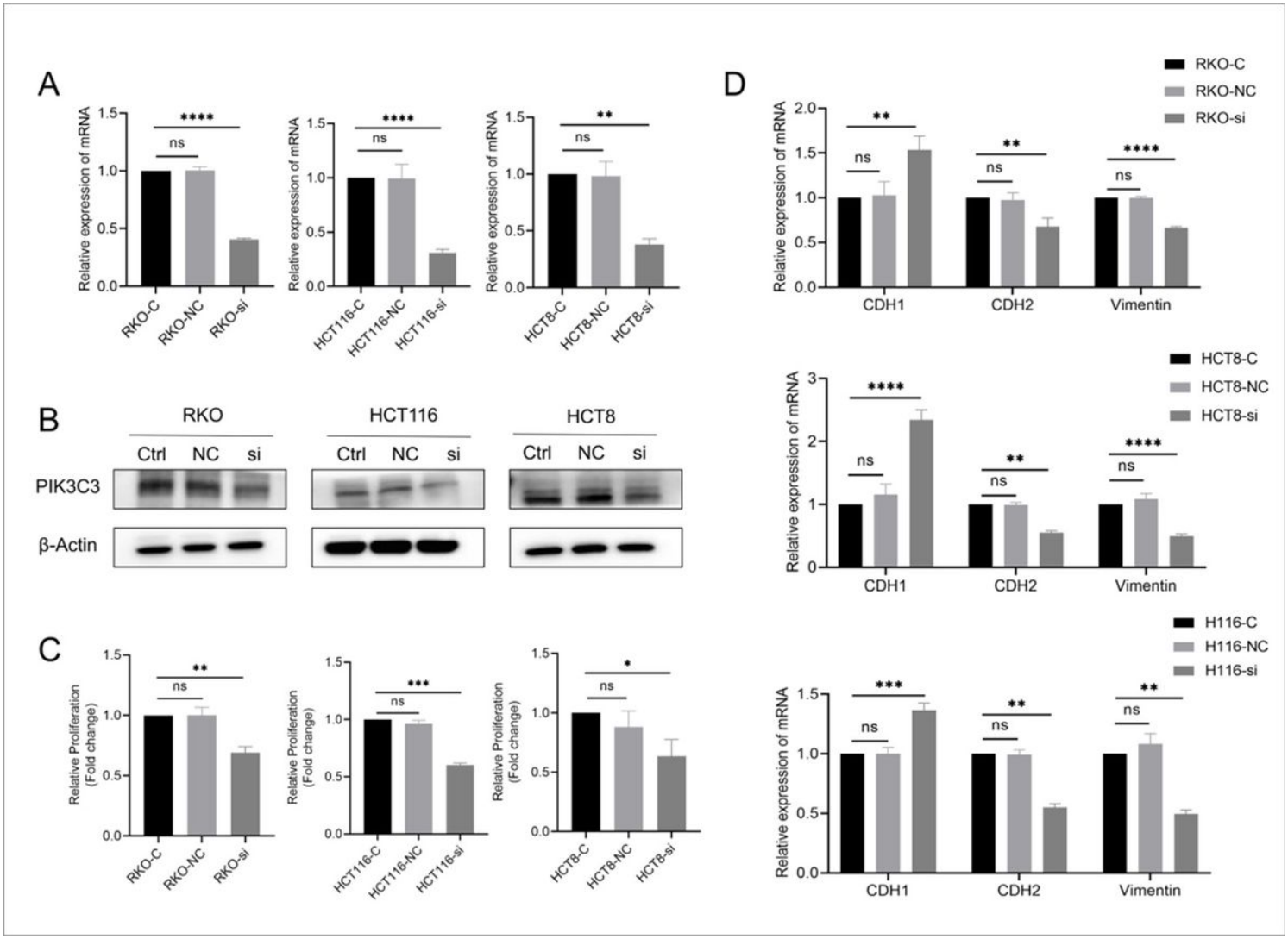

Figure 9

Knockdown of PIK3C3 inhibited proliferation and migration. **(A, B)** mRNA expression and protein expression of PIK3C3 in HCT8, HCT116 and RKO cell lines after knockdown of PIK3C3. **(C)** CCK8 assay to detect the proliferation of HCT8, HCT116 and RKO cells. **(D)** RT-PCR assay to detect the expression of EMT-related genes in HCT8, HCT116 and RKO cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarymaterials.docx