Integrative Analysis of Pinin as A Prognostic Factor in Digestive Tract Cancers

Hui Zhang

Ningbo Medical Treatment Centre Li Huili Hospital

Ming Jin

Ningbo Medical Treatment Centre Li Huili Hospital

Meng Ye

The Affiliated Hospital of Medical School of Ningbo University

Yanping Bei

Ningbo Medical Treatment Centre Li Huili Hospital

Shaohui Yang

Ningbo Medical Treatment Centre Li Huili Hospital

Kaitai Liu (✉ liukaitai@nbu.edu.cn)

Lihuili Hospital

Research

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Abstract

Background: Pinin (PNN) was originally identified for acting an essential role in epithelial cell-cell adhesion. We aim to illuminate the expression profile, mutation feature, methylation status of PNN and its prognostic value in digestive tract cancers.

Methods: Expression and methylation data of PNN, as well as clinical information on esophagus cancer (ESCA), gastric adenocarcinoma (STAD), colon adenocarcinoma (COAD) and rectal adenocarcinoma (READ) were acquired from The Cancer Genome Atlas database. The value of PNN expression, mutation feature and methylation status in prognosis were assessed. GO enrichment and Gene set enrichment analysis were performed to investigate the enriched biological functions and pathways of PNN in COAD. Tumor Immune Estimation Resource and CIBERSORT were applied for evaluating the effects of PNN on tumor immune infiltrating cells.

Results: PNN was significantly overexpressed in digestive tract cancers and was remarkably related to tumor stage. Highly expressed of PNN was positively related to poor free survival (PFS) and overall survival (OS) in COAD. Additionally, 10 CpG sites methylation in PNN had significant effects on survival. PNN expression was confirmed as an independent prognostic factor for predicting the OS in COAD. GO and GSEA analyses revealed that PNN participates in multiple biological processes underlying carcinogenicity in COAD. PNN was significantly associated with TIICs. Moreover, a promising prognostic nomogram incorporating the PNN expression and clinicopathological characteristics was established for predicting OS probability in COAD.

Conclusions: Our comprehensive bioinformatics study demonstrated that PNN was highly expressed in digestive tract cancers, which could act as an independent prognostic factor for COAD.

Background

Gene PNN codifies protein pinin, a desmosome-associated molecule, which was originally found acting an essential role in epithelial cell-cell adhesion(1), was further confirmed to be involved in development of malignant tumors (2). Shi et al reported that PNN was relatively low expressed in several cancer samples and some cancer cell lines [3]. The results showed that PNN might act an antioncogene in some tumors such as renal cell carcinoma. Increasing PNN expression could significantly inhibit cancer cell proliferation. However, in recent years, more studies observed that PNN was higher expressed compared with its corresponding normal tissues, contributing to a poor prognosis in several cancer types including ovarian carcinoma, hepatocellular carcinoma and colorectal cancer (2–4). Likewise, a research revealed high PNN expression in renal cell carcinoma (RCC) tissues and RCC cells. The results demonstrated the oncogenic role of PNN, which had positive effect on cell proliferation and migration in OS-RC-2 and Caki-1, two human clear cell lines [6]. Mini et al. explored the role of PNN in patients diagnosed with local advanced colorectal cancer (CRC), who received fluorouracil-based chemotherapy (5). The results demonstrated that colorectal patients with high PNN expression benefit less from this regime, result in an

unfavorable disease-free survival (DFS). The authors concluded that PNN can act as a predictive biomarker in those patients. Therefore, the PNN expression and its physiological function in cancers are still inconsistent.

Digestive tract cancer is a common malignancy worldwide and accounts for a large proportion of cancer occurrence and death (6). Although some helpful diagnostic biomarkers have been identified, more reliable and effective biomarkers are expected in cancer management. However, few studies have systematically explored the PNN profile in digestive tract cancers up to now. The present study aims to illuminate the expression profile, mutation feature and methylation status of PNN, as well as its prognostic value in four digestive tract cancers, including esophagus cancer (ESCA), gastric adenocarcinoma (STAD), colon adenocarcinoma (COAD) and rectal adenocarcinoma (READ) by analyzing The Cancer Genome Atlas (TCGA) and several acknowledged open-access bioinformatics databases.

Results

Expression features of PNN in digestive tract cancers

The target data included in the gene transcripts acquired in this study were as follows: 162 ESCA and 11 adjacent normal samples, 375 STAD and 32 adjacent normal samples, 480 COAD and 41 adjacent normal samples, 167 READ and 10 adjacent normal samples. The PNN mRNA expression were observed significantly upregulated in four types of digestive tract cancers, including ESCA, STAD, COAD and READ, compared with corresponding normal tissues (Figure 1A). Correlations between PNN mRNA expression and tumor clinical stages in ESCA, STAD, COAD, READ were also analyzed respectively. In general, the expression of PNN were positively associated with tumor stages in digestive tract cancers (Figure 1B). Patients with advanced clinical stage tended to have higher expression of PNN and the highest expression was observed in stage III. However, we noticed that the expression of PNN in stage IV were lower than in stage III, it may be due to the small sample number in stage IV.

Relationships between DNA methylation and PNN expression

Studies have suggested that gene promoter region methylation is a significant cause affecting gene expression, contributing to the progression of human cancer. Current research assessed the effect of promoter DNA methylation on PNN expression in four types of digestive tract cancers using Pearson correlation. We found a significant negative correlation between PNN expression and promoter region methylation level, especially in STAD (Figure A). It indicated that in STAD, abnormal methylation of promoter region is one of the important causes of PNN gene overexpression, but in other digestive tract cancers, there may exist other more regulatory mechanisms influencing the expression of PNN.

The prognostic value of PNN in digestive tract cancers

Kaplan-Meier analyses indicated that PNN played different prognostic role in different types of digestive tract cancers (Figure 3). In ESCA, patients with higher PNN expression level associated with poorer OS ($p=0.076$) and PFS ($p=0.044$). In STAD, patients with high PNN expression had a better prognosis, but unfortunately, there was no statistically difference observed. In COAD, higher expression of PNN was significantly associated with poorer OS ($p=0.003$) and poorer PFS ($p=0.009$). And in patients with READ, the higher expression level of PNN was correlated with longer OS ($p=0.02$), but there was no statistical difference in PFS ($p=0.082$).

We further explored the genetic alterations in PNN and their effects on prognosis in patients with digestive tract cancers by utilizing cBioPortal database. The proportion of various genetic alterations of PNN in different digestive tract cancers were similar: 6% in ESCA, 8% in STAD and 6% in CRC (Figure 4). It was worth noting that the types of genetic alteration were diverse. Amplification and deep deletion were more common in ESCA, missense mutation and truncating mutation were more frequent in STAD and CRC. It was with regret that there was no significant correlation between PNN genetic alterations and OS in these cancers.

Moreover, we investigated the DNA methylation of PNN CpG sites and corresponding prognostic effects in four digestive tract cancers by the MethSurv database. The results were illustrated in Table 1. We observed that cg18648343, cg12087797, cg15592059, cg20337385 were remarkably associated with the prognosis in patients with STAD. In COAD, cg15592059, cg24034629 and cg10250651 were indicated as significant factors for prognosis. As for READ, the meaningful cg sites in prognosis included cg02969452, cg18648343 and cg12087797. However, there were no statistically significant DNA methylation CpG sites observed for predicting OS in ESCA.

Finally, considering the significantly prognostic effect of PNN expression in COAD, we performed univariate and multivariate cox regression analyses to assess the independent prognostic effect in COAD. After controlling the clinical parameters, the univariate cox regression analysis suggested that the expression of PNN had significant effect both in survival ($p=0.004$) and recurrence ($p=0.016$) (Table 2, 3). Subsequently, the expression of PNN was identified as an independent prognostic biomarker for predicting the OS in patients with COAD with multivariate cox regression analysis ($p=0.041$, HR=1.7, 95% CI 1.02-2.8, Figure 5).

Table 1

The prognostic effect of CpGs in PNN

	PNN-TSS200-Island-cg10035432	1.214	0.42
	PNN-Body-Island- cg15592059	0.583	0.025*
	PNN-Body-Island-cg20337385	1.068	0.81
	PNN-Body-Island-cg24034629	0.423	0.0078*
	PNN-Body-S_Shore-cg03045079	0.793	0.35
	PNN-Body-S_Shelf-cg06918918	0.832	0.51
	PNN-TSS1500-N_Shore-cg10250651	0.595	0.041*
	PNN-TSS1500-N_Shore-cg16408528	0.796	0.38
	PNN-TSS1500-N_Shore-cg19599972	1.36	0.21
	PNN-TSS1500-N_Shore-cg24138021	1.245	0.37
Rectum Adenocarcinoma	PNN-5'UTR;1stExon-Island-cg02969452	0.154	0.016*
	PNN-5'UTR;1stExon-Island-cg18648343	3.34	0.034*
	PNN-TSS200-Island-cg10035432	0.412	0.082
	PNN-Body-Island-cg12087797	0.318	0.048*
	PNN-Body-Island-cg15592059	1.515	0.4
	PNN-Body-Island-cg20337385	0.674	0.44
	PNN-Body-Island-cg24034629	0.756	0.58
	PNN-Body-S_Shore-cg03045079	0.716	0.5
	PNN-Body-S_Shelf-cg06918918	1.677	0.3
	PNN-TSS1500-N_Shore-cg10250651	0.385	0.055
	PNN-TSS1500-N_Shore-cg16408528	0.42	0.14
	PNN-TSS1500-N_Shore-cg19599972	0.304	0.071
	PNN-TSS1500-N_Shore-cg24138021	0.424	0.087
* p<0.05.			

Table 2

Univariate cox regression analysis of PNN expression as survival predictors in COAD

Parameter	Univariate analysis		
	Hazard Ratio	95% CI	<i>P</i> value
Age	1.023	1.005-1.042	0.012*
Gender	1.162	0.769-1.757	0.476
T stage	2.777	1.842-4.187	<0.001*
N stage	2.550	1.673-3.886	<0.001*
M stage	3.519	2.312-5.356	<0.001*
PNN expression	2.064	1.256-3.392	0.004*

Table 3

Univariate cox regression analysis of PNN expression as recurrence predictors in COAD

Parameter	Univariate analysis		
	Hazard Ratio	95% CI	<i>P</i> value
Age	1.000	0.986-1.015	1.000
Gender	1.192	0.831-1.711	0.339
T stage	2.750	1.947-3.883	<0.001*
N stage	2.551	1.772-3.672	<0.001*
M stage	3.088	2.145-4.445	<0.001*
PNN expression	1.767	1.112-2.806	0.016*

Functional enrichment analysis of PNN expression in COAD

Owing to the independent prognostic value of PNN expression in recurrence and survival, we explored the biological functions of PNN in COAD by GO analysis based on Metascape. In this research, GO pathway and process enrichment analysis included: molecular functions (MFs, functional set), biological processes (BPs, pathway) and cellular components (CCs, structural complex). Top 15 clusters were displayed in Figure 6A, CCs, including GO: 0016607 (nuclear speck) and GO: 0000226 (microtubule cytoskeleton organization); MFs such as GO:0006397 (mRNA processing), GO:1903313 (positive regulation of mRNA metabolic process), GO: 0031124 (mRNA 3'-end processing), GO: 0018023 (peptidyl-lysine trimethylation), and GO: 0006354 (DNA-templated transcription, elongation); BPs, such as GO 0033044: (regulation of chromosome organization), GO 0009314 (response to radiation), GO 0051056

(regulation of small GTPase mediated signal transduction) and GO 0061136 (regulation of proteasomal protein catabolic process).

The GSEA was performed for evaluating the underlying signaling pathways, which were involved in carcinogenesis of PNN in COAD. The study indicated that PNN high expression was positively associated with “spliceosome”, “basal transcription factors”, “WNT signaling pathway”, “ERBB signaling pathway”, “mTOR signaling pathway” and “adherens junction” (Figure 6B).

Associations between TIICs and PNN in COAD

In the last few years, increasing researches have revealed the crucial relationships between the immune microenvironment and cancer progression. In the current study, we investigated the effects of different PNN expression levels on tumor-infiltrating immune cells in COAD by using CIBERSORT algorithm. The results illustrated that the distribution of 22 immune cell types in each COAD sample varied markedly (Figure 7A). Furthermore, we observed that CD8⁺ T cells, regulatory T cells (Tregs) and resting dendritic cells were significantly enriched in low PNN expression group, nevertheless, resting NK cells and naive CD4⁺ T cells were markedly enriched in high PNN expression group (Figure 7B). Moreover, TIMER database analysis demonstrated that high expression of PNN was positively associated with several types of TIICs, including B cells, CD8⁺ T cells, CD4⁺ T cells, macrophages, neutrophils and dendritic cells (Figure 7C).

A prognostic nomogram for patients with COAD

A prognostic nomogram incorporating the PNN expression and common clinicopathological characteristics was successfully established for predicting the 3, 5, 10-year overall survival probability, which might be promisingly applied in clinical evaluation of patients with COAD (Figure 8).

Discussion

PNN was initially reported as a novel factor involved in the mature desmosomes of the epithelia cell(1). Studies have revealed that PNN participated in apoptosis, proliferation, and migration regulation by affecting mRNA splicing and gene transcription (7). PNN was once described as a potential cancer suppressor factor in RCC via PNN/DRS/memA and upregulated expression of PNN resulted in inhibition of cell growth (8). Conversely, PNN has been found to increase cell growth. High PNN expression had negative effect on survival in breast cancer cells. With more and more studies, the bio-function of PNN was gradually disclosed. Previous research has revealed that PNN was overexpressed in nasopharyngeal cancer, associating with poor overall survival (9). Yang et al. reported the similar result in hepatocellular carcinoma (HCC) (2). They found that PNN could promote cell proliferation and suppress glucose deprivation-induced apoptosis. However, few studies have systematically explored the PNN profile in digestive tract cancers up to now. In our study, we comprehensively explored the expression picture of

PNN in digestive tract cancers. Our findings showed that PNN were highly expressed in ESCA, STAD, COAD and READ, compared with corresponding normal tissues. And we further analyzed the PNN expression status in different clinical stage for each types of digestive tract cancer. The study demonstrated that PNN was overexpressed in all stages of tumor than corresponding normal tissues. Besides, we observed that advanced stage tumors tended to have higher PNN expression in digestive tract cancers.

Researches have shown that abnormal DNA methylation participated in gene expression. DNA methylation can be used as a biomarker for cancer diagnosis and prognosis (10). For example, Li et al. found that abnormal DNA methylation of MCC gene were associated with the progression of esophageal adenocarcinoma via epigenetic regulation (11). Homma et al. revealed that promoter region hypermethylation resulted in frequent gene silencing of RUNX3 in gastric cancer occurrence and development (12). Melotte et al. suggested that N-Myc downstream-regulated gene 4 (NDRG4) promoter methylation could be a potential biomarker for the detection of colorectal cancer (13). Liang et al. found some methylation-regulated differentially expressed genes play an important role in colon cancer (CC) progression [22]. Wang et al. reported that hypomethylated and hypermethylated differentially methylated CpG sites could be used as diagnostic and prognostic biomarkers in CC [23]. Thus, in present study, we analyzed the correlations between PNN mRNA expression and the DNA methylation level of cg sites in the promoter regions in different digestive tract cancers. The result showed that the PNN expression was significantly negatively associated with the DNA methylation level in gastric cancer. It intensively indicated that abnormal methylation of promoter region is one of the important causes of PNN gene overexpression in STAD. Moreover, our study revealed that methylation of several PNN CpG sites showed significantly prognostic effects in ESCA, STAD and COAD. These results may provide a clue that the PNN promoter regions methylation could be a candidate prognostic biomarker in patients with these cancers.

The prognostic value of PNN expression have been investigated in several cancers. Wei et al. suggested that the overexpression of PNN was significantly correlated with the aggressive clinical characteristics in CRC patients (3). PNN could promote cell proliferation and metastasis via activating egfr/erk signaling pathway in colorectal cancer. The upregulated PNN was also confirmed as an independent adverse prognostic factor in hepatocellular carcinoma patients (2). In ovarian cancer, PNN high expression was observed to increase cell adhesion and clonogenicity of epithelial cell (4). Besides, Tang et al. found increased expression of PNN promoted migration and invasion of nasopharyngeal cancer cells in vitro, as well as metastasis in vivo(9). In addition, recently, a study demonstrated that patients with stage III colorectal cancer would not benefit from the adjuvant fluoropyrimidine-based chemotherapy who expressing high mRNA levels of PNN (5). In the current study, we found that PNN high expression had significantly poor OS and DFS in colon cancer. Further analysis confirmed that PNN expression was an independent prognostic factor for predicting the OS in colon cancer. Also, we found high expression of PNN was significantly related with poorer PFS in esophageal cancer. However, on the contrary, the upregulated PNN expression was markedly related with longer OS, and tend to have longer PFS in rectal cancer. But beyond that, our study rejected the independent prognostic effect of PNN in esophageal, gastric, and rectal cancer. Since previous studies has not subdivided colorectal cancer, the controversial

results may contribute by heterogeneity of tumor site, or insufficient sample numbers of rectal cancer in TCGA.

It was commonly known that the prognosis and drug response of colorectal cancer patients were closely associated with specific gene mutation status, such as KRAS, BRAF and PIK3CA (14, 15). Thus, we further explored whether PNN mutation features affect the prognosis of digestive tract cancers. Our results turned out to be disappointed, although the types of genetic alteration were diverse, there were no significant correlations between the PNN mutation status and the overall survival in different digestive tract cancers.

The molecular mechanism of PNN has been illustrated by several studies, however, it still remains uncertain. Study have revealed that PNN played a key role of cell-cell adhesion by inducing desmoglein-2(DSG2) and E-cadherin(E-ca) in human corneal epithelial cells (16, 17). Another study observed that overexpressed PNN promoted DSG2 in colorectal cancer cells, and DSG2 facilitated cell proliferation, viability, invasion and adhesion of CRC through EGFR/ERK signaling pathway (3). Yang and colleagues found that PNN inhibited cell apoptosis by maintaining ERK phosphorylation and preventing PARP cleavage in HCC (2). And recently, studies showed that PNN participated in histone modification and pre-mRNA splicing events (18, 19). In our study, the results of functional enrichment analysis demonstrated that PNN was involved in "spliceosome", "adherens junction and mRNA processing". The KEGG analysis showed PNN affect cell-cell adhesion and tumor invasion and metastasis via variety signaling pathways (e.g., WNT signaling pathway, ErbB signaling pathway, mTOR signaling pathway). WNT signaling pathway is known as one of the most important signaling pathways, its activation is very common during development of many tumors by facilitating cell differentiation, polarization and migration (20). The ErbB belongs to the receptor tyrosine kinase receptor family, includes four distinct members: EGFR (also known as ErbB-1/HER1), ErbB-2 (HER2), ErbB-3 (HER3) and ErbB-4 (HER4). The ErbB pathway is one of the most extensively studied areas of signal transduction, which best exemplifies the pathogenic power of aberrations in biological information transfer (21, 22). The mTOR signaling pathway is frequently activated in cancer, regulates cell growth and various cellular metabolic processes (23).

Until now, few studies have investigated the effect of PNN expression on tumor-infiltrating immune cells. A study reported that PNN was markedly related to T cell receptor signaling pathway in renal cell carcinoma, and has a positive correlation with TIICs (24). At present study, we found that overexpressed PNN was significantly associated with resting NK cells and naive CD4 + T cells in COAD, conversely, PNN low expression were more enriched with CD8 + T cells, regulatory T cells (Tregs) and resting dendritic cells. Therefore, we considered that regulating the tumor immune microenvironment may be a crucial underlying mechanism by which PNN promotes tumor progression in COAD, which deserves further study. Since our study validated a powerful efficiency of prognostic value of PNN in predicting the OS in patients with COAD, a promising prognostic nomogram incorporating the PNN expression and common clinicopathological characteristics was successfully established for predicting 3, 5, 10-year overall survival probability, which might be well applied in clinical evaluation.

However, there were still several limitations in this study. First, we only got the results through bioinformatics and database analysis, further experimental verification is required. Second, the limited sample size of sub-group may affect the result. Finally, the prognostic nomogram for patients with COAD needs more clinical verification. However, our study has its convincing power for its larger sample-based study by TCGA database.

Conclusions

In conclusion, our bioinformatics analyses demonstrated that PNN was highly expressed in digestive tract cancers, which could act an independent prognostic factor for COAD. Furthermore, the potential molecular mechanisms by which PNN participated in carcinogenicity and its correlations with TIICs need to be investigated further.

Methods

Data resource

Data of PNN mRNA expression in four digestive tract cancers including ESCA, STAD, COAD, READ and corresponding normal tissues in TCGA were acquired from the Genomic Data Commons (GDC) Data Portal (<https://portal.gdc.cancer.gov>). The high-throughput sequencing (HTSeq) gene transcript data with normalization in Fragments Per Kilobase of transcript per Million mapped reads (FPKM) were downloaded by Genomic Data Commons (GDC) Data transfer tool. Besides, the clinicopathologic data for four types of cancer such as age, gender, pathological stage, tumor (T) status, node (N) status and metastasis (M) status, as well as survival information were extracted from TCGA database. Since there was no personal identifying information was used in the current study, it was granted an exemption from ethics approval from the Institutional Review Board of *Blinded for peer review*.

Expression profile of PNN in digestive tract cancers

The mRNA expression levels of PNN in four digestive tract cancers were extract and structure from the HTSeq data by using Perl software. We assessed the differential expression of PNN in ESCA, STAD, COAD and READ compared with corresponding normal tissues by the *limma* package. Besides, we further analyze the disparities of PNN expression level according to different clinical stages in four digestive tract cancers. The results were represented by box plots, which were conducted by the *ggpubr* package in R software.

DNA methylation and PNN expression in digestive tract cancers

The Illumina Human Methylation 450K data of TCGA-EACA, TCGA-STAD, TCGA-COAD, and TCGA-READ samples were obtained from the open-access exploration platform (<https://xena.ucsc.edu>). The DNA methylation status of cg sites in promoter region of PNN in four digestive tract cancers were recognized. Subsequently, we investigated the associations between PNN expression and DNA methylation in four digestive tract cancers by utilizing the Pearson correlation. The file used for annotating the information on cg sites was obtained from the official website of Illumina. The *corrplot* package in R software was employed for the analyses.

Prognosis analyses in regard to PNN in digestive tract cancers

The effects of PNN mRNA expression on prognosis were evaluated according to not only progression-free survival (PFS) but also overall survival (OS) in four digestive tract cancers by Kaplan-Meier method. Thereafter, we analyzed the correlations of PNN mRNA expression and clinicopathologic characteristics with OS and PFS among ESCA, STAD, COAD and READ patients respectively using univariate Cox regression analyses. Furthermore, we conducted multivariate Cox regression analyses to assess whether PNN expression was an independent factor in four digestive tract cancers. All analyses were conducted by *survival* and *survminer* packages of R software, and the forest plot was drawn by the *ggplot* package.

CpG sites methylation of PNN and its prognostic effect in digestive tract cancers

We evaluated the DNA methylation of PNN CpG sites and its prognostic value for OS in ESCA, STAD, COAD and READ by the excellent online MethSurv database. The MethSurv is an online bioinformatics platform for multivariable prognosis assessment according to the massive DNA methylation data (<https://biit.cs.ut.ee/methsurv/>) (25).

Genetic Mutations of PNN and its prognostic effect in digestive tract cancers

We explored the genetic mutation features of PNN in digestive tract cancers, including ESCA, STAD, colorectal cancer (CRC) by utilizing the open-access cBioPortal database (v3.6.12; <http://www.cbioportal.org>). The cBioPortal is a preeminent public online tool for exploring, analyzing and visualizing comprehensive cancer genomics data (26). Data in TCGA PanCancer Atlas of ESCA, STAD and CRC were involved in the study, with selected genomic profiles as follows: mutations, structural variant, putative copy-number alterations from Genomic Identification of Significant Targets in Cancer (GISTIC), and mRNA Expression z-scores relative to diploid samples (RNASeqV2RSEM). Besides, the

correlations between genetic mutations of PNN and OS in patients with digestive tract cancers were assessed in turn via cBioportal. The z-score threshold was set to ± 1.8 .

Functional enrichment analysis in COAD

In order to evaluate the underlying gene functions and pathways, by which PNN participated in colon adenocarcinoma, we performed Gene Ontology (GO) analysis by utilizing the open-access online Metascape database (<http://metascape.org>) (27). Before this, we recognized the top 50 similar genes of PNN in COAD through an interactive open-access bioinformatics platform: gene expression profiling interactive analysis (GEPIA) (<http://gepia.cancer-pku.cn>) (27). In the present GO analysis, we only considered human species and the enrichment analysis was conducted with the custom settings of thresholds in “min overlap 3”, “p-value 0.05”, and “min enrichment 3”. Furthermore, we performed gene set enrichment analysis (GSEA) to unfolding the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways related to PNN expression in COAD. The GSEA software (version 4.0.1) was downloaded from the website (<http://software.broadinstitute.org/gsea/index.jsp>) and the annotated gene set file (c2.cp.kegg.v7.0.symbols.gmt) was acquired from the Msig database (28). The median value of gene expression was taken as the cut-off point, by which the software divided all samples into high and low groups. The model of “high vs low” and a random combination of at least 1,000 permutations were selected for pathway enrichment analysis. A false discovery rate (FDR) < 0.05 were the criteria for the identification of the enriched pathways.

PNN miRNA expression and Tumor Immune Infiltrating Cells (TIICs) in COAD

According to the optimal cutoff value of PNN mRNA expression in the survival analysis based on the Kaplan-Meier method, the COAD samples were divided into two groups, high and low expression. CIBERSORT was applied to estimate the relationships between PNN and the subsets of each immune cell types in COAD. The result was visualized by the *vioplot* package of R software. CIBERSORT is an online platform to estimate the proportions of 22 tumor-infiltrating lymphocyte subsets in the tumor microenvironment (<https://cibersort.stanford.edu/>) (29). Furthermore, we utilized the Tumor Immune Estimation Resource (TIMER) to evaluate the effect of PNN expression on six major TIICs (CD4⁺ T-cells, CD8⁺ T-cells, B-cells, macrophages, neutrophils and dendritic cells) (<https://cistrome.shinyapps.io/timer/>). TIMER is an online helpful platform for systematically analyzing of infiltrating immune cells in cancers (30).

A prognostic nomogram for patients with COAD

For better clinical application of PNN expression, we further developed a nomogram incorporating the PNN expression and clinicopathological characteristics to estimate the survival probability of COAD

patients by *rms* package of R software. The 3-, 5-, and 10-year OS rates served as endpoints in the nomogram. The clinicopathological characteristics involved in the nomogram including age, gender, stage, T stage and N stage.

Statistical analysis

Perl software 5.32 was used to extract and structure the HTSeq FPKM data, DNA methylation data and GESA preparation documents. R 4.0.3 software with specific packages was used to perform analyses for gene differential expression, Pearson correlation, prognostic value evaluation and nomogram development. The comparisons of intergroup variables were conducted by using the chi-square test with SPSS software 20.0 (IBM, Chicago, USA). $P < 0.05$ was considered to be statistically significant.

Declarations

Ethical approval statement

Since there was no personal identifying information was used in the current study, it was granted an exemption from ethics approval from the Institutional Review Board of the Affiliated Lihuili Hospital, Ningbo University.

Consent for publication

Not applicable.

Data availability statement

The data used for bioinformatics analyses in this study are freely available on The Cancer Genome Atlas (TCGA) program website (<https://xena.ucsc.edu>; <https://portal.gdc.cancer.gov/>) and other open-access bioinformatics databases. The interpretation and reporting of these data are the sole responsibility of the authors.

Conflicts of interest

The authors declare that they have no competing interests.

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Authors' contributions

KTL and HZ conceived and designed the study. MJ, HZ, MY, YPB performed the analyses. HZ and SHY prepared all tables and figures. KTL, HZ and MJ wrote the main manuscript. KTL and MY contributed to the revised manuscript. All authors approved the final version of the manuscript.

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Figures

Figure 1

(A) The expression level of PNN in ESCA, STAD, COAD, READ and matched normal tissues. (B) Correlation between PNN expression and tumor stages in ESCA, STAD, COAD, READ.

Figure 2

Pearson correlation between methylation and PNN expression in (A) ESCA, (B) STAD, (C), COAD and (D) READ.

Figure 3

Prognostic value of PNN expression in four digestive tract cancers. (A) PFS. (B) OS.

Figure 4

(A) Genetic alterations of PNN in different digestive tract cancers and (B) its correlation with OS in ESCA, STAD and CRC.

Figure 5

Multivariate survival analyses of patients with COAD represented in a forest plot. *stands for P < 0.05; **stands for p < 0.01; ***stands for P < 0.001;

Figure 6

(A) GO analysis of PNN in COAD. B GSEA analysis of PNN in COAD.

Figure 7

(A) Distribution of 22 immune cells in each COAD sample represented by a stacked bar chart. (B) Relationships between PNN expression and the subsets of each immune cell types in COAD. (C) Correlations between PNN expression and the tumor infiltrating immune cells (TILs: B cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils, and dendritic cells) in COAD.

Figure 8

A prognostic nomogram for patients with COAD.