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Analysis of Expression Levels and Functions of MDK and PTN Genes in Colorectal Cancer

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1. Abstract

1.1. Background: Colorectal cancer (CRC) is the third most common and the fourth most lethal type of cancer. Current therapies for CRC mainly include chemotherapy, radiotherapy, and surgery. However, the therapeutic effects of these therapies are not satisfactory for advanced CRC patients.

1.2. Purpose: Therefore, there has been ongoing research looking for better diagnostic targets that may give rise to more efficient interventions. Midkine (MDK) and pleiotrophin (PTN) are two important heparin-binding cytokines. These proteins are highly expressed in many human tumor cells, but have low or no expression in normal tissues.

1.3. Methods: To identify novel diagnostic targets for CRC, we herein analyzed the expression patterns of MDK and PTN genes in colorectal cancer and normal samples by using The Cancer Genome Atlas (TCGA) and tumor immune estimation resource (TIMER) databases.

1.4. Results: We found that both genes were abnormal expressed in CRC samples and involved in the regulation of immune response and cell metabolism, and the expression levels of these two genes had significant prognostic value.

1.5. Conclusion: In conclusion, our results provide a comprehensive understanding of the functions of MDK and PTN genes in colorectal cancer.

2. Introduction

Colorectal cancer (CRC) is the third most common and the fourth most lethal type of cancer [1-3]. During early stages, CRC can be effectively managed via surgical resections. In fact, patients with early-stage CRC have a long survival period and can even be completely cured. Data show that the 5-year survival rate of patients with stage I CRC is higher than 85% [4]. However, for patients with stage IV CRC, 65% may experience recurrence after surgical resections [5] and most of them exhibit disease progression due to resistance to chemotherapies and targeted drugs [6]. As a result, the 5-year survival rate of patients with stage IV CRC is less than 10% [4]. Therefore, exploring novel targets for adjuvant therapy for CRC has become more and more important.

Midkine (MDK) and pleiotrophin (PTN) are two members of the heparin-binding cytokines family. The amino acid sequences of mammalian MDKs show 50% homology to those of mammalian PTNs [7, 8]. In humans, MDK and PTN are abnormally expressed in many kinds of cancer cells [9], such as neuroblastoma [10], bladder cancer [11], lung cancer [12, 13], breast cancer [14, 15] and thyroid papillary carcinoma [16, 17]. In addition, studies have shown that both MDK and PTN are abnormally expressed in many inflammatory diseases [18]. In a previous study, we found that the MDK gene was highly expressed in the CRC tissue relative to the normal tissue by immunohistology analysis, and the expression of MDK was close-

ly related to the clinical stage and degree of malignancy of CRC. This finding was consistent with those of some other studies, which indicated that MDK was abnormally expressed in CRC; in addition, according to these studies, PTN was also expressed abnormally in CRC [19, 20]. However, according to existing reports, the expression patterns of MDK and PTN in CRC remain controversial. To clarify the expression patterns of these two genes and unveil their underlying regulatory mechanisms, in this study, by comprehensively analyzing the RNA-seq data on MDK and PTN genes, we explored the expression characteristics and potential biological functions of the two genes in CRC. The findings were then combined with the clinical characteristics of CRC patients to guide the establishment of new prognostic markers for CRC.

3. Methods

3.1. Retrieval and Processing of mRNA Expression Data

We collected RNA□ seq profiles (level 3 HTSeq-FPKM format) from the CRC cohort deposited in The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/), which contains 454 tumor samples and 41 normal samples. Then the FPKM (Fragments Per Kilobase per Million reads) data were converted into the TPM (Transcripts Per Million reads) format and log2-transformed. For data filtering, the clinical information was retained and duplicate samples were removed. R (version 3.6.3) was used for statistical analysis of data, and the "ggplot2" (version 3.3.3) package in R was used for data visualization.

3.2. Characterization of the Expression Levels of MDK and PTN Genes

Differences in mRNA expression levels of MDK and PTN genes between the CRC and normal samples of the TCGA cohort were identified by t□ test. R (version 3.6.3) was used for statistical analysis of data, and the "ggplot2" (version 3.3.3) package in R was used for data visualization.

3.3. Gene Set Enrichment Analysis (GSEA) of MDK and PTN Genes

The LinkedOmics database (http://www.linkedomics.org/) [21] was used for GSEA. Genes significantly correlated with the expression of MDK and PTN were filtered by Pearson correlation analysis using the "LinkFinder" module of LinkedOmics. Then, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed for the selected positively or negatively correlated genes by using the "LinkInterpreter" module of LinkedOmics.

3.4. Analysis of the Correlation between MDK and PTN Expression Levels and Immune Infiltration

The tumor immune estimation resource (TIMER; https://cistrome.shinyapps.io/timer/) [22] database was used to analyze the correlation between MDK and PTN expression and the levels of infiltrating immune cells, such as dendritic cells, CD4+ T cells, neutrophils, CD8+ T cells, macrophages, and B cells. The stromal score (that captures the presence of stroma in tumor tissue), immune score (that represents the infiltration of immune cells in tumor tissue), and ESTIMATE (Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data) score[23] of each CRC patient were also analyzed by corresponding R packages to determine the infiltration levels of immune cells and stromal cells in CRC tissues [24]. Statistical analysis was performed by adopting Pearson's correlation coefficients.

3.5. Survival Analysis of MDK and PTN Expression Levels

Overall survival analysis was performed by R (3.6.3). The "survival" package (version 3.2.10) in R was used for statistical analysis of survival data, while the "survivinер" package (version 0.4.9) was used for visual analysis of the survival data. For data filtering, the duplicate samples were removed. The grouping method with the minimum p value was adopted for survival analysis. The prognostic data were provided by Liu Jianfang et al.'s article [25]. Receiver operating characteristic (ROC) curves were established by the "pROC" package (version 1.17.0.1) and visualized by the "ggplot2" package (version 3.3.3). For data filtering, duplicate samples were removed.

3.6. Statistical Analysis

All statistical analyses were conducted using R (version 3.6.3) software in this study. The significance of the expression of MDK/PTN in tumor and normal tissues was analysis using the Student's t test (two-tailed), the Mann-Whitney test (two-tailed), or one-way analysis of variance (ANOVA). Overall survival (OS) curves were analyzed by the log-rank test. The correlation analysis was conducted using Pearson correlation test or Spearman rank correlation test. $P<0.05$ was considered statistically significant.

4. Results

4.1. MDK and PTN Exhibited Abnormal Expression Levels in CRC Samples

We first analyzed data from the TCGA cohort and found that MDK was significantly upregulated ($P<0.0001$), while PTN was significantly downregulated ($P<0.0001$) in CRC samples versus normal tissues (Figure 1A and 1B). Pearson correlation analysis showed no statistically significant correlation between the mRNA expression levels of PTN and MDK ($r=0.050$, $P=0.279$), suggesting that the expression of PTN and MDK may be subjected to unrelated regulatory mechanisms during the occurrence and development of CRC (Figure 2).

4.2. MDK and PTN were Associated with Multiple Biological Pathways in CRC

To further elucidate the biological functions of MDK and PTN in CRC, we performed GO and KEGG signaling pathway enrichment analyses based on the LinkedOmics database. The results showed that MDK was mainly enriched with immune response and inflammatory response pathways in CRC and was also involved in RNA metabolic process and cell cycle regulation (Figure 1C and 1D). Meanwhile, PTN was mainly enriched with metabolic process and DNA replication pathways (GO pathways) in CRC (Figure 1E and 1F).

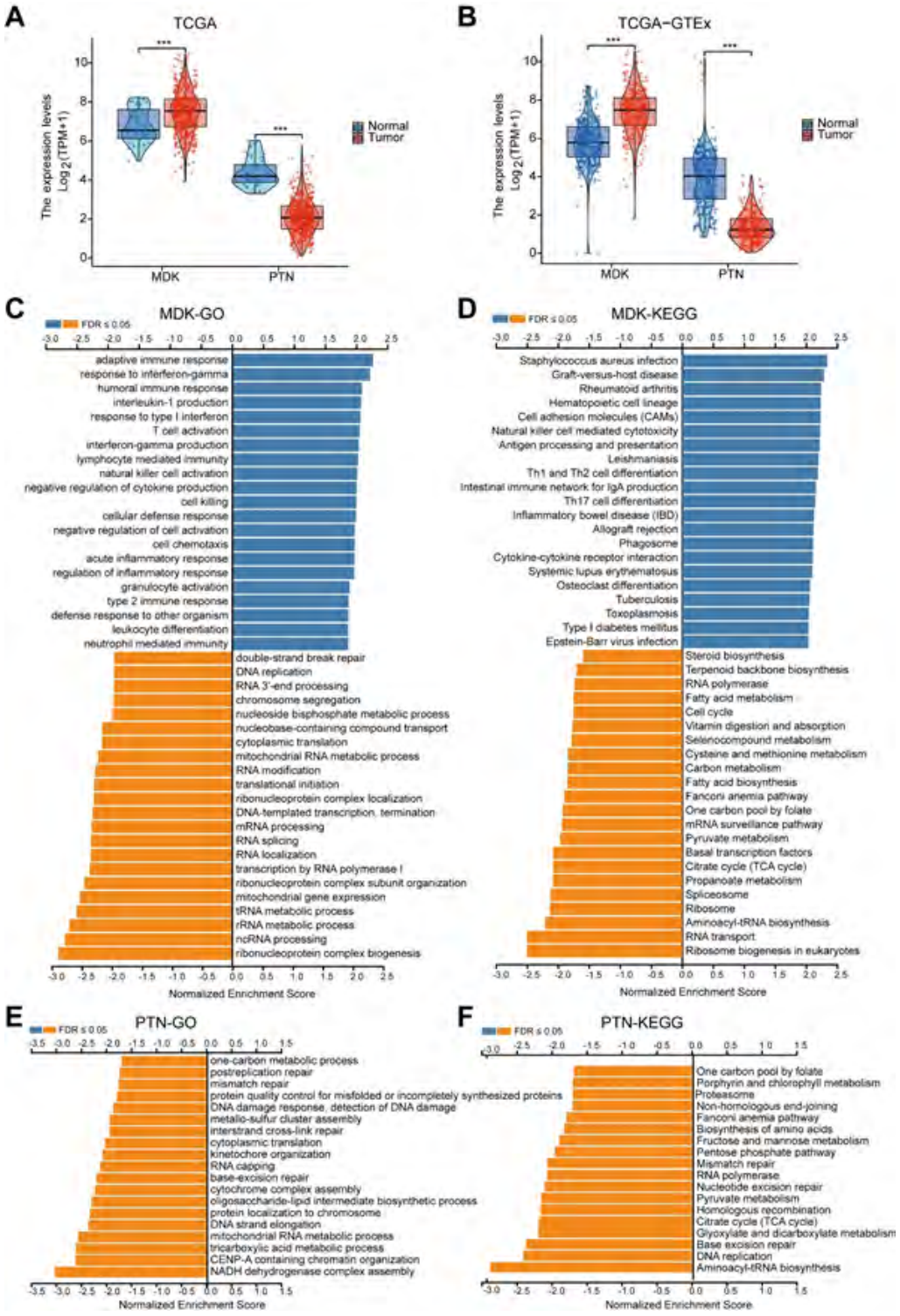

Figure 1: Expression levels and functions of MDK and PTN in CRC. Expression levels of MDK and PTN in CRC according to TCGA (A) and TCGA-GTEx (B) databases. GO analysis of MDK (C) and PTN (E) and KEGG analysis of MDK (D) and PTN (F) based on the LinkedOmics database.

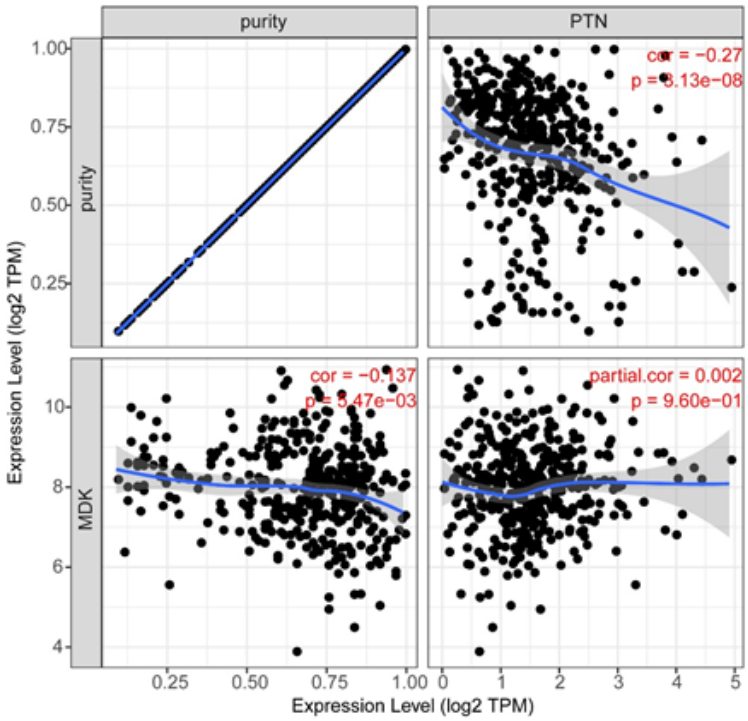

Figure 2: Correlations between expression levels of MDK and PTN and cancer purity.

4.3. MDK and PTN were Correlated with Immune Functions in CRC

Based on the LinkedOmics database, we performed GSEA on MDK and PTN in CRC. The results showed that the expression of MDK was significantly positively correlated with that of genes involved in humoral immune response, adaptive immune response, T cell activation, lymphocyte-mediated immunity, cell killing, regulation of inflammatory response, natural killer (NK) cell-mediated cytotoxicity, differentiation of Th1 and Th2 cells, intestinal immune network for IgA production, Th17 cell differentiation, inflammatory bowel disease, and autoimmune thyroid disease, and so on, in CRC ($p<0.05$, false discovery rate [FDR]<0.05; Figure 3). Furthermore, the expression of PTN was significantly negatively correlated with that of genes implicated in NADH dehydrogenase complex assembly, tricarboxylic acid metabolic process, mitochondrial RNA metabolic process, protein localization to chromosome, cytoplasmic translation, DNA damage response, detection of DNA damage, pentose phosphate pathway, glyoxylate and dicarboxylate metabolisms, pyruvate metabolism, DNA replication, fructose and mannose metabolisms, citrate cycle (TCA cycle), and so on, in CRC ($p<0.05$, FDR<0.05; Figure 4).

4.4. Associations of MDK and PTN with Immune Infiltration

Since our GSEA found that MDK and PTN played important immunological roles in CRC samples, we next sought to determine the relationships between MDK and PTN expression and the levels of infiltrating immune cells. Based on the TIMER database, we discovered that the expression of MDK was negatively correlated with tumor purity, and positively correlated with B cell, neutrophil and dendritic cell (DC) infiltration in CRC tissues (Figure 5a). Meanwhile, PTN expression was also negatively correlated with tumor purity, but positively correlated with B cell, CD8+ T cell, CD4+ T cell, macrophage, neutrophil and dendritic cell infiltration in CRC tissues (Figure 5A). Based on the "GSVA" package (1.34.0 version) of R (3.6.3 version), the correlations of MDK and PTN expression with the infiltration of various subtypes of aDCs (activated DCs), B cells, CD8+ T cells, cytotoxic cells, DCs, eosinophils, iDCs (immature DCs), macrophages, mast cells, neutrophils, NK cells, CD56bright cells [26], NK CD56dim cells [26], NK cells, pDCs (plasmacytoid DCs), T cells, T helper cells, Tcm (T central memory) cells, Tem (T effector memory) cells, Tfh (T follicular helper) cells, Tgd (T gamma delta) cells, Th1 cells, Th17 cells, Th2 cells, and Tregs (regulatory T cells) were comprehensively analyzed. The results (Figure 5B, Table 1) were consistent with those obtained based on the TIMER database (Figure 5A). We also calculated the immune score, stromal score, and ESTIMATE score for each CRC patient by using the "estimate" package (version 1.0.13) in R (version 3.6.3). Mann-Whitney U analysis revealed that the expression levels of MDK and PTN were both positively correlated with immune score, stromal score, and ESTIMATE score of the CRC patients (Figure 5C).

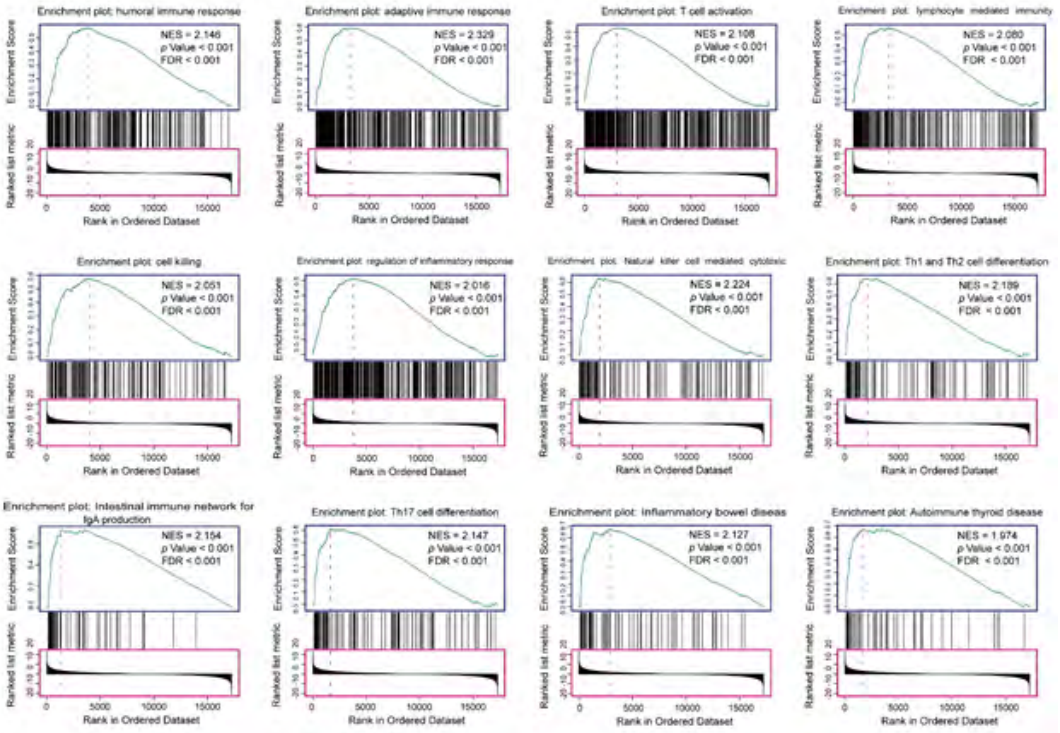

Figure 3: GSEA of MDK in CRC.

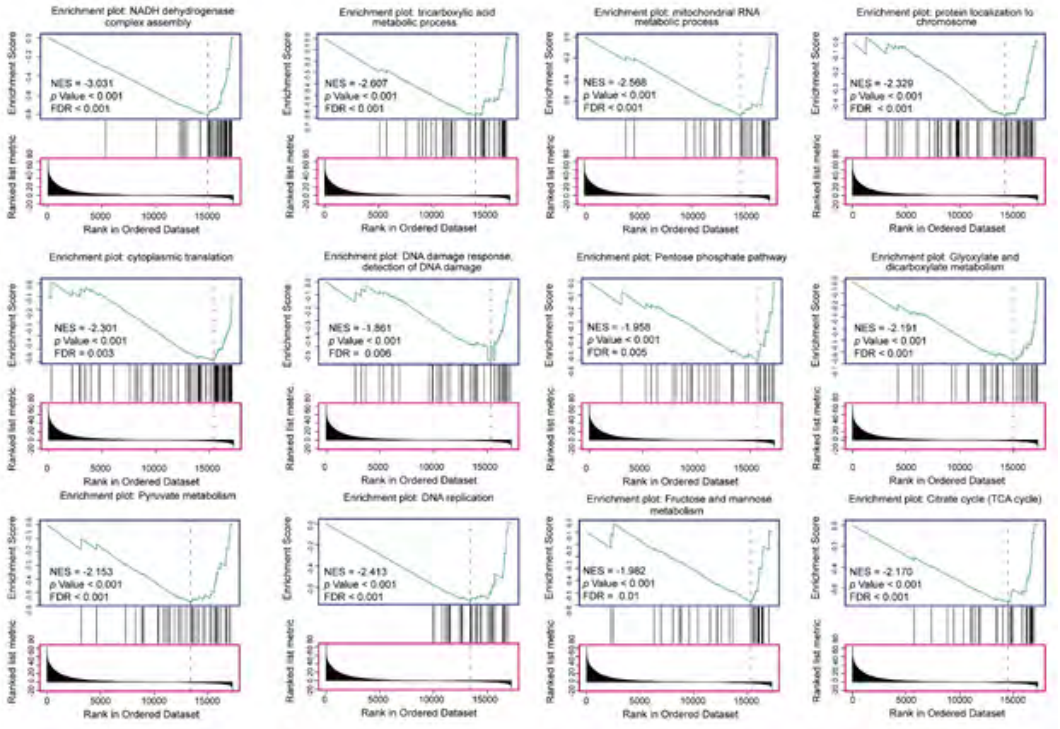

Figure 4: GSEA of PTN in CRC.

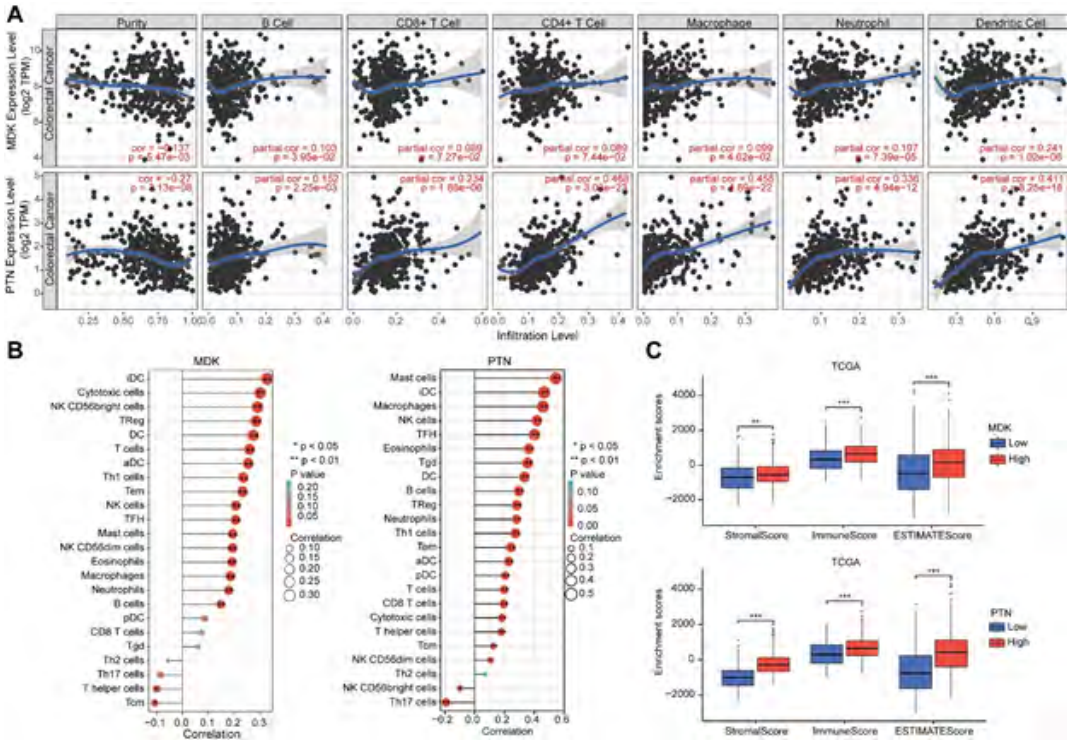

Figure 5: Correlations between expression levels of MDK and PTN in immune infiltration. (A) Correlations between expression levels of MDK and PTN and tumor purity, B cell infiltration, neutrophil infiltration and dendritic cell infiltration in CRC. (B) Correlations between expression levels of MDK and PTN and the infiltration of various subtypes of immune cells. (C) Correlations between expression levels of MDK and PTN and immune score, stromal score, and ESTIMATE score for each CRC patient.

Table 1: Correlation analysis between MDK and PTN expression and the infiltration of immune cells in CRC

Cell type	*PTN*		*MDK*	
	Correlation coefficient (Pearson)	***p* value (Pearson)**	**Correlation coefficient (Pearson)**	***p* value (Pearson)**
aDCs	0.192	<0.001	0.275	<0.001
B cells	0.298	<0.001	0.157	<0.001
CD8 T cells	0.238	<0.001	0.119	0.011
Cytotoxic cells	0.17	<0.001	0.335	<0.001
DCs	0.332	<0.001	0.248	<0.001
Eosinophils	0.355	<0.001	0.19	<0.001
iDCs	0.459	<0.001	0.328	<0.001
Macrophages	0.455	<0.001	0.178	<0.001
Mast cells	0.562	<0.001	0.199	<0.001
Neutrophils	0.262	<0.001	0.168	<0.001
NK CD56bright cells	-0.109	0.021	0.301	<0.001
NK CD56dim cells	0.096	0.041	0.242	<0.001
NK cells	0.454	<0.001	0.238	<0.001
pDCs	0.197	<0.001	0.084	0.074
T cells	0.205	<0.001	0.28	<0.001
T helper cells	0.222	<0.001	-0.139	0.003
Tcm cells	0.153	0.001	-0.127	0.007
Tem cells	0.268	<0.001	0.244	<0.001
Tfh cells	0.405	<0.001	0.214	<0.001
Tgd cells	0.378	<0.001	0.037	0.432
Th1 cells	0.292	<0.001	0.252	<0.001
Th17 cells	-0.18	<0.001	-0.101	0.031
Th2 cells	0.084	0.073	-0.097	0.038
Tregs	0.29	<0.001	0.3	<0.001

4.5 Prognostic Analysis of MDK and PTN in CRC Samples

We analyzed the overall survival (OS), disease-free survival (DSS) and progression-free interval (PFI) of CRC patients and found that low MDK expression was associated with a better prognosis in terms of OS (hazard ratio [HR]=1.58, 95% confidence interval (CI): 1.00-2.49; p=0.052) (Figure 6A) and DSS (HR=2.26, 95% CI: 1.18-4.33; p=0.016) (Figure 6B), but was not significantly associated with the PFI of CRC patients (HR=1.34, 95% CI: 0.93-1.94; p=0.116) (Figure 6C). We also analyzed the associations between MDK expression and OS of CRC patients in the T1/T2 and T3/T4 stages. The results indicated that MDK expression correlated negatively with the OS of T1/T2-stage CRC patients (HR=2.99, 95% CI: 0.57-15.55; p = 0.194) (Figure 6D) but positively with the OS of T3/T4-stage CRC patients (HR=1.62, 95% CI: 1.01-2.61; p=0.048) (Figure 6E). Meanwhile, high PTN expression was associated with a better prognosis in terms of OS (HR=0.65, 95% CI: 0.44-0.96; p=0.031) (Figure 6F), but was not significantly associated with the DSS (HR=0.66, 95% CI: 0.40-1.09; p=0.103) (Figure 6G) and PFI (HR=0.85, 95% CI: 0.58-1.23; p=0.378) (Figure 6H) of CRC patients. We also analyzed the correlations between PTN expression and OS of CRC patients in the T1/T2 and T3/T4 stages. The findings revealed that PTN expression had no significant correlation with the OS of patients with T1/T2-stage CRC (HR=0.74, 95% CI: 0.14-3.80; p=0.715) (Figure 6I) but had a negative correlation with the OS of patients with T3/T4-stage CRC (HR=0.67, 95% CI: 0.44-1.00; p=0.052) (Figure 6J). In addition, the area under the ROC curve (AUC) values for the expression of MDK and PTN were 0.682 and 0.970, respectively, in CRC patients (Figure 6K and 6L), indicating that the expression levels of MDK and PTN were closed related with the prognosis of CRC. Therefore, these two genes are potential biomarkers for CRC.

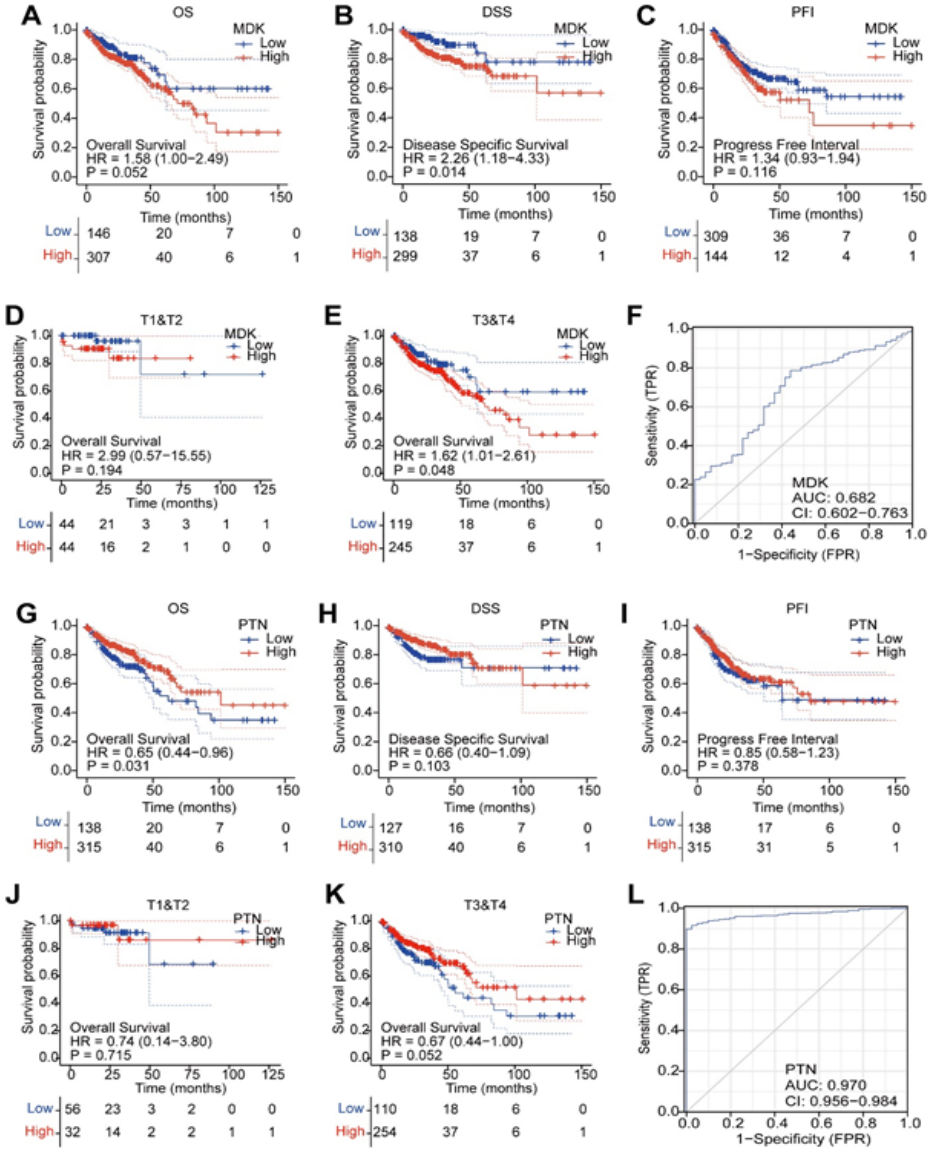

Figure 6: Prognostic value of MDK and PTN in CRC. The OS (A), DSS (B), and PFI (C) of CRC patients with different levels of MDK expression. The OS of T1/T2-stage CRC (D) and T3/T4-stage CRC (E) patients with different levels of MDK expression. The AUC of MDK expression in CRC patients (F). The OS Gg), DSS (H), and PFI (I) of CRC patients with different levels of PTN expression. The OS of T1/T2-stage CRC (J) and T3/T4-stage CRC (K) patients with different levels of PTN expression. The AUC of PTN expression in CRC patients (L).

5. Discussions

MDK and PTN are two members of the heparin-binding growth factor family of cytokines. These two proteins are highly expressed in multiple embryonic and malignant tissues. MDK is a cysteine-rich protein with a molecular weight of 13 kDa [20, 27, 28], while PTN is a secretory protein with a molecular weight of 18 kDa [8]. Prior evidence suggests that MDK and PTN can interact with several proteins, such as Anaplastic Lymphoma Kinase (ALK), syndecans (SDCs), Receptor-Type Protein-Tyrosine Phosphatase (RPTP), low-density Lipoprotein Receptor Related Protein (LRP), integrins, Neuroglycan C (NGC), and Notch [18], all of which are involved in promoting tumor growth, tumor invasion and angiogenesis [9, 29].

Many studies have demonstrated that both MDK and PTN are abnormally expressed in CRC tissues (Table 2). Aridome, K. et al. [27] analyzed the expression of MDK in various gastrointestinal carcinomas, including gastric cancer, liver cancer, pancreatic cancer, duodenal cancer, and esophageal cancer. They found that the mRNA and protein expression levels of MDK in CRC tissues were much higher than those in adjacent tissues. Barderas, R. et al. [20] found via stable isotope labeling by amino acids in cell culture (SILAC), a high-throughput proteomic analysis approach, that the protein level of MDK in KM12SM, a highly metastatic CRC cell line, was significantly higher than that in KM12C, a lowly metastatic CRC cell line. After the expression of MDK in KM12SM cells was knocked down by siRNA interference, the metastatic ability of the cells was significantly reduced. In addition, MDK gene had also been involved in a six-gene prognostic signature (including CD137L, CTSS, SOSTDC1, ZG16B, EFNA3 and MDK) for CRC [20]. It was found that the high expression of this signature was significantly correlated with a poor prognosis of CRC. The higher the expression of the signature genes, the shorter the survival and the worse the prognosis of CRC patients. However, some studies have also found that the expression of MDK gene was low in the serum of CRC patients. Kemik, O. et al. [28] found that the serum levels of albumin, MDK, adiponectin and ghrelin in esophageal, gastric, pancreatic, colon and rectal cancer patients were lower than those in the healthy control group. These findings were confirmed by another study from the same research group [30].

PTN has also been implicated in CRC. According to Yamakawa, T. et al. [19] and Mikelis, C. et al. [29], the mRNA levels of PTN and protein tyrosine phosphatase zeta (PTP zeta) were decreased in CRC tissues compared with adjacent noncancerous tissues. However, another study found that the expression of PTN in CRC tissues was much higher than that in normal colorectal tissues [31]. Consistent with this study, serum levels of PTN in CRC patients were found to be significantly higher than those of healthy volunteers [31]. In the same study, PTN expression was also found to be associated with CRC prognosis and tumor node metastasis classification (TNM) stage [31]. Additionally, high levels of PTN were accompanied by high expression of vascular endothelial growth factor (VEGF) and were predictive of a poor prognosis in CRC patients [31].

According to the aforementioned existing studies on MDK and PTN, these two genes seem to have different expression patterns in CRC-most studies found that MDK was highly expressed and PTN was lowly expressed in CRC. This is consistent with the findings of our analysis of data from TCGA database-the expression of MDK in CRC tissues was significantly higher than that in adjacent normal tissues, while the expression of PTN in CRC tissues was significantly lower than that in adjacent noncancerous tissues. Although PTN and MDK share similar amino acid sequences [7] and have many common biological functions, such as those associated with neurodevelopment and tumor growth, but they have different expression patterns in CRC and play different roles in the regulation of CRC development. It is interesting that opposite expression patterns of MDK and PTN also exist in many other tumors, such as breast cancer, lung adenocarcinoma, esophageal carcinoma, kidney renal papillary cell carcinoma, and so on. The mechanism of this phenotype has not been confirmed, but we hypothesize that the different expression patterns may be associated with different tumor environments, and MDK and PTN may have participated in different biological processes. The GSEA results showed that MDK and PTN were associated with significantly different biological pathways in CRC. MDK was mainly involved in immune-related signaling pathways, while PTN was mainly involved in metabolic signaling pathways in CRC.

It has been reported that the PTN and MDK are aberrantly expressed under many inflammatory conditions, such as acute injury [32], hypoxia [33], atherosclerosis [34], and rheumatoid arthritis [35, 36]. However, it is not yet clear how they contribute to the inflammatory environment of cancer. In this study, the correlations between MDK and PTN expression and the infiltration of immune cells in CRC were analyzed based on the TIMER database. Interestingly, we found that the expression levels of MDK and PTN had significant correlations with the infiltration of different types of immune cells. For example, the infiltration of T helper cells and Tcm cells was negatively correlated with the expression of MDK but positively correlated with the expression of PTN. The infiltration of pDCs and CD8+ T cells was positively correlated with the expression of PTN but negatively correlated with the expression of MDK. In addition, the infiltration of CD8+ T cells, CD4+ T cells and macrophages was positively correlated with the expression of PTN but had no significant correlation with the expression of MDK. It is well known that CD8+ T cells, CD4+ T cells and macrophages can kill tumor cells. Among them, CD8+ T cells are the most lethal T cells that can kill antigen-expression tumor cells, and are important effector cells in anti-virus infection, acute allograft rejection and tumor cell elimination. Therefore, the number of CD8+ T cells directly determines the body's ability of eliminating tumor cells [37]. This may explain why high PTN expression was associated with a higher survival rate of CRC patients in our study.

Table 2: Existing studies on MDK and PTN in CRC

Related studies and journals they are published in	Year of publication	Gene investigated	Main findings	Gene expression level
Mol Cell Proteomics [20]	2013	*MDK*	Knockdown of *MDK* caused a significant decrease in the migration and invasion abilities of highly metastatic cells.	High
Hum Exp Toxicol [30]	2012	*MDK*	*MDK* was lowly expressed in gastric cancer patients' serum.	Low
Int J Colorectal Dis [31]	2012	*MDK*	*MDK* was lowly expressed in colon cancer tissues.	Low
World J Surg Oncol [28]	2010	*MDK*	*MDK* was lowly expressed in colon cancer patients' serum.	Low
Jpn J Cancer Res [27]	1995	*MDK*	The increased expression of *MDK* in gastric carcinoma was more significant in well- and moderately-differentiated adenocarcinomas than in poorly-differentiated adenocarcinomas and signet ring cell carcinomas.	High
PLoS One [38]	2017	*PTN*	PTN is a secretory cytokine expressed in various cancer cell lines and human tumors such as colon cancer, lung cancer, gastric cancer and melanoma. It plays significant roles in angiogenesis, metastasis, cell differentiation and cell growth.	High
Int J Colorectal Dis [31]	2012	*PTN*	High *PTN* expression levels are accompanied by high VEGF expression and are predictive of a poor prognosis in CRC patients.	High
Recent Pat Anticancer Drug Discov [29]	2007	*PTN*	*PTN* is expressed at lower levels in CRC tissues than in adjacent normal mucosae.	Low
Cancer Lett [19]	1999	*PTN*	*PTN* and *PTPzeta* mRNA levels were decreased in CRC tissues as compared with adjacent normal mucosae.	Low
J Natl Cancer Inst [39]	1998	*PTN*	pancreatic cancer (n = 41; P<0.0001) and colon cancer (n = 65; P=0.0079) but not in patients with stomach cancer (n = 87; P=0.42)	High

6. Conclusions

Our study has several limitations. First, the exact mechanisms of action of MDK and PTN in CRC remain unclear. In addition, we are not sure if immune cells are the targets or the "providers" of abnormal expression levels of MDK and PTN in CRC. Due to these limitations, our study is still far from clarifying the detailed functions of MDK and PTN in CRC. Despite these limitations, our study lays a foundation for exploring the mechanisms of action of MDK and PTN in CRC, providing further support for the development of novel targeted drugs.

7. Funding

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