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Research article

Bioinformatics analysis identified MMP14 and COL12A1 as immune-related biomarkers associated with pancreatic adenocarcinoma prognosis

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Abstract: *Background:* Pancreatic adenocarcinoma (PAAD) is one of the most common malignant tumors with high mortality rates and a poor prognosis. There is an urgent need to determine the molecular mechanism of PAAD tumorigenesis and identify promising biomarkers for the diagnosis and targeted therapy of the disease. *Methods:* Three GEO datasets (GSE62165, GSE15471 and GSE62452) were analyzed to obtain differentially expressed genes (DEGs). The PPI networks and hub genes were identified through the STRING database and MCODE plugin in Cytoscape software. GO and KEGG enrichment pathways were analyzed by the DAVID database. The GEPIA database was utilized to estimate the prognostic value of hub genes. Furthermore, the roles of MMP14 and COL12A1 in immune infiltration and tumor-immune interaction and their biological functions in PAAD were explored by TIMER, TISIDB, GeneMANIA, Metascape and GSEA. *Results:* A total of 209 common DEGs in the three datasets were obtained. GO function analysis showed that the 209 DEGs were significantly enriched in calcium ion binding, serine-type endopeptidase activity, integrin binding, extracellular matrix structural constituent and collagen binding. KEGG pathway analysis showed that DEGs were mainly enriched in focal adhesion, protein digestion and absorption and ECM-receptor interaction. The 14 genes with the highest degree of connectivity were defined as the hub genes of PAAD development. GEPIA revealed that PAAD patients with upregulated MMP14 and COL12A1 expression had poor prognoses. In addition, TIMER analysis revealed that MMP14 and COL12A1 were closely associated with the infiltration levels of macrophages, neutrophils and dendritic cells in PAAD. TISIDB revealed that MMP14 was strongly positively correlated with CD276,

TNFSF4, CD70 and TNFSF9, while COL12A1 was strongly positively correlated with TNFSF4, CD276, ENTPD1 and CD70. GSEA revealed that MMP14 and COL12A1 were significantly enriched in epithelial mesenchymal transition, extracellular matrix receptor interaction, apical junction, and focal adhesion in PAAD development. *Conclusions:* Our study revealed that overexpression of MMP14 and COL12A1 is significantly correlated with PAAD patient poor prognosis. MMP14 and COL12A1 participate in regulating tumor immune interactions and might become promising biomarkers for PAAD.

Keywords: pancreatic adenocarcinoma; MMP14; COL12A1; prognosis; biomarker; bioinformatics analysis

Abbreviations: PAAD: pancreatic adenocarcinoma; GEO: Gene Expression Omnibus; DEGs: differentially expressed genes; STRING: Search Tool for the Retrieval of Interacting Genes; MCODE: Molecular Complex Detection; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DAVID: Database for Annotation, Visualization, and Integrated Discovery; GEPIA: Gene Expression Profiling Interactive Analysis; TIMER: Tumor Immune Estimation Resource; GSEA: Gene Set Enrichment Analysis; ECM: extracellular matrix; PPI: protein-protein interaction; MF, molecular function; BP: biological process; CC: cellular component; TCGA: The Cancer Genome Atlas; GTEx: genotype-tissue expression.

1. Introduction

Pancreatic adenocarcinoma (PAAD) is the fourth leading cause of cancer deaths due to its high degree of malignancy, high recurrence rate and poor prognosis [1]. According to published statistics, there were nearly 56,770 new cases in the United States in 2019, and the 5-year survival rate is less than 10% [2]. The mechanisms of PAAD involve complex biological processes, including genomic, epigenetic and metabolic changes. The symptoms of PAAD are atypical, the disease develops rapidly, and there are no sensitive early diagnosis biomarkers or appropriate clinical treatments [3]. Potential biomarkers to assist in the diagnosis and treatment of PAAD have long been a research focus [4]. In recent years, many microarray analysis studies have been conducted to identify key genes involved in the progression of PAAD [5]. However, the key drivers of tumorigenesis remain unclear, which limits the early detection and personalized treatment of PAAD. Thus, it is urgent to reveal the underlying molecular pathogenesis of PAAD and identify effective therapeutic targets.

In this research study, we first used three Gene Expression Synthesis (GEO) datasets (GSE62452, GSE15471 and GSE62165) to obtain differentially expressed genes (DEGs). Next, we constructed a protein-protein interaction (PPI) network using the Search Tool for the Retrieval of Interacting Genes (STRING) database and applied Cytoscape to identify significant modules and hub genes. Subsequently, the Database for Annotation, Visualization, and Integrated Discovery (DAVID) was utilized to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of the DEGs. We then estimated the prognostic value of the hub genes using the Gene Expression Profiling Interactive Analysis (GEPIA) database. Finally, we identified two genes (MMP14 and COL12A1) that were associated with the prognosis of PAAD patients. We further explored the roles of MMP14 and COL12A1 in tumor-immune interactions and their biological

functions in PAAD.

In summary, the results of this study provide new clues to further our understanding of PAAD development and identify new immune-related biomarkers for personalized therapy and prognostic monitoring of PAAD patients.

2. Materials and methods

2.1. Identification of differentially expressed genes

The GEO is a large public genomic database containing array and sequence-based study data. The following keywords were used to search the GEO database: (pancreas OR pancreatic) AND (cancer OR carcinoma OR tumor OR neoplas* OR malignan* OR adenocarcinoma). The inclusion criteria were as follows: (1) patients diagnosed with PAAD; (2) the samples in microarrays were human tissues from patients with PAAD instead of animal tissues and cell lines; (3) the patients with PAAD were not treated with chemotherapy, radiotherapy, or any other methods; and (4) the microarray profiles included PAAD samples and nontumor tissue samples. The exclusion criteria were as follows: (1) cancer or non-cancer groups with small sample sizes ($n < 10$) and (2) poor‑quality profiling expression data (0, 0.1 or 1) that accounted for > 30% of the total expression data. Three transcriptome expression profiles, GSE62452, GSE15471 and GSE62165, were obtained from the GEO. The GSE62452 dataset included 69 pancreatic cancer samples and 61 normal pancreatic samples. The GSE15471 dataset consisted of 36 pairs of matched pancreatic tumor and nontumor tissues. The GSE62165 dataset contained 118 PAAD samples and 13 control samples. The three datasets were analyzed using the limma package in R software to identify DEGs. $|\log2FC| > 1$ and adjusted P value < 0.05 were set as the cutoffs.

2.2. PPI network construction and module analysis

The STRING [6] database (https://string-db.org/) was utilized to construct a PPI network. A total interaction score > 0.4 was considered statistically significant. Cytoscape [7] is a bioinformatics software used to analyze and visualize the interaction networks between molecules. We used the Molecular Complex Detection (MCODE) plugin to determine the densest and significant module in the PPI network. The criteria were as follows: degree cutoff = 2, node score cutoff = 0.2, max depth = 100 and K-score = 2. We selected the genes with the highest node score and the strongest connectivities as the PAAD hub genes.

2.3. GO and KEGG pathway analyses of the DEGs

DAVID [8] (https://david.ncifcrf.gov/) is an integrated online database that can provide researchers with comprehensive gene functional annotation information to help them understand biological processes. GO analysis annotates genes and analyzes the molecular function (MF), biological process (BP) and cellular component (CC) of these genes [9]. KEGG is another powerful database for the functional interpretation of genomic sequences [10]. We used the DAVID database to analyze the GO and KEGG pathways of the DEGs. All significant GO and KEGG enrichment pathways were visualized with the ggplot package.

2.4. Relationship between hub genes and clinical parameters in PAAD patients

GEPIA [11] (http://gepia.cancer-pku.cn/) is a user-friendly database that can be used to analyze and visualize large The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) datasets. GEPIA can perform gene expression comparisons between tumor and control samples, gene correlation analysis and survival analysis. The GEPIA database was utilized to explore the prognostic value of PAAD hub genes. In addition, we downloaded the clinical data of PAAD patients from the TCGA database and analyzed the relationship between hub gene expression and different pathological parameters by the chi-square test. A P value < 0.05 was considered statistically significant.

2.5. Correlation between MMP14 and COL12A1 and immune molecules

The Tumor Immune Estimation Resource (TIMER) [12] (https://cistrome.shinyapps.io/timer/) systematically analyzes immune infiltration in cancer samples based on TCGA database data with its own algorithm. We used the TIMER database to explore the correlation between MAD14 and COL12A1 and the infiltration level of 6 types of immune cells; specifically, B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells. Besides, TISIDB [13] (http://cis.hku.hk/TISIDB/), an online database for exploring tumor-immune system interactions, was used to assess the correlation between MMP14 and COL12A1 and the immune system in PAAD. A P value < 0.05 obtained from the Spearman's test was considered statistically significant.

2.6. Functional annotations of MMP14 and COL12A1

GeneMANIA [14] (http://www.genemania.org), a user-friendly website, can display gene or gene lists that share the same functions as submitted genes and provide a PPI network. The genes interacting with MMP14 and COL12A1, as determined by GeneMANIA, were all input into Metascape [15] for further functional annotations and analyses. Meanwhile, we conducted Gene Set Enrichment Analysis (GSEA) [16] to reveal the functional pathways of MMP14 and COL12A1 in PAAD by using transcription data from the TCGA. A permutation test (1000x) was applied to identify the most significantly involved pathways. NOM p-value < 0.05 and FDR q-value < 0.25 were used as the screening criteria.

3. Results

3.1. Analysis of DEGs in PAAD

After standardization of the microarray results, we identified 4063, 1794 and 296 DEGs from GSE62165, GSE15471 and GSE62452 respectively. Figures 1A–1C show the differential expression of genes in the tumor and normal samples in the three microarrays. A total of 209 common DEGs (153 upregulated and 56 downregulated) in the three datasets were obtained through the online tool Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) (Figure 1D). The PPI network of DEGs was constructed using the STRING database and then visualized by Cytoscape software, as shown in Figure 1E. The most significant module, with 14 nodes and 83 edges, was obtained based on the PPI network of the DEGs. A total of 14 upregulated genes were identified as hub PAAD genes with a high

degree of connectivity, FN1, BGN, THSB2, ASPN, COL11A1, FBN1, COL12A1, COL6A3, SPARC, COL1A1, COL3A1, COL5A2, MMP14 and POSTN (Figure 1F).

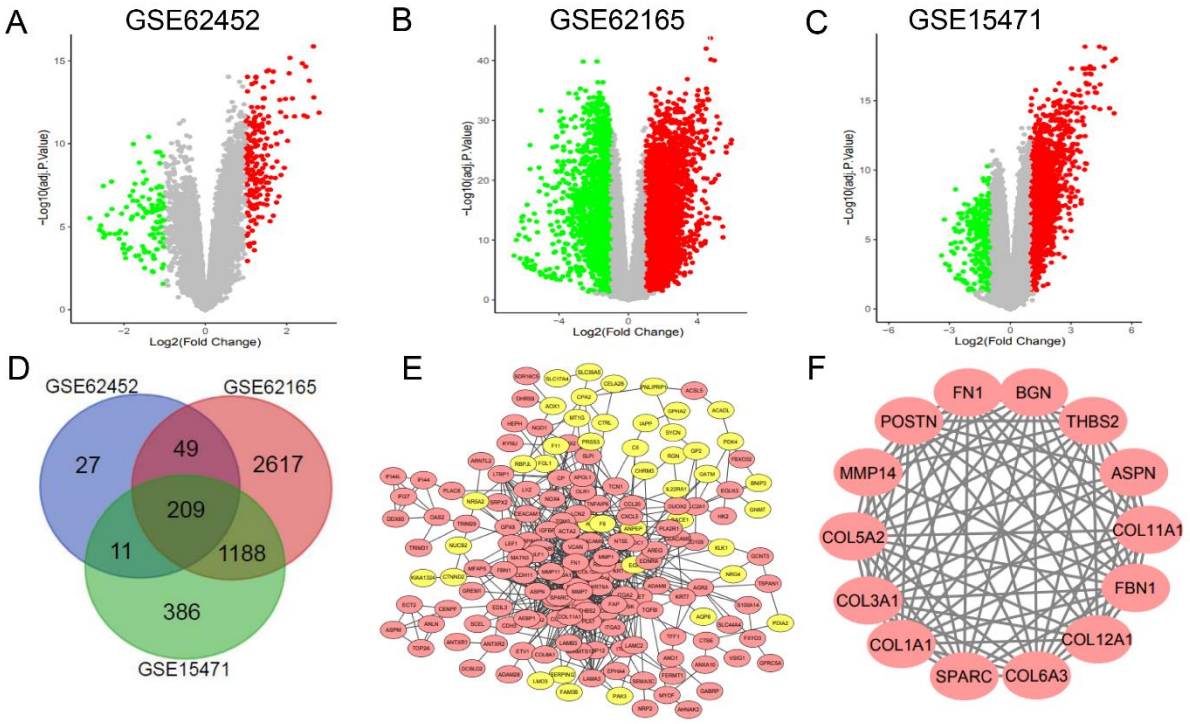

Figure 1. Identification and analysis of DEGs in PAAD. (A)–(C) Differential expression of genes in the tumor and normal sample in GSE62452, GSE62165 and GSE15471. Upregulated genes with adjusted P value < 0.05 and log2FC > 1 were marked in red. Downregulated genes with adjusted P value < 0.05 and log2FC < −1 were marked in green. The gray points represented genes with no significant difference. (D) An overlap of 209 common DEGs were showed in GSE62165, GSE15471 and GSE62452 datasets. (E) The PPI network of DEGs was visualized by Cytoscape. Upregulated genes were marked in red. Downregulated genes were marked in yellow. (F) The most significant module was obtained from PPI network.

3.2. GO and KEGG enrichment pathways of DEGs

The GO analysis results showed that the DEGs were significantly enriched in the biological processes of proteolysis, cell adhesion, skeletal system development, extracellular matrix organization, extracellular matrix disassembly, collagen fibril organization, collagen catabolic process and endodermal cell differentiation (Figure 2A). Changes in cellular components were significantly enriched in extracellular exosomes, extracellular regions, cell surfaces, extracellular space, endoplasmic reticulum lumen, proteinaceous extracellular matrix, collagen trimers and extracellular matrix (Figure 2B). With regard to molecular function, DEGs were mainly enriched in calcium ion binding, serine-type endopeptidase activity, integrin binding, extracellular matrix structural constituent and collagen binding (Figure 2C). KEGG pathway analysis showed that the DEGs were mainly enriched in focal adhesion, protein digestion and absorption and (extracellular matrix) ECM-receptor

interaction (Figure 2D).

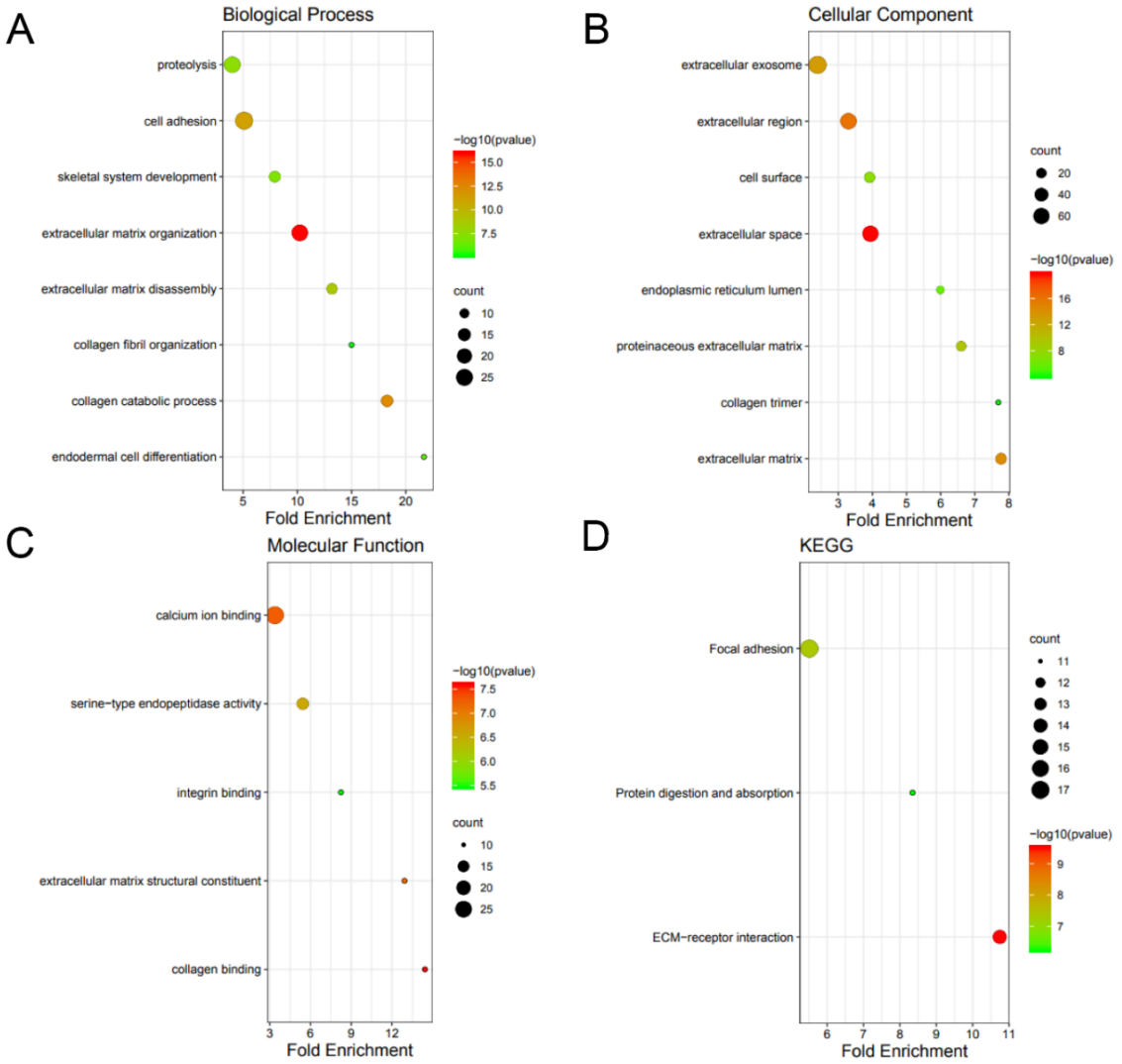

Figure 2. Significant GO and KEGG enrichment pathways of DEGs in PAAD. (A) BP terms of GO. (B) CC terms of GO. (C) MF terms of GO. (D) KEGG pathways.

3.3. Clinical value of hub genes in PAAD

Using GEPIA data, we noted that among the 14 hub genes, only MMP14 and COL12A1 were associated with the prognosis of PAAD patients. Patients with high MMP14 expression had a worse overall (P = 0.033, HR = 1.6) and disease-free survival rates (P = 0.0035, HR = 1.9) (Figure 3A,B). High expression of COL12A1 indicated a poor overall survival outcome (P = 0.0055, HR = 1.8), but it had no effect on the patient's disease-free survival rate (P = 0.057, HR = 1.5) (Figure 3C,D). In addition, as shown in Table 1, MMP14 expression was correlated with tumor grade and M classification, and COL12A1 expression was associated with tumor grade, stage, and T, N and M classification in PAAD (P value < 0.05). Taken together, these results suggested that the hub genes MMP14 and COL12A1 have clinical relevance in PAAD. Thus, we further explored the roles of MMP14 and COL12A1 in tumor-immune interactions and their biological functions in PAAD.

Table 1. Relationship between MMP14 and COL12A1 expression and clinicopathological parameters of PAAD.

Parameters	Overall	COL12A1 expression		P	MMP14 expression		P
	(n = 178)	low (89)	high (89)		high (89)	low (89)	
Primary site (%)				0.262			0.295
Body of Pancreas	14 (7.9)	9 (10.1)	5 (5.6)		9 (10.1)	5 (5.6)	
Head of Pancreas	139 (78.1)	65 (73.0)	74 (83.1)		69 (77.5)	70 (78.7)	
Tail of Pancreas	14 (7.9)	7 (7.9)	7 (7.9)		8 (9.0)	6 (6.7)	
Other	11 (6.2)	8 (9.0)	3 (3.4)		3 (3.4)	8 (9.0)	
Pathological type (%)				0.094			0.094
Other Type	25 (14.1)	18 (20.2)	7 (8.0)		7 (8.0)	18 (20.2)	
Ductal Type	147 (83.1)	69 (77.5)	78 (88.6)		78 (88.6)	69 (77.5)	
Colloid Carcinoma	4 (2.3)	2 (2.2)	2 (2.3)		2 (2.3)	2 (2.2)	
Undifferentiated Carcinoma	1 (0.6)	0 (0.0)	1 (1.1)		1 (1.1)	0 (0.0)	
Grade (%)				**0.044**			**0.018**
G1	31 (17.4)	22 (24.7)	9 (10.1)		8 (9.0)	23 (25.8)	
G2	95 (53.4)	40 (44.9)	55 (61.8)		51 (57.3)	44 (49.4)	
G3	48 (27.0)	24 (27.0)	24 (27.0)		29 (32.6)	19 (21.3)	
G4	2 (1.1)	1 (1.1)	1 (1.1)		1 (1.1)	1 (1.1)	
GX	2 (1.1)	2 (2.2)	0 (0.0)		0 (0.0)	2 (2.2)	
Tumor stage (%)				**0.004**			0.28
Stage I	21 (12.0)	18 (20.7)	3 (3.4)		7 (8.0)	14 (16.1)	
Stage II	147 (84.0)	66 (75.9)	81 (92.0)		76 (86.4)	71 (81.6)	
Stage III	3 (1.7)	2 (2.3)	1 (1.1)		2 (2.3)	1 (1.1)	
Stage IV	4 (2.3)	1 (1.1)	3 (3.4)		3 (3.4)	1 (1.1)	
T classification (%)				**0.004**			0.444
T1	7 (4.0)	7 (8.0)	0 (0.0)		2 (2.2)	5 (5.7)	
T2	24 (13.6)	17 (19.3)	7 (7.9)		10 (11.2)	14 (15.9)	
T3	142 (80.2)	61 (69.3)	81 (91.0)		75 (84.3)	67 (76.1)	
T4	3 (1.7)	2 (2.3)	1 (1.1)		2 (2.2)	1 (1.1)	
TX	1 (0.6)	1 (1.1)	0 (0.0)		0 (0.0)	1 (1.1)	
N classification (%)				**0.08**			0.977
N0	49 (27.7)	30 (34.1)	19 (21.3)		24 (27.0)	25 (28.4)	
N1	124 (70.1)	55 (62.5)	69 (77.5)		63 (70.8)	61 (69.3)	
NX	4 (2.3)	3 (3.4)	1 (1.1)		2 (2.2)	2 (2.3)	
M classification (%)				**0.022**			**0.046**
M0	80 (44.9)	32 (36.0)	48 (53.9)		47 (52.8)	33 (37.1)	
M1	4 (2.2)	1 (1.1)	3 (3.4)		3 (3.4)	1 (1.1)	
MX	94 (52.8)	56 (62.9)	38 (42.7)		39 (43.8)	55 (61.8)	

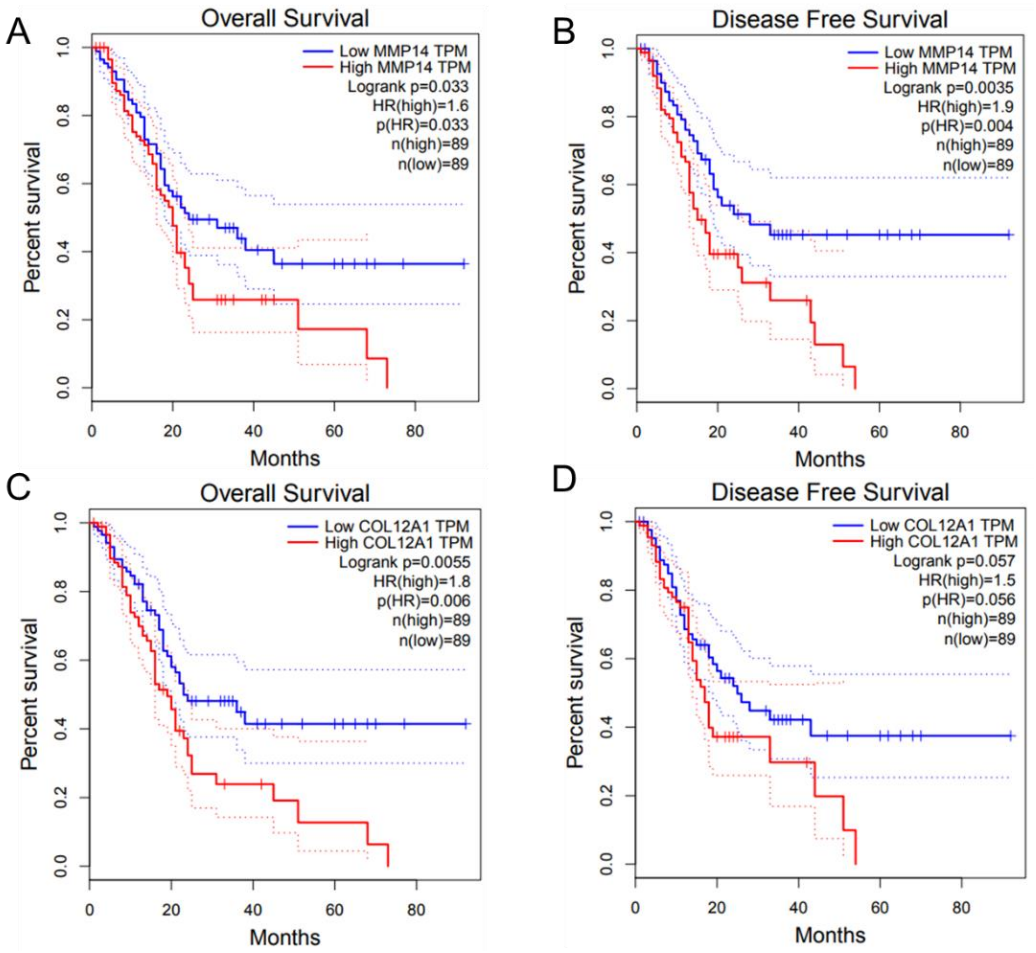

Figure 3. Prognostic value of MMP14 and COL12A1 in PAAD patients analyzed by GEPIA. Logrank $P < 0.05$ was statistically significant. (A)–(B) High MMP14 expression was associated with worse overall and disease-free survival in PAAD. (C)–(D) High expression of COL12A1was significantly associated with worse overall survival while disease-free survival was not statistically significant in PAAD.

3.4. Correlation between MMP14 and COL12A1 with immunity

TIMER analysis revealed that MMP14 had significant positive associations with infiltrating levels of CD8+ T cells ($r = 0.251$, $p = 9.24e{-4}$), macrophages ($r = 0.417$, $p = 1.42e{-8}$), neutrophils ($r = 0.46$, $p = 2.5e{-10}$) and dendritic cells ($r = 0.463$, $p = 1.77e{-10}$) in PAAD (Figure 4A). COL12A1 significantly correlated with B cells ($r = 0.183$, $p = 1.67e{-2}$), CD8+ T cells ($r = 0.386$, $p = 1.87e{-7}$), macrophages ($r = 0.537$, $p = 3.58e{-14}$), neutrophils ($r = 0.509$, $p = 1.24e{-12}$) and dendritic cells ($r = 0.526$, $p = 1.55e{-13}$) in PAAD (Figure 4B). Specifically, both MMP14 and COL12A1 were strongly correlated with macrophage, neutrophil and dendritic cell infiltration.

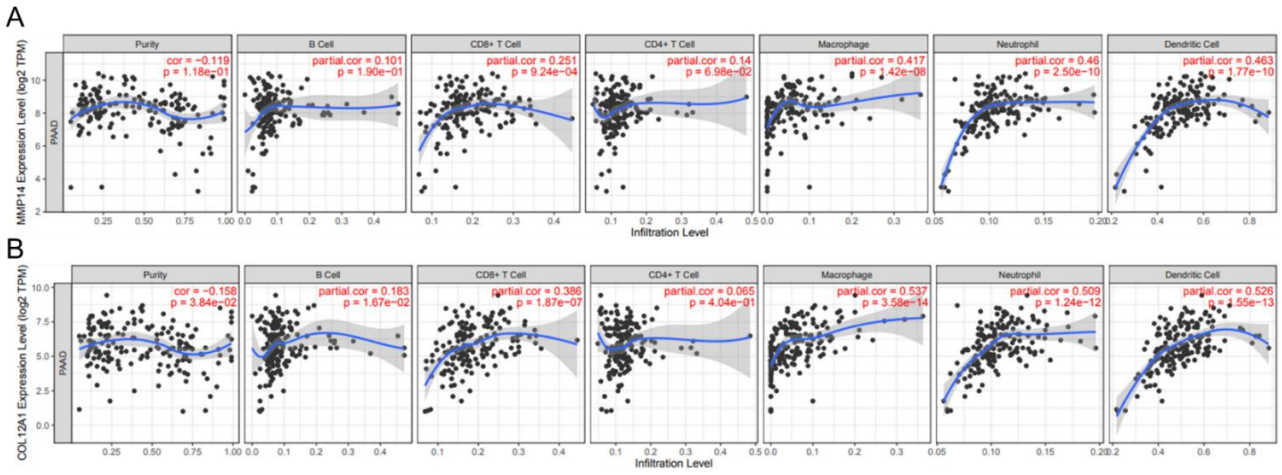

Figure 4. The correlation between MMP14. (A) and COL12A1 (B) with immune cell infiltration levels in PAAD analyzed by TIMER.

Meanwhile, TISIDB was utilized to comprehensively analyze the relationship between MMP14 and COL12A1 and immune molecules in PAAD. With regard to tumor infiltrating lymphocytes (Figure 5), MMP14 was found to positively correlate with Tcm CD4+ T cells (rho = 0.526, P < 2.26E−16), Act DCs (rho = 0.451, P = 3.6E−10), NK cells (rho = 0.447, P = 5.38E−10) and Tcm CD8+ T cells (rho = 0.432, P = 2.23E−9), while COL12A1 was positively correlated with NK cells (rho = 0.524, P < 2.2E−16), Tcm CD8+ T cells (rho = 0.502, P < 2.2E−16), Treg cells (rho = 0.481, P = 7.19E−12) and Tgd cells (rho = 0.475, P = 2.14E-11). With regard to immunoinhibitors (Figure 6), MMP14 was found to positively correlate with TGFB1 (rho = 0.584, P < 2.26E−16), PDCD1LG2 (rho = 0.366, P = 5.69E−7), HAVCR2 (rho = 0.325, P = 1.01E−5) and TGFBR1 (rho = 0.252, P = 0.000682) while COL12A1 was positively correlated with PDCD1LG2 (rho = 0.583, P < 2.2E−16), TGFBR1 (rho = 0.431, P = 2.46E−9), HAVCR2 (rho = 0.407, P = 2.18E−8) and CD274 (rho = 0.346, P = 2.4E−6). With regard to immunostimulators (Figure 7), MMP14 was strongly positively correlated with CD276 (rho = 0.861, P < 2.2E−16), TNFSF4 (rho = 0.605, P < 2.2E−16), CD70 (rho = 0.573, P < 2.2E−16) and TNFSF9 (rho = 0.426, P = 4.02E−9) while COL12A1 was strongly positively correlated with TNFSF4 (rho = 0.758, P < 2.2E−16), CD276 (rho = 0.594, P < 2.2E−16), ENTPD1 (rho = 0.577, P < 2.2E−16) and CD70 (rho = 0.512, P < 2.2E−16). In conclusion, these results indicate functions in the immune process of PAAD.

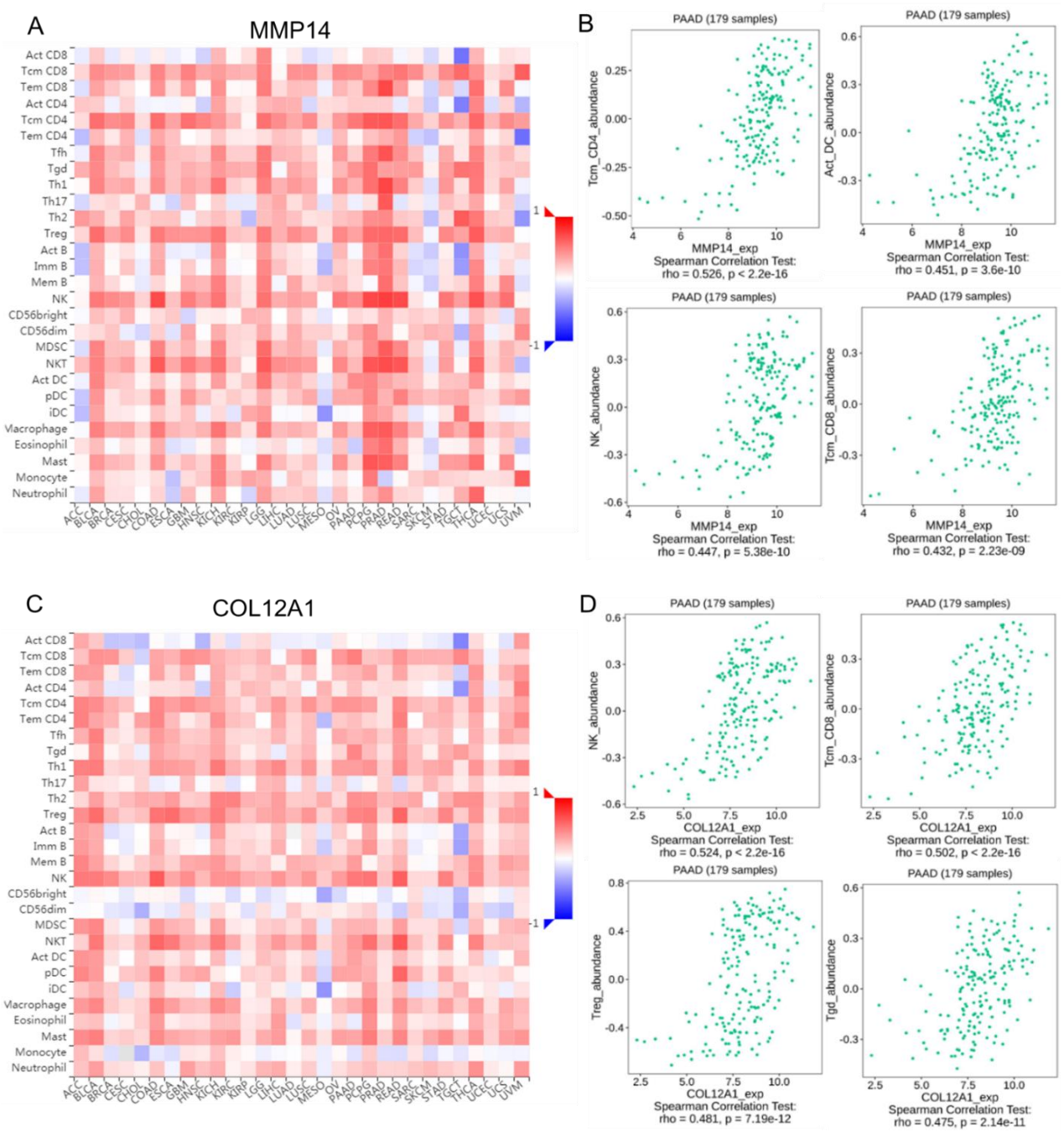

Figure 5. The correlation between MMP14 (A) and COL12A1 (C) with tumor infiltrating lymphocytes in 30 types of tumors analyzed by TISIDB. The top 4 tumor infiltrating lymphocytes with the strongest positive correlation with MMP14 (B) and COL12A1 (D) in PAAD.

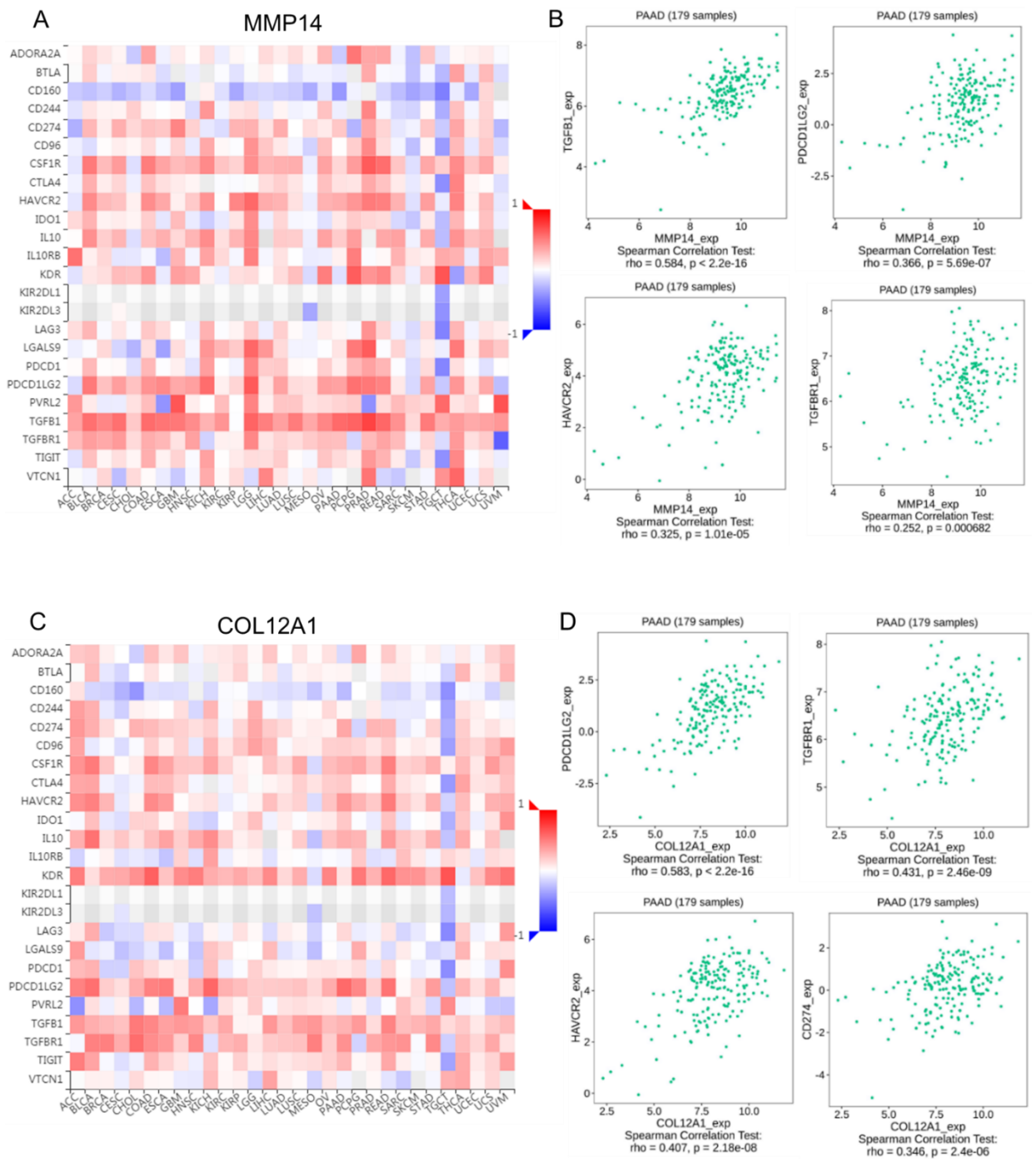

Figure 6. The correlation between MMP14 (A) and COL12A1 (C) with immunoinhibitors in 30 types of tumors analyzed by TISIDB. The top 4 immunoinhibitors with the strongest positive correlation with MMP14 (B) and COL12A1 (D) in PAAD.

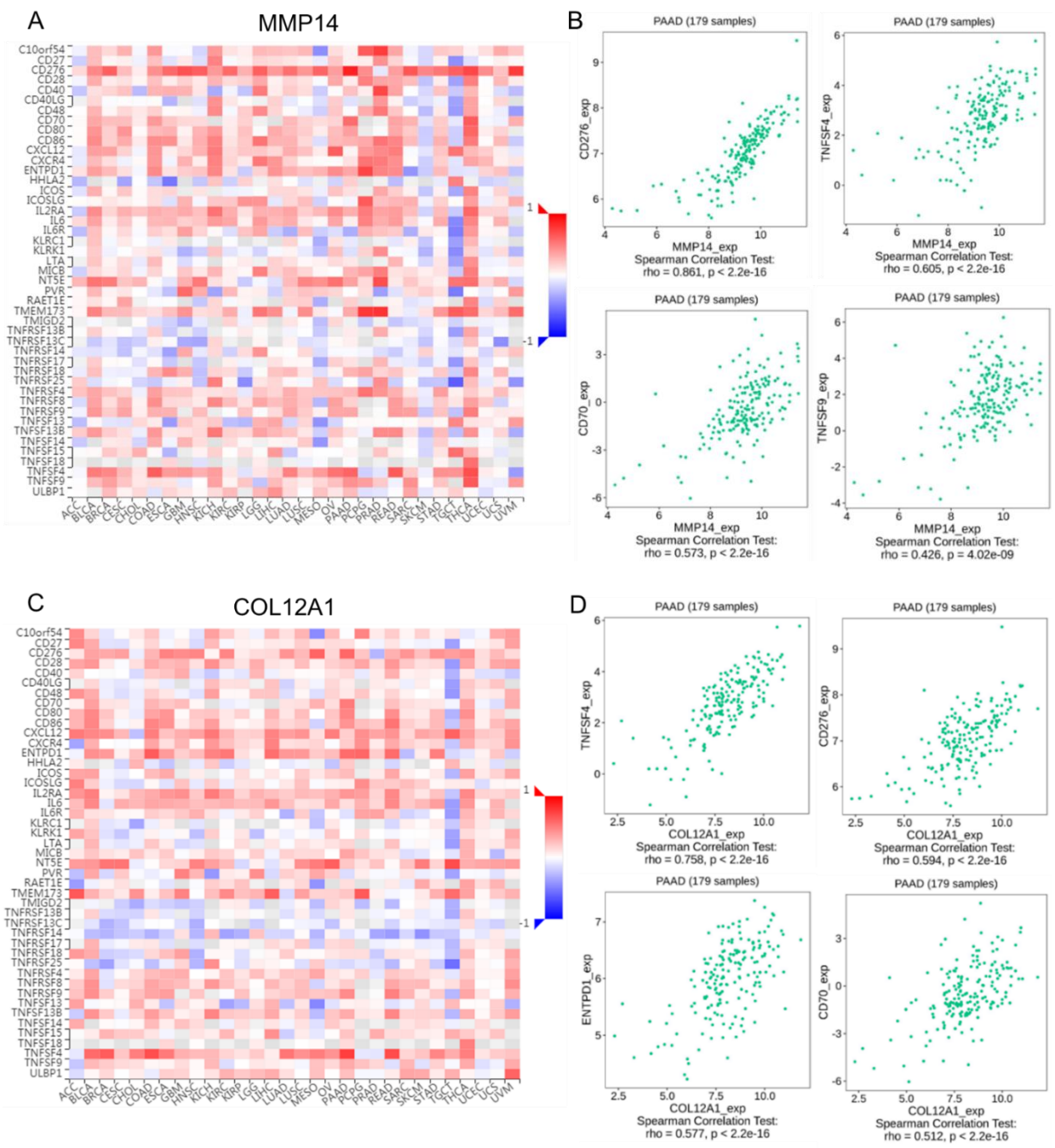

Figure 7. The correlation between MMP14 (A) and COL12A1 (C) with immunostimulators in 30 types of tumors analyzed by TISIDB. The top 4 immunostimulators with the strongest positive correlation with MMP14 (B) and COL12A1 (D) in PAAD.

3.5. *Functions and mechanisms of MMP14 and COL12A1 in PAAD*

Figure 8A,B shows the interaction network and biological functions of MMP14 and COL12A1 and their related genes, respectively. MMP14 was found to be involved in multiple biological processes in PAAD, such as extracellular structure organization, blood vessel development, negative regulation

of metallopeptidase activity and amyloid-beta clearance (Figure 8C). COL12A1 was mainly involved in extracellular matrix organization, the pid integrin1 pathway, integrin cell surface interactions, and MET activated PTK2 signaling in PAAD (Figure 8D). Moreover, GSEA was utilized to conduct hallmark and KEGG analyses for MMP14 and COL12A1 expression. The results indicated that the most significant pathways involving MMP14 included epithelial mesenchymal transition, apical junction, ECM receptor interaction, and focal adhesion (Table 2). The most significant pathways involving COL12A1 included epithelial mesenchymal transition, apical junction, focal adhesion, ECM receptor interaction, and regulation of actin cytoskeleton (Table 3).

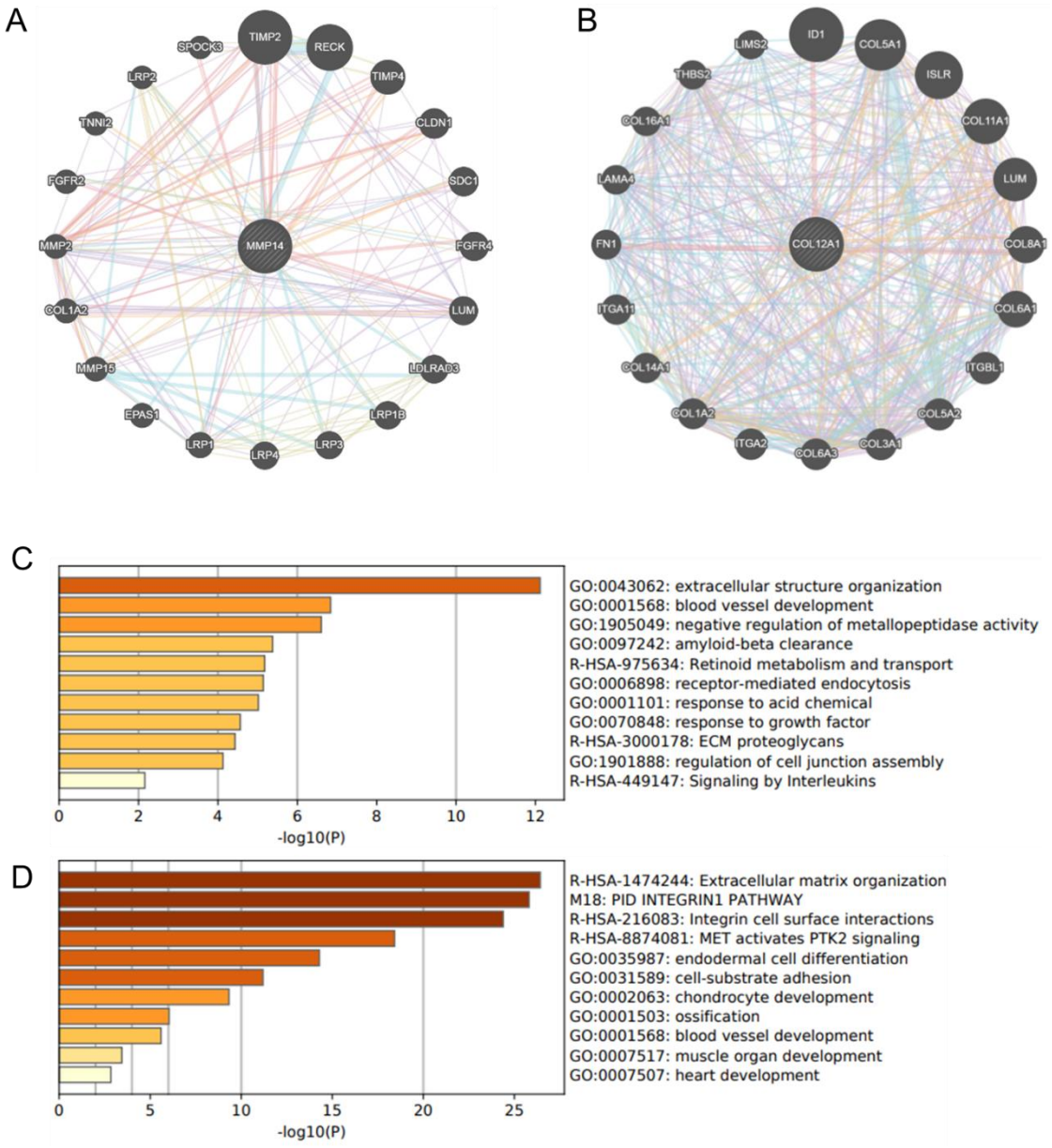

Figure 8. The interaction network of MMP14 (A) and COL12A1 (B) and their related genes. The biological functions of MMP14 (C) and COL12A1 (D) and their related genes analyzed by Metascape.

Table 2. The top 10 significant hallmarks and KEGG pathways in the data sets of high MMP14 expression in PAAD.

Name	ES	NES	NOM p-value	FDR q-value
Hallmark-epithelial mesenchymal transition	0.76	2.25	≤ 0.001	≤ 0.001
Hallmark-apical junction	0.52	2.24	≤ 0.001	≤ 0.001
Hallmark-apical surface	0.52	1.90	≤ 0.001	0.04
Hallmark-mitotic spindle	0.47	1.83	0.01	0.06
Hallmark-hypoxia	0.47	1.82	0.01	0.06
Hallmark-TGF-β signaling	0.56	1.82	0.01	0.05
Hallmark-angiogenesis	0.57	1.80	0.01	0.05
Hallmark-WNT-β catenin signaling	0.47	1.69	0.02	0.09
Hallmark-apoptosis	0.41	1.69	0.01	0.08
Hallmark-coagulation	0.40	1.65	0.01	0.08
KEGG-ECM receptor interaction	0.69	2.17	≤ 0.001	≤ 0.001
KEGG-focal adhesion	0.58	2.15	≤ 0.001	≤ 0.001
KEGG-pathway in cancer	0.46	1.98	≤ 0.001	0.03
KEGG-basal cell carcinoma	0.58	1.96	≤ 0.001	0.03
KEGG-glycosaminoglycan biosynthesis chondroitin sulfate	0.71	1.92	≤ 0.001	0.04
KEGG-regulation of actin cytoskeleton	0.44	1.91	≤ 0.001	0.04
KEGG-small cell lung cancer	0.54	1.90	≤ 0.001	0.03
KEGG-pathogenic Escherichia coli infection	0.55	1.83	0.01	0.06
KEGG-bladder cancer	0.54	1.83	0.01	0.06
KEGG-axon guidance	0.43	1.75	≤0.001	0.10

*Note: ES: enrichment score; NES: normalized enrichment score; NOM: nominal. FDR: false discovery rate.

Table 3. The top 10 significant hallmarks and KEGG pathways in the data sets of high COL12A1 expression in PAAD.

Name	ES	NES	NOM p-value	FDR q-value
Hallmark-epithelial mesenchymal transition	0.78	2.41	≤ 0.001	≤ 0.001
Hallmark-apical junction	0.48	2.10	≤ 0.001	0.01
Hallmark-UV response DN	0.58	2.09	≤ 0.001	0.01
Hallmark-TGF-β signaling	0.59	1.97	≤ 0.001	0.02
Hallmark-angiogenesis	0.61	1.93	0.01	0.02
Hallmark-inflammatory response	0.53	1.86	0.01	0.04
Hallmark-coagulation	0.44	1.78	≤ 0.001	0.05
Hallmark-apoptosis	0.42	1.78	0.01	0.05
Hallmark-complement	0.45	1.76	0.01	0.05
Hallmark-notch signaling	0.52	1.70	0.02	0.07
KEGG-focal adhesion	0.64	2.33	≤ 0.001	≤ 0.001

Continued on next page

Name	ES	NES	NOM p-value	FDR q-value
KEGG-ECM receptor interaction	0.71	2.20	≤ 0.001	≤ 0.001
KEGG-regulation of actin cytoskeleton	0.50	2.19	≤ 0.001	≤ 0.001
KEGG-pathway in cancer	0.50	2.19	≤ 0.001	≤ 0.001
KEGG-TGF-β signaling	0.55	2.11	≤ 0.001	≤ 0.001
KEGG-glycosaminoglycan biosynthesis chondroitin sulfate	0.77	2.10	≤ 0.001	≤ 0.001
KEGG- small cell lung cancer	0.56	1.99	≤ 0.001	0.01
KEGG- basal cell carcinoma	0.57	1.99	≤ 0.001	0.01
KEGG- melanoma	0.48	1.88	≤ 0.001	0.04
KEGG- adherens junction	0.53	1.87	0.01	0.04

*Note: ES: enrichment score; NES: normalized enrichment score; NOM: nominal. FDR: false discovery rate.

4. Discussion

PAAD is one of the most common digestive system malignancies worldwide. The treatments available for advanced PAAD are limited, and the patient prognosis is poor. The mechanisms leading to malignancy in PAAD remain poorly characterized. Moreover, biomarkers for targeted therapy and prognosis monitoring are still elusive. In many cancers, the level of tumor immune infiltration is closely associated with patient prognosis. Thus, there is an urgent need to identify new immune-associated biomarkers that are associated with prognosis and treatment in PAAD.

In this study, we identified 209 DEGs in PAAD using three microarray datasets from the GEO database. Through GO and KEGG enrichment analyses, we determined the biological functions and pathways of these DEGs in the development of PAAD. GO functional annotations identified many biological pathways associated with the DEGs in PAAD, including cell adhesion, ECM organization, proteolysis, collagen fibril organization, collagen catabolic process and endodermal cell differentiation. KEGG pathway analysis revealed that the DEGs were strongly associated with focal adhesion, ECM-receptor interaction and protein digestion and absorption. These pathways were found to be the most significant pathways in relation to PAAD occurrence and development. Cell adhesion is known to be a key mediator of invasiveness, metastasis, and immune suppression during malignancy since homeostasis in normal tissues is dependent on cell-to-cell adhesion [17]. Mutations and changes in the expression of cell adhesion molecules cadherins, integrins, selectins, and immunoglobulins have been associated with cancer progression in many studies [18]. The dysregulation of the ECM has recently been recognized as a key driver in the development and progression of cancer, as its organization and disassembly control malignant cell behavior and differentiation [19]. Proteolysis is the most fundamental property of malignancy, and its inhibition may be used to attenuate tumor invasion, angiogenesis and migration [20]. Perumal et al found that proteolysis of collagen fibrils is a key process in normal growth, development, repair, and cell differentiation, and in cancerous tumor progression [21]. Collagen is a major component of the ECM, and increasing evidence suggests that it influences tumor cell progression and metastasis through integrins, discoidin domain receptors, tyrosine kinase receptors, and some signaling pathways [22]. Bastidas-Ponce et al described the cellular process of pancreatic morphogenesis and demonstrated that pancreatic formation and development were closely related to endodermal cell differentiation [23]. The functional enrichment results obtained by analyzing the DEGs identified in this study provided novel insights into the mechanisms of PAAD occurrence

and development. Based on the PPI network analyzed by Cytoscape, a total of 14 hub genes involved in PAAD development were identified as follows: FN1, BGN, THSB2, ASPN, COL11A1, FBN1, COL12A1, COL6A3, SPARC, COL1A1, COL3A1, COL5A2, MMP14 and POSTN.

To determine whether these hub genes have clinical relevance in PAAD, we used GEPIA to analyze their prognostic value. We found that only MMP14 and COL12A1 were significantly associated with the survival outcomes of PAAD patients among the 14 hub genes. Our results revealed that high expression of MMP14 and COL12A1 tended to predict poor PAAD patient prognosis. In addition, MMP14 expression was correlated with tumor grade and M classification, while the expression of COL12A1 was related to tumor grade, stage, and T, N and M classification. Taken together, these results suggested that the hub genes MMP14 and COL12A1 had clinical relevance in PAAD. Thus, we selected MMP14 and COL12A1 as true hub genes for further analysis. Functional enrichment analysis and GSEA showed that MMP14 and COL12A1 significantly were involved in cancer hallmarks and KEGG pathways, including epithelial mesenchymal transition, ECM receptor interaction, apical junction, and focal adhesion. Proteolytic degradation of ECM and epithelial mesenchymal transition are known to be crucial steps in the development and progression of cancer [19,24]. The results of this study further confirmed that MMP14 and COL12A1 play important roles in the tumorigenesis and progression of PAAD.

MMP14 is a member of the membrane-type matrix metalloprotease (MMP) family with previously known roles in ECM degradation or proteolytic protein processing [25]. Previous studies demonstrated that MMP14 affects cancer cell proliferation and invasion and is usually associated with poor patient prognosis in those with cancers such as bladder cancer, gastric cancer and hepatocellular carcinoma[26–28]. A meta-analysis comprehensively evaluated the expression levels of MMP14 and its prognostic value in digestive system carcinomas, such as gastric cancer, esophageal carcinoma, oral cancer and hepatocellular carcinoma [29]. However, there were no data on its prognostic value in PAAD. Previous results indicated that MMP14 mainly exerts its proto-oncogenic functions by regulating processes such as ECM degradation and remodeling, cell invasion, and cancer metastasis [30]. Wei Jiang et al reported that MMP14 expression is required for the induction of epithelial mesenchymal transition and the activation of an invasive program in pancreatic cancer [31]. These results revealed the importance of MMP14 in PAAD and indicated that MMP14 is a potential therapeutic target for PAAD.

COL12A1 encodes the alpha chain of collagen XII, which is a member of the fibril-associated collagen family with interrupted triple helices [32]. Collage XII is an important dominant component of the ECM and interacts with other ECM molecules to provide structural support for cells [33]. As an ECM-related gene, COL12A1 has received increasing attention due to its critical role in human cancer. Some studies have explored the prognostic value of COL12A1 in different cancer types, such as breast cancer, gastric cancer and colorectal cancer [34–36]. IDO1 and COL12A1 synergistically participate in the metastasis of gastric cancer via the MAPK pathway[37]. The expression of COL12A1 was found to be increased in ovarian cancer and associated with the drug resistance of cancer cells[38]. In another study, it was revealed that type IV collagen promotes pancreatic cancer cell proliferation and migration and inhibits apoptosis through an autocrine loop[39]. The expression of Snail, a well-known regulator of epithelial-mesenchymal transition, was found to increase in pancreatic cancer cells in the presence of a collagen-rich milieu, suggesting that the desmoplastic reaction actively contributes to cancer progression[40]. Taken together, these results indicate that COL12A1 might serve as an effective biomarker and therapeutic target for PAAD.

Significant progress has been made in the treatment of malignancies, especially via the use of immunotherapies. In this study, we conducted a correlation analysis between MMP14 and COL12A1 and immune-infiltrating cells in PAAD. Our results demonstrated that the expression levels of both MMP14 and COL12A1 were significantly positively associated with the infiltration levels of macrophages, neutrophils, dendritic cells and NK cells in PAAD (all $P < 0.05$, all cor > 0.4). Strong evidence has indicated that these tumor-infiltrating cells play significant roles in the development and progression of PAAD. Macrophages, which are the main components of the tumor microenvironment, are known to promote the initiation, progression, angiogenesis, invasion and metastasis of PAAD [41]. A meta-analysis of 1699 patients showed that a high infiltration level of macrophages was significantly associated with poor overall and disease-free survival rates in pancreatic cancer patients [42]. Many studies have established that neutrophils also promote the initiation, development and progression of pancreatic cancer and that high levels of neutrophils predict poor patient prognoses [43]. Dendritic cells are professional antigen-presenting cells and are involved in the induction and regulation of antitumor immune responses. The levels of dendritic cells have been found to be a prognostic factor in pancreatic cancer [44]. Recent clinical evidence has shown that NK cells play a critical role in the antigen-independent immune response against malignant cells and that high NK infiltration predicts poor survival in patients with advanced pancreatic cancer [45]. Patients with a high abundance of these tumor-infiltrating cells had poor survival outcomes, which is consistent with the relationship between high expression of MMP14 and COL12A1 and PAAD patient survival. Previous studies have reported that MMPs promote cancer disease progression, new blood vessel formation, invasion, metastasis, the activity of inflammatory cytokines and chemokines, and avoidance of immune surveillance [46]. MMP14 controls macrophage immune function by modulating inflammatory responses, which triggers the activation of PI3Kδ signal cascades and the remodeling of the Mi-2/NuRD nucleosome complex [47]. In addition, the results of other recent studies suggested that high collagen density can modulate the activity and functions of tumor-infiltrating macrophages and T cells to support cancer cell immune escape, which was associated with poor prognosis in patients with several types of tumor[48,49]. Thus, we hypothesize that the MMP14- and COL12A1-regulated changes in the abundance and functions of tumor immune cells, especially macrophages, neutrophils, dendritic cells and NK cells may lead to invasion, immune system avoidance, and metastasis, which is responsible for the poor prognosis of PAAD patients. However, further experiments and prospective studies are warranted to validate the exact link between these tumor-infiltrating cells and MMP14 and COL12A1 in PAAD.

We also explored the association between MMP14 and COL12A1 and immune markers in PAAD. Among the immunostimulators, there was a high correlation coefficient between the expression of MMP14 and CD276, TNFSF4, CD70, and TNFSF9. There was also a high correlation coefficient between the expression of COL12A1 and TNFSF4, CD276, ENTPD1, and CD70 in PAAD. In terms of immunoinhibitors, MMP14 expression was positively correlated with TGFB1, PDCD1LG2, HAVCR2 and TGFBR1 while COL12A1 was positively correlated with PDCD1LG2, TGFBR1, HAVCR2 and CD274 in PAAD. Macrophages, neutrophils and dendritic cells are an essential component of innate immunity and play a vital role in various inflammatory diseases as well as in tumor progression. Evidence indicates that tumor-infiltrating lymphocytes play a significant role in the promotion or inhibition of tumor growth [50]. Du et al reported that CAR-T cells targeting CD276/B7-H3 effectively controlled the growth of pancreatic ductal adenocarcinoma, ovarian cancer and neuroblastoma in vitro and in mice without obvious toxicity [51]. CD70 is a costimulatory molecule that participates in the survival of regulatory T cells by interacting with its ligand CD27. Many studies

have indicated that anti-CD70 therapy is likely beneficial for stimulating antitumor immune responses [52]. TNFSF4 is a costimulatory checkpoint protein that has been proven to enhance the antitumor activity of T cells [53]. TNFSF9 is highly expressed in PAAD tissues and is related to M1 polarization of macrophages [54]. Chen Liang demonstrated that TGFB1 could affect the progression of pancreatic cancer based on the status of SMAD4 [55]. The PD-1 receptor and its ligands PD-L1/CD274 and PD-L2/PDCD1LG2 play key roles in T cell inhibition and exhaustion. Monoclonal antibodies that block the PD-1/PD-L1 pathway have been developed for cancer immunotherapy and enhance T cell function, especially in melanoma, non-small cell lung cancer, renal cell carcinoma and bladder cancer [56]. The interactions between MMP14 and COL12A1 and immune markers have rarely been reported. Our results provide evidence for a strong correlation between MMP14 and COL12A1 and the above immune molecules in PAAD, indicating that MMP14 and COL12A1 may serve as immune-related therapy targets for PAAD.

Our study has some limitations. This study is based on bulk RNA sequencing data used to identify biomarkers for PAAD and gene expression in rare clonal cells may have been missed. Due to the heterogeneity of cancer cells, multiple research methods are needed to reveal the molecular mechanisms of PAAD development. Emerging single-cell RNA sequencing technology, which can study every individual cell in a tumor, has become an important tool for analyzing cell biology, discovering the evolutionary relationships among cells, and revealing the heterogeneity of tumor cells [57]. Single-cell RNA sequencing technology provides a new perspective on the regulatory relationship between gene expression and the cell microenvironment [58]. Moreover, single-cell RNA sequencing technology helps identify biomarkers for predicting the prognosis and therapeutic response of tumors [59]. Recent studies have revealed the value of the application of single-cell RNA sequencing for characterizing cell heterogeneity and identifying specific cell-type biomarkers in pancreatic cancer [60,61]. In future studies, we will integrate and analyze more available data, including single-cell sequencing genomic, transcriptome and proteomic data, to provide new insights into the mechanisms of PAAD development and diagnostic and therapeutic strategies for PAAD patients.

5. Conclusions

This study identified 14 hub genes and the molecular mechanisms of PAAD development by using bioinformatics analysis. Among the 14 hub genes, we found that overexpression of MMP14 and COL12A1 was significantly correlated with poor PAAD patient prognosis. Furthermore, the expression of MMP14 and COL12A1 is strongly associated with the level of immune cell infiltration and the levels of multiple immunomodulators in PAAD. Our study demonstrates that MMP14 and COL12A1 play an important role in the tumorigenesis and progression of PAAD and might become promising biomarkers and therapeutic targets.

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Conflict of interest

The authors declare there is no conflict of interest.

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