



Activating miRNA-mRNA network in gemcitabine-resistant pancreatic cancer cell associates with alteration of memory CD4⁺ T cells

Jianyou Gu^{1#}, Junfeng Zhang^{2#}, Wenjie Huang^{1,3}, Tian Tao¹, Yaohuan Huang¹, Ludi Yang⁴, Jiali Yang², Yingfang Fan¹, Huaizhi Wang⁵

¹Department of Hepatobiliary Surgery, Zhujiang Hospital, Southern Medical University, Guangzhou 510280, China; ²Institute of Hepatopancreatobiliary Surgery, Southwest Hospital, Army Medical University (Third Military Medical University), Chongqing 400038, China; ³Affiliated Cancer Hospital & Institute of Guangzhou Medical University, Guangzhou 510000, China; ⁴State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing 400716, China; ⁵Institute of Hepatopancreatobiliary Surgery, Chongqing General Hospital, University of Chinese Academy of Sciences, Chongqing 400038, China

Contributions: (I) Conception and design: J Gu, J Zhang; (II) Administrative support: Y Fan, H Wang; (III) Provision of study materials or patients: J Gu, J Zhang; (IV) Collection and assembly of data: W Huang, T Tao, Y Huang, L Yang, J Yang; (V) Data analysis and interpretation: J Gu, J Zhang, W Huang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Huaizhi Wang, MD, PhD. Professor of Surgery, Chairman of Institute of Hepatopancreatobiliary Surgery, Chongqing General Hospital, University of Chinese Academy of Sciences, Chongqing 400038, China. Email: whuaizhi@gmail.com; Dr. Yingfang Fan. Department of Hepatobiliary Surgery, Zhujiang Hospital, Southern Medical University/The Second School of Clinical Medicine, Southern Medical University, Industrial Road No. 253, Guangzhou, Guangdong 510280, China. Email: fanxifan@smu.edu.cn.

Background: To identify key microRNAs (miRNAs) and their target mRNAs related to gemcitabine-resistant pancreatic cancer (PC) and investigate the association between gemcitabine-resistant-related miRNAs and mRNAs and immune infiltration.

Methods: Expression profiles of miRNAs and mRNAs were obtained from the Gene Expression Omnibus (GEO) database. The differentially expressed miRNAs and mRNAs (referred to as “DEmiRNAs” and “DEmRNAs”, respectively) were distinguished between gemcitabine-resistant PC cells and its parental cells. The DEmRNAs targeted by the DEmiRNAs were retrieved using miRDB, microT, and Targetscan. Furthermore, GO and KEGG pathway enrichment analysis and GSEA were performed. The Kaplan-Meier plotter was used to analyze the prognosis of key DEmiRNAs and DEmRNAs on PC patients. The relationship between the key DEmRNAs and tumor-infiltrating immune cells in PC was investigated using CIBERSORT method using the LM22 signature as reference. Key infiltrating immune cells were further analyzed for the associations with prognosis of TCGA PAAD patients.

Results: Four DEmiRNAs, including hsa-miR-3178, hsa-miR-485-3p, hsa-miR-574-5p, and hsa-miR-584-5p, were identified to target seven DEmRNAs, including MSI2, TEAD1, GNPDA1, RND3, PRKACB, TRIM68, and YKT6, individually, in gemcitabine-resistant PC cells versus parental cells. Gemcitabine-resistant PC cells were enriched in proteasome-related, immune-related, and memory CD4⁺ T cell-related pathways, indicating a gemcitabine therapeutic effect on PC cells. All four DEmiRNAs and almost all DEmRNAs had an impact on the prognosis of PC patients. All seven DEmRNAs had remarkable effects on CD4⁺ memory T cells, which were affected by the gemcitabine therapeutic effect. Effector memory CD4⁺ T cells rather than central memory CD4⁺ T cells predicted a good prognosis according to the TCGA PAAD dataset.

Conclusions: Gemcitabine resistance can alter the fraction of memory CD4⁺ T cells via hsa-miR-3178, hsa-miR-485-3p, hsa-miR-574-5p and hsa-miR-584-5p targeted MSI2, TEAD1, GNPDA1, RND3, PRKACB, TRIM68, and YKT6 network in PC.

Keywords: Gemcitabine resistance; microRNAs; tumor-infiltrating immune cells; CIBERSORT; CD4⁺ memory T cells

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Introduction

Pancreatic cancer (PC) is the fourth most lethal malignancy, with a poor prognosis and a 5-year relative survival rate of only 9% (1). In patients with inoperable or unresectable PC, gemcitabine treatment is the frontline chemotherapy in PDA, though its mechanism of resistance remains unclear.

MicroRNAs (miRNAs), a class of small, endogenous single-stranded RNAs of approximately 22 nucleotides in length initially discovered in 1993 (2), often play a negative regulatory effect in animals and plants via targeting specific mRNAs for degradation or translation suppression (3). However, the emerging role of miRNAs as activators of gene expression by targeting gene regulatory sequences (4) has been fully recognized just recently. Place *et al.* described this novel miRNA-induced RNA activation (miRNAa) phenomena and discovered that miR-373 bound the E-cadherin promoter sequences and induced gene expression (5). Interestingly, its mechanism has since then been elucidated by several subsequent studies. A series of such studies were followed to elucidate the mechanism. Xiao *et al.* reported that miR-24-1 could serve as an enhancer trigger by modifying chromatin status favorable for transcriptional gene activation (6). In support to this view, Huang *et al.* revealed an endogenous function for miRNA in gene activation as miR-744 and miR-1186 could induce CCNB1 expression and reinforce cancer cell growth (7).

Notably, aberrant expression of miRNAs is linked to gemcitabine sensitivity/resistance (8,9). Wang *et al.* validated the role of miR-30a in PC sensitization to gemcitabine (10). Another study reported that gemcitabine-resistant cells exhibited upregulated miR-301 expression and downregulated gemcitabine-induced apoptosis (11).

The differential expression of miRNAs has also been reported in modulation of immune infiltration. Frank *et al.* provided evidence for tumor-immune cell interactions shaping the immune cell phenotype and miR-375 acting as a crucial regulator of phagocyte infiltration and the subsequent development of a tumor-promoting microenvironment (12). In addition, Pyfferoen *et al.*

reported that dendritic cell-derived miR-31 promoted lung cancer progression (13).

Unfortunately, the underlying mechanisms of gemcitabine resistance in PC are poorly understood, and the impact of gemcitabine resistance in tumor-associated immune cells is implicit as well.

CIBERSORT is utilized to explore the relationship between immune infiltration and gemcitabine resistance. The bioinformatics tool of CIBERSORT was developed to deconvolve the expression matrix of immune cell subtype based on the principle of linear support vector regression (14). This deconvolution algorithm characterizes cell composition of complex tissues based on their gene expression profiles. In this study, CIBERSORT was used to assess the relative proportions of 22 tumor-infiltrating immune cells in PC and to investigate the relationship between the composition of tumor-infiltrating immune cells and gemcitabine therapeutic effect.

Here, we identified four differentially expressed miRNAs and their targeted DE mRNAs in gemcitabine resistant PC cells from the Gene Expression Omnibus (GEO) database using integrated bioinformatics analysis. Subsequently, through the bioinformatics tool of CIBERSORT, we explored the relationship between immune infiltration and gemcitabine resistance according to the gene expression profiles of PC from the TCGA database and found that gemcitabine therapeutic effects were closely associated to memory CD4⁺ T cells. Collectively, this study showed a T cell immune-related miRNAa regulatory network. Additionally, our findings provide insights into the role of memory CD4⁺ T cells in PC chemotherapy and can potentially help in the design of future treatments.

Methods

Cell lines and cell culture

Gemcitabine-resistant PC cells (PANC-1-GEM) and its parental cells (PANC-1) used in this research were purchase from Suyan Co (Guangzhou, China). All cells

were maintained in RPMI medium 1640 (Gibco Invitrogen, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) (Gibco Invitrogen, Grand Island, NY, USA) and 1% antibiotics, in a humidified atmosphere containing 5% carbon dioxide (CO₂) and 95% air at 37 °C. Additionally, the PANC-1-GEM cells were cultured with 80 μmol/L gemcitabine to maintain drug resistance and gemcitabine was removed from RPMI medium 48 hours before any experiment was performed.

RNA extraction and real-time PCR (qRT-PCR)

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used for total RNA isolation from PC cells according to the manufacturer's instruction. After extraction, RNA concentration was detected and the RNA was maintained at -80 °C refrigerator to avoid degradation. miRNAs expressions were determined using Mir-X miR FirstStrand Synthesis Kit (TaKaRa) and SYBR Premix Ex Taq II (TaKaRa) and RNU6-2 was used as an internal control. The 2^{-ΔΔCT} method was used to calculate the relative expressions of miRNAs. Genecopoeia Co. (Guangzhou, China) designed the bulge-loop miRNA qRT-PCR Primer Sets specific for four miRNAs.

miRNAs overexpression and silencing

miRNAs inhibitor, inhibitor negative control (Inhi-NC), miRNAs mimics and mimic negative control (Mim-NC) were purchased from Genecopoeia Co. (Guangzhou, China). Transfection was performed using Lipofectamine 3000 (Invitrogen) according to the protocol.

Cell viability assay

Cell viability ability was determined by using CCK-8 assay. CCK-8 assay was performed under the manufacturer's instructions (Dojindo, Shanghai, China). PANC-1-GEM and PANC-1 cells with transfection were seeded in 96-well plates at 5×10³ cells per well and incubated with gemcitabine for 48 h. CCK-8 (10 μL per 100 μL) was added to each well and incubated for 2 hours at 37 °C before detection.

Data collection

The mRNA and miRNA expression data used in this study were obtained from the GEO database. miRNA expression data were acquired from GSE74562, GSE74565, and GSE79234. mRNA expression data were obtained derived

from GSE80617 and GSE79953.

Identification of DEmRNAs and DE miRNAs in gemcitabine-resistant PC cells compared with its parental cells

We firstly filtered and deleted the difficultly detected mRNAs or miRNAs with read count value =0 or missing values in more than 33.3% of samples. Then, the k-nearest neighbor algorithm (KNN) was used for filling in the missing values. The differentially expressed mRNAs ("DEmRNAs") and miRNAs ("DE miRNAs") between gemcitabine resistant PC cells and their parental cells were screened out individually using the limma package in the R 3.3.2 software. The threshold for the expression of DEmRNAs and DE miRNAs was P<0.05 and fold change >1.2. The DEmRNAs and DE miRNAs whose expression trend was inconsistent in two mRNAs datasets or three miRNAs datasets were excluded. With R package *pheatmap*, hierarchical clustering analysis of DEmRNAs and DE miRNAs were conducted.

Prediction of DE miRNAs target mRNAs and construction of the DE miRNAs-DEmRNAs interaction pairs

Three bioinformatics algorithms, including miRDB, microT, and Targetscan were used to predict the targeted DEmRNAs. In the predicted results by Targetscan, the "Total context+ score" less than -0.2 was selected. Only mRNAs predicted by all three databases were considered as candidate mRNAs. The candidate mRNAs that intersected with DEmRNAs were screened out, and a miRNA-target gene network was constructed. The Cytoscape (version 3.7.1) software was utilized to draw this network.

Potential functional enrichment analysis of key DEmRNAs related to gemcitabine-resistant PC

Functional enrichment analysis of the key DEmRNAs in this network was performed based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to identify the genes' functions and pathways regarding gemcitabine-resistant PC. Metascape tool was used to display network relationships among DEmRNAs.

Gene set enrichment analysis (GSEA)

GSEA was performed using GSEA 3.0 (JAVA) using

GSE79953 dataset. Datasets were divided into two subgroups, gemcitabine-resistant group and parental group. Immune-related gene sets were downloaded in MSigDB, including “go response to interferon gamma”, “memory CD4 T cell vs. B cell up”, “naive vs. memory CD4 T cell up”, “hallmark IL-6 JAK STAT3 signaling”, “immune response”, “IL27 pathway”, “reactome cytokine signaling in immune system”, “go cellular response to interferon gamma”, etc. The number of permutations was set up to 1,000.

Protein-protein interaction (PPI) network construction

GeneMANIA online tool (<http://genemania.org/>) was used to construct and visualize the PPI network of seven targeted genes, including *MSI2*, *TEAD1*, *GNPDA1*, *RND3*, *PRKACB*, *TRIM68*, and *YKT6*.

Survival analysis of key DEmiRNAs, targeted DEmRNAs, and memory CD4⁺ T cells

The Kaplan-Meier plotter is a web server for a meta-analysis based on discovery and validation of survival biomarkers (15). Its mRNA subsystems include 54k genes from 21 cancer types, and miRNA subsystems include 11k samples from 20 different cancer types. Gene expression data and relapse free and overall survival (OS) information are derived from GEO, EGA, and TCGA. The survival analysis tool in Kaplan Meier plotter was utilized to analyze the prognostic significance of DEmiRNAs and targeted DEmRNAs. Survival analysis was performed using the Kaplan-Meier method, and the hazard ratio with 95% confidence intervals and $\log_{\text{rank}} P$ value were calculated.

Besides, survival analysis of high central memory CD4⁺ T cell patients and low central memory CD4⁺ T cell patients and high effector memory CD4⁺ T cell patients and low effector memory CD4⁺ T cell patients was performed on The Cancer Immunome Atlas (<https://tcia.at/home>) using TCGA PAAD dataset.

CIBERSORT deconvolution algorithm

To explore the relationship between the targeted DEmRNAs and immune cell infiltration in PC, the CIBERSORT method with 1,000 permutations was applied in R 3.3.2 using the LM22 signature as reference. Samples whose P value of CIBERSORT results were higher than

0.05 were excluded. The proportion of 22 immune cell types in each sample was obtained, including macrophages M2, T cells CD4⁺ memory resting, monocytes, dendritic cells resting, neutrophils, mast cells resting, B cells naive, eosinophils, B cells memory, NK cells resting, macrophages M1, T cells CD4⁺ naive, NK cells activated, T cells follicular helper, T cells regulatory, T cells gamma delta, Macrophages M0, dendritic cells activated, plasma cells, mast cells activated, T cells CD4⁺ memory activated, and T cells CD8. Pearson correlation among 22 immune cell types was calculated, and the visualization was presented using *corrplot* R package.

Results

DEmiRNAs in gemcitabine-resistant PC cells compared to their parental cells

Compared with the parental cells, a total of 198 (108 up- and 90 down-regulated miRNAs), 339 (194 up- and 145 down-regulated miRNAs), and 189 (115 up- and 74 down-regulated miRNAs) DEmiRNAs were identified from GSE74562, GSE74565, and GSE79234 in gemcitabine-resistant PC cells (*Figure 1A,B,C,D,E,F*), respectively. Unsupervised hierarchical clustering analysis was performed for the top 100 up-regulated and top 100 down-regulated miRNAs. The heatmaps showed that the gemcitabine-resistant PC cell samples could be clearly distinguished from the parental cells with respect to the expression of these differentially expressed miRNAs (*Figure 1D,E,F*).

Further intersection analysis showed that 13 miRNAs overlapped in three datasets (*Figure 1G*). We excluded the DEmiRNAs whose expression trend was inconsistent, and four miRNAs (hsa-miR-3178, hsa-miR-485-3p, hsa-miR-574-5p, and hsa-miR-584-5p) were defined as key miRNAs related to PC gemcitabine resistance (*Figure 1H,I,J,K*).

Consistently, qRT-PCR analyses confirmed that the four miRNAs were upregulated in gemcitabine-resistant PC cells (PANC-1-GEM) compared with its parental cell (PANC-1) (*Figure 1L*). We next studied the effect of these four miRNAs on the sensitivity of gemcitabine in PC gemcitabine resistance and found that all of these four miRNAs conferred gemcitabine resistance in PANC-1 cell (*Figure 1M*) and knockdown of four miRNAs re-sensitized PANC-1-GEM cells (*Figure 1N*).

The four DEmiRNAs recognized above were mapped into miRDB, microT, and Targetscan databases to search for their target mRNAs according to the inclusion

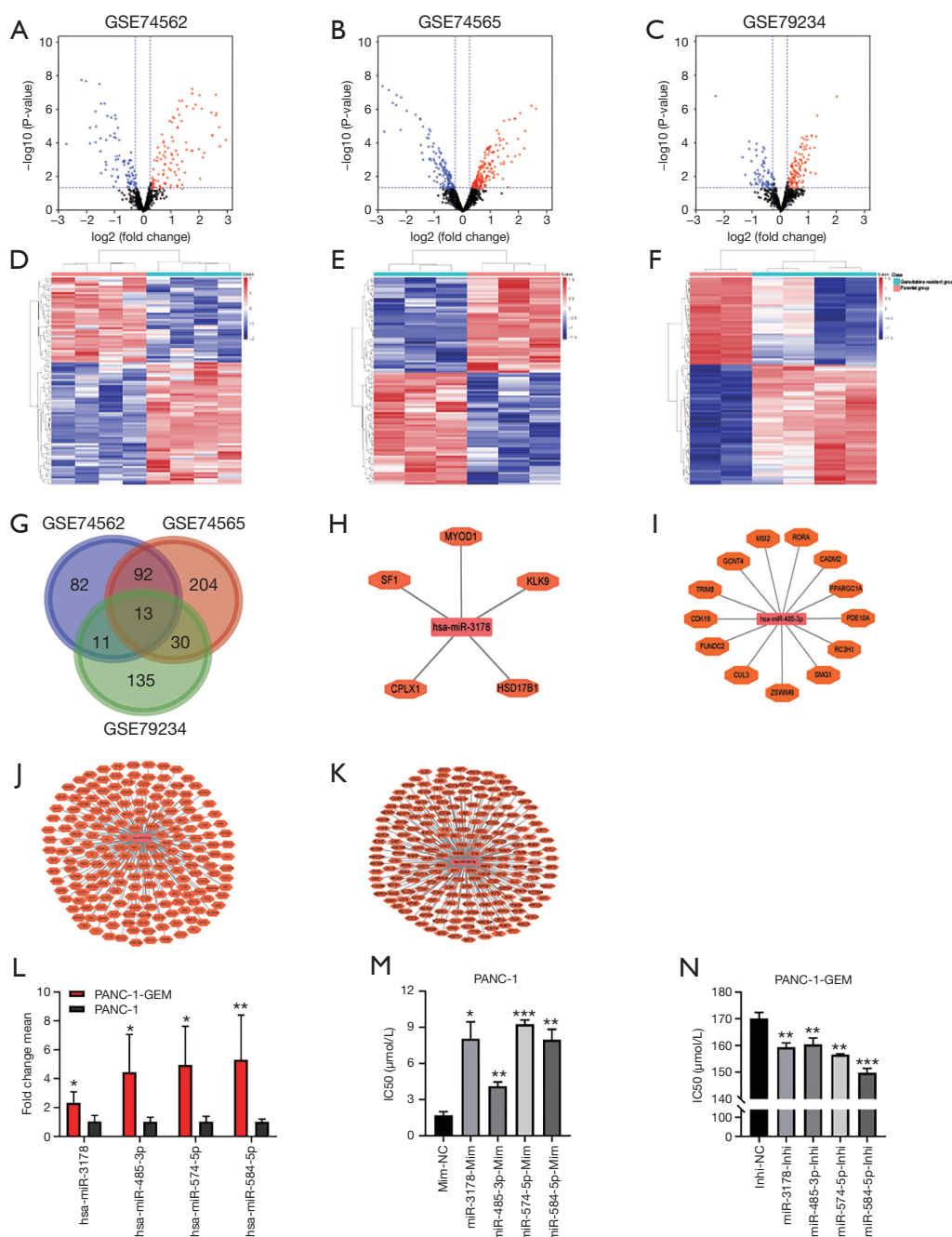


Figure 1 DE miRNAs between gemcitabine-resistant pancreatic cancer cells and parental cells. (A-C) Volcano plots showed DE miRNAs in GEO datasets GSE79234, GSE74565, and GSE74562; (D-F) the cluster heatmap showed the top 100 up-regulated and top 100 down-regulated miRNAs in GEO datasets GSE79234, GSE74565, and GSE74562. Red color indicates high expression level, and green color indicates low expression level; (G) Venn diagram showed that 13 DE miRNAs overlapped in GSE79234, GSE74565, and GSE74562; (H-K) miRNA-target gene network of hsa-miR-3178, hsa-miR-485-3p, hsa-miR-574-5p, and hsa-miR-584-5p, which had a consistent expression trend in three GEO datasets. The targeted genes were mapped into miRDB, microT, and Targetscan databases; (L) qRT-PCR analyses showed that the four miRNAs were upregulated in gemcitabine-resistant PC cells (PANC-1-GEM) compared with its parental cells (PANC-1); (M) miRNAs transfection in PANC-1 cells led to increased IC₅₀ to gemcitabine compared to control cells; (N) knockdown of miRNAs in PANC-1-GEM cells led to decreased IC₅₀ to gemcitabine compared to control cells. Each bar represents the mean \pm SD. *, P<0.05; **, P<0.01; ***, P<0.001. PANC-1-GEM, gemcitabine-resistant PANC-1 cells; IC₅₀, half-inhibitory concentration; Inhi-NC, inhibitor negative control; Mim-NC, mimic negative control.

criteria. Their respective miRNA-target gene network was generated using Cytoscape software (Figure 1H,I,J,K).

DEmRNAs in gemcitabine-resistant PC cells compared with their parental cells

Compared with the parental cells, a total of 5,340 (3,084 up- and 2,256 down-regulated mRNAs) and 5,774 (3,518 up- and 1,715 down-regulated mRNAs) DEmRNAs were identified from GSE79953 and GSE80617 in gemcitabine-resistant PC cells (Figure 2A,B,C,D), respectively. Further intersection analysis showed that 1,565 mRNAs overlapped in two datasets (Figure 2E). Similarly, DEmRNAs with inconsistent expression trend were excluded. Furthermore, the remaining 952 DEmRNAs were performed for next analysis.

Functional enrichment analysis

Functional enrichment analysis based on 952 DEmRNAs showed that the DEmRNAs related to PC gemcitabine resistance were involved in biological processes (BPs) such as regulation of cellular amino acid metabolic process and negative regulation of ubiquitin-protein ligase activity and molecular functions (MFs) such as protein channel activity in GO database (Figure 2F). In addition, they had an impact on cell components (CCs) such as proteasome complex, proteasome accessory complex, proteasome regulatory particle, proteasome regulatory particle, and lid subcomplex (Figure 2F). As for the KEGG database, proteasome and ribosome biogenesis in eukaryotes and RNA polymerase-related pathways were significantly enriched (Figure 2G). Consistent with GO and KEGG results, hsa03050:proteasome was enriched best using the tool of Metascape (Figure 2H,I).

Interestingly, GSEA revealed that chemotherapy resistance to gemcitabine in PC was positively correlated with gene signatures of immune related and memory CD4⁺ T cell-related pathways (Figure 3). “Go response to interferon gamma” (Normalized Enrichment Score, NES =1.85, P=0), “memory CD4 T cell vs. B cell up” (NES =1.43, P=0.010), “hallmark IL-6 JAK STAT3 signaling” (NES =1.58, P=0.001), “immune response” (NES =1.57, P=0), “IL-27 pathway” (NES =1.54, P=0.016), “reactome cytokine signaling in immune system” (NES =1.55, P=0), and “go cellular response to interferon gamma” (NES

=1.81, P=0) were significantly enriched in the gemcitabine-resistant group, whereas “naive vs. memory CD4 T cell up” (NES =-1.32, P=0.009) was well enriched in the parental group.

The DEmiRNAs-DEmRNAs interaction pairs

The candidate mRNAs targeted by the four DEmiRNAs were intersected with the 952 DEmRNAs, and zero, one, five, and one target mRNAs were identified by hsa-miR-3178, hsa-miR-485-3p, hsa-miR-574-5p, and hsa-miR-584-5p, respectively. Particularly, the expression tendency of all four DEmiRNAs and their target mRNAs has increased in gemcitabine-resistant PC cells compared with their parental cells, which was consistent with miRNAa phenomena. The PPI network of seven targeted genes is displayed in Figure 4.

Survival analysis of four DEmiRNAs and seven targeted DEmRNAs

The prognostic significance of four DEmiRNAs and seven targeted DEmRNAs was analyzed using Kaplan-Meier plotter. As shown in Figure 5, data of 177 PAAD patients were incorporated in the survival analysis. Three DEmiRNAs, hsa-miR-3178 ($\log_{\text{rank}} P=0.0018$, HR =2.27), hsa-miR-485-3p ($\log_{\text{rank}} P=0.039$, HR =0.61), and hsa-miR-584-5p ($\log_{\text{rank}} P=0.00072$, HR =2.09) were significantly associated with the OS of PAAD patients. Another DEmiRNA, hsa-miR-574-5p ($\log_{\text{rank}} P=0.043$, HR =0.5), was significantly linked to OS of female PDAC patients. Among seven targeted DEmRNAs, three DEmRNAs, MSI2 ($\log_{\text{rank}} P=0.042$, HR =0.64), RND3 ($\log_{\text{rank}} P=0.013$, HR =1.83), and TRIM68 ($\log_{\text{rank}} P=0.012$, HR =0.58) were significantly associated with the OS of PDAC patients. Two DEmRNAs, TEAD1 ($\log_{\text{rank}} P=0.021$, HR =1.98) and YKT6 ($\log_{\text{rank}} P=0.046$, HR =1.81), were significantly linked to OS of male PAAD patients. In addition, PRKACB and GNPDA1 were not qualified predictors for PAAD patient survival.

Immune infiltration analysis of TCGA PAAD patients

Given that gemcitabine might affect immune-related pathways of PC, we investigated whether immune cell fractions were altered by DEmRNAs. TCGA PAAD

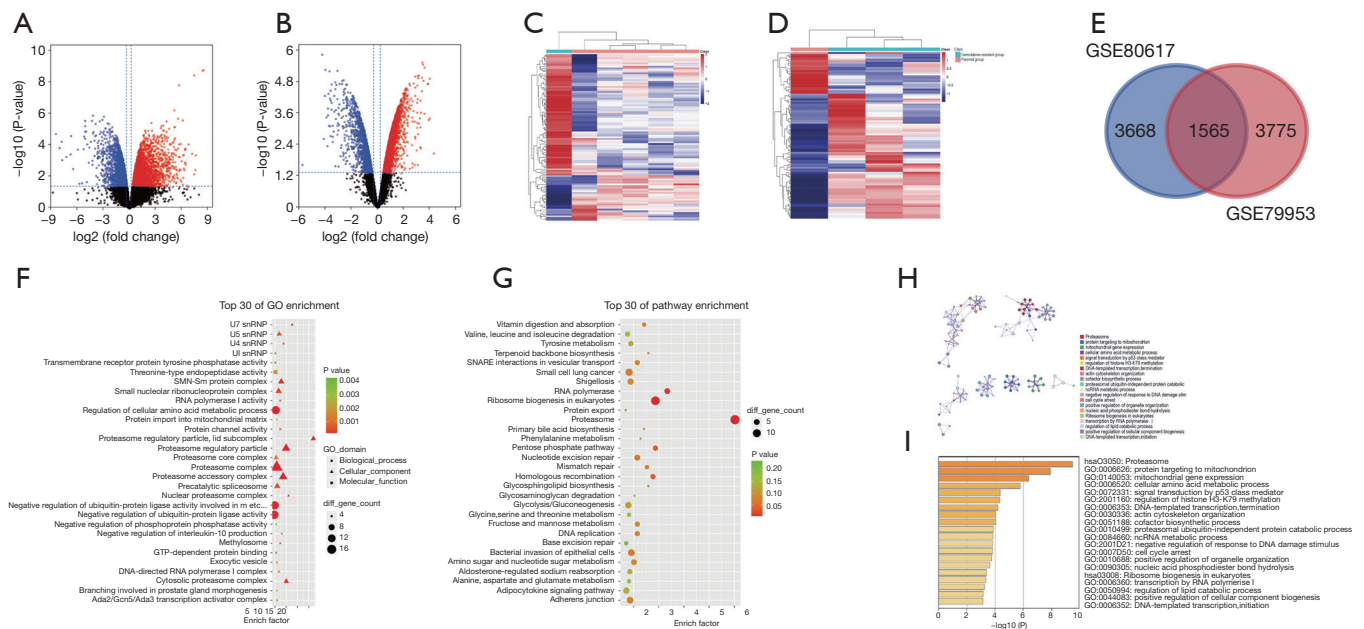


Figure 2 DE mRNAs between gemcitabine-resistant pancreatic cancer cells and parental cells and significantly enriched GO terms and KEGG pathways of DE mRNAs. (A,B) Volcano plots showed DE mRNAs in GEO datasets GSE79953 and GSE80617; (C,D) the cluster heatmap showed the top 100 up-regulated and top 100 down-regulated mRNAs in GEO datasets GSE79953 and GSE80617, respectively. Red color indicates high expression level, and green color indicates low expression level; (E) Venn diagram demonstrated 1,565 DE mRNAs overlapped in GSE79953 and GSE80617; (F-I) considerably enriched GO terms and KEGG pathways based on the 952 DE mRNAs with a consistent expression trend in GSE79953 and GSE80617 datasets.

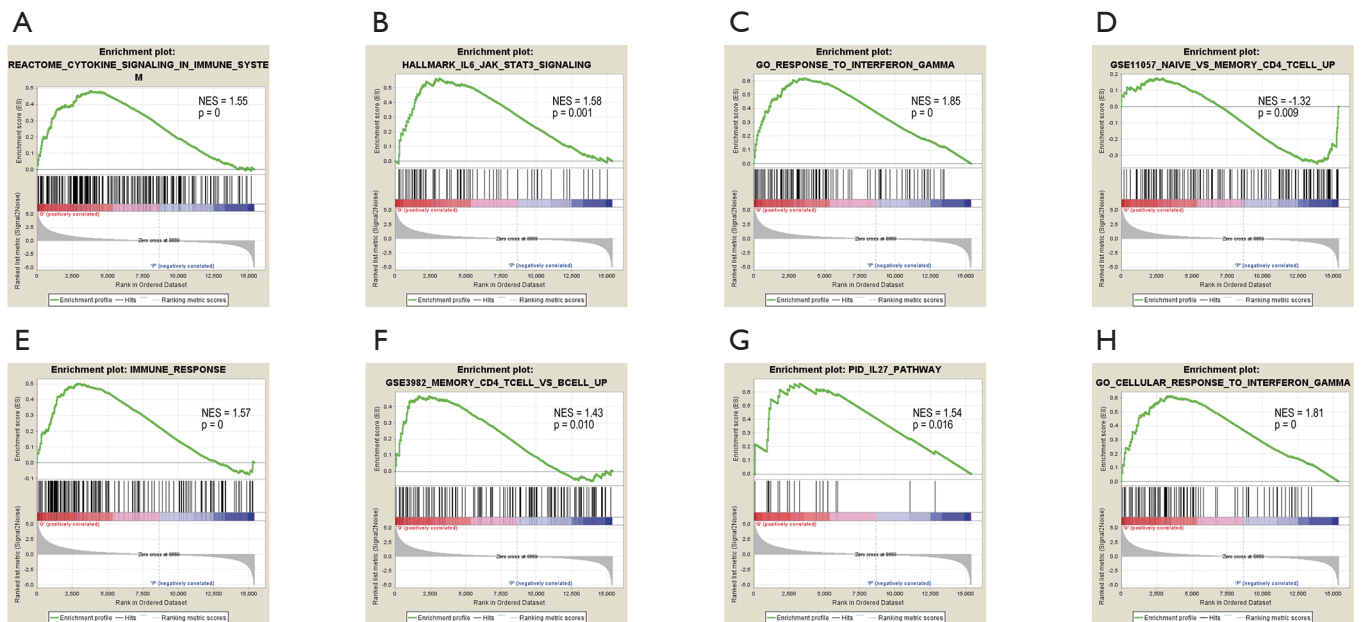


Figure 3 Gene set enrichment analysis using GSE79953 dataset revealed that gemcitabine resistance to PC strongly correlated positively with gene signatures associated with immune related and memory CD4⁺ T cell-related pathways. NES, normalized enrichment score.

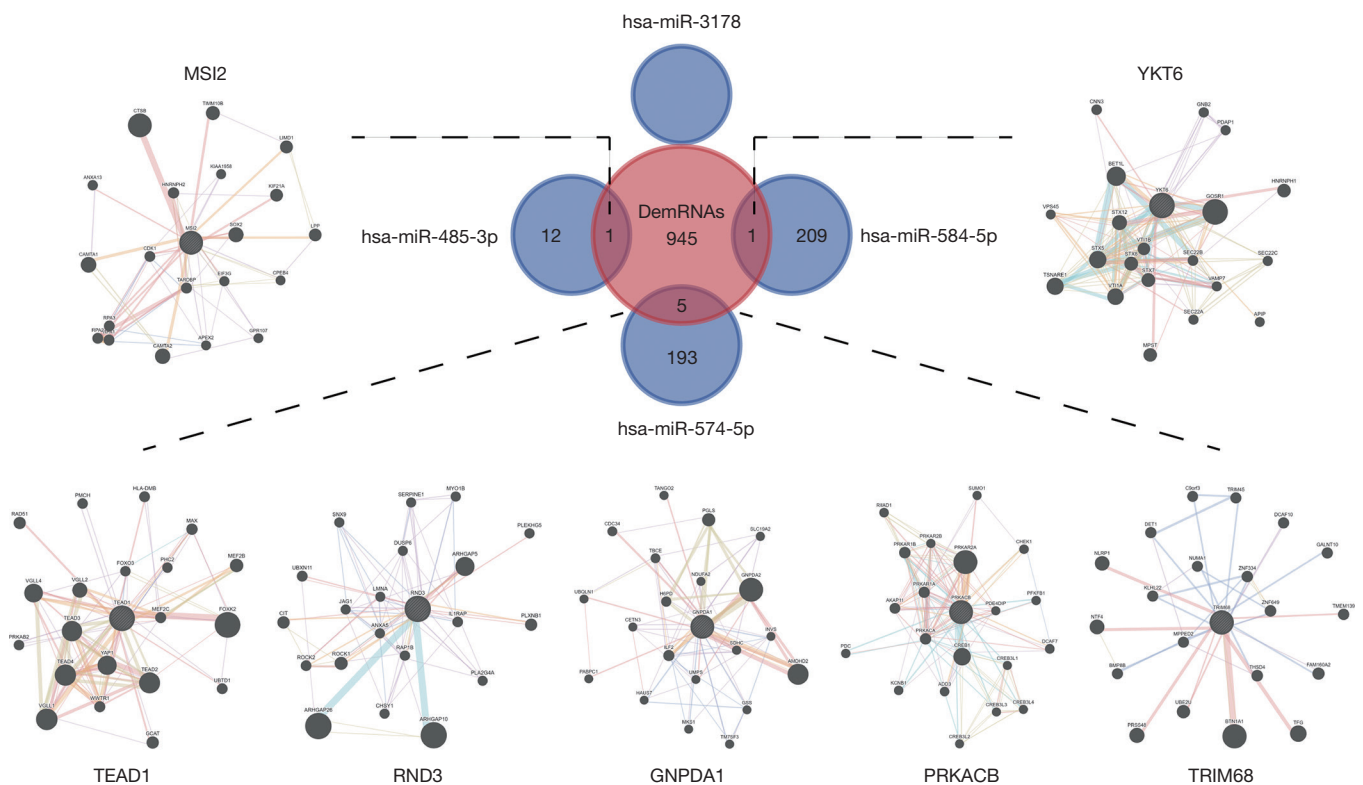


Figure 4 The targeted genes of four DE miRNAs and the PPI network of these targeted genes. These targeted genes predicted by miRDB, microT, and Targetscan databases of four DE miRNAs were intersected with the 952 DE miRNAs, and zero, one (MSI2), five (TEAD1, RND3, GNPDA1, PRKACB, and TRIM68), one (YKT6) target mRNAs were identified by hsa-miR-3178, hsa-miR-485-3p, hsa-miR-574-5p, and hsa-miR-584-5p, respectively.

patients' immune infiltration status was analyzed using CIBERSORT using RNA-seq data (Figure 6A). Most patients exhibited heterogeneity of immune infiltration. However, some of immune cell types showed high correlation with the others (Figure 6B). Activated memory CD4⁺ T cells showed positive correlation with CD8 T cells, whereas resting memory CD4⁺ T cells negatively correlated with macrophages M0, T cells follicular helper, B cells naïve, and T cells regulatory, indicating totally different roles of activated and resting memory CD4⁺ T cells. Regrouping PAAD dataset by level of seven DE miRNAs expression, we found that the fraction of CD4⁺ memory T cells significantly changed (Figure 6C,D,E,F,G,H,I). Resting memory CD4⁺ T cells were significantly altered by MSI2, TEAD1, GNPDA1, PRKACB, TRIM68, and YKT6, whereas activated memory CD4⁺ T cells were significantly altered by RND3.

Besides, fractions of macrophages M1, NK cells resting, activated memory CD4⁺ T cells, and resting memory CD4⁺

T cells were altered in the presence of gemcitabine according to TCGA PAAD patients' RNA-seq data who took gemcitabine or not (Figure 7A). Finally, we tested whether memory CD4⁺ T cell fraction could influence prognosis of PC patients and found that effector memory CD4⁺ T cells had a great effect on prognosis, whereas central memory CD4⁺ T cells had no effect (Figure 7B).

As Figure 8 shows (16), gemcitabine resistance could alter memory CD4⁺ T cell fraction of PC patients via regulating MSI2, TEAD1, GNPDA1, RND3, PRKACB, TRIM68, and YKT6 by miRNAs. Gemcitabine therapeutic effect could lead to a better prognosis in high effector memory CD4⁺ T cell fraction patients.

Discussion

Three notable findings stand out in the present study. First, our study identified four DE miRNAs (hsa-miR-3178, hsa-miR-485-3p, hsa-miR-574-5p, and hsa-miR-584-5p)

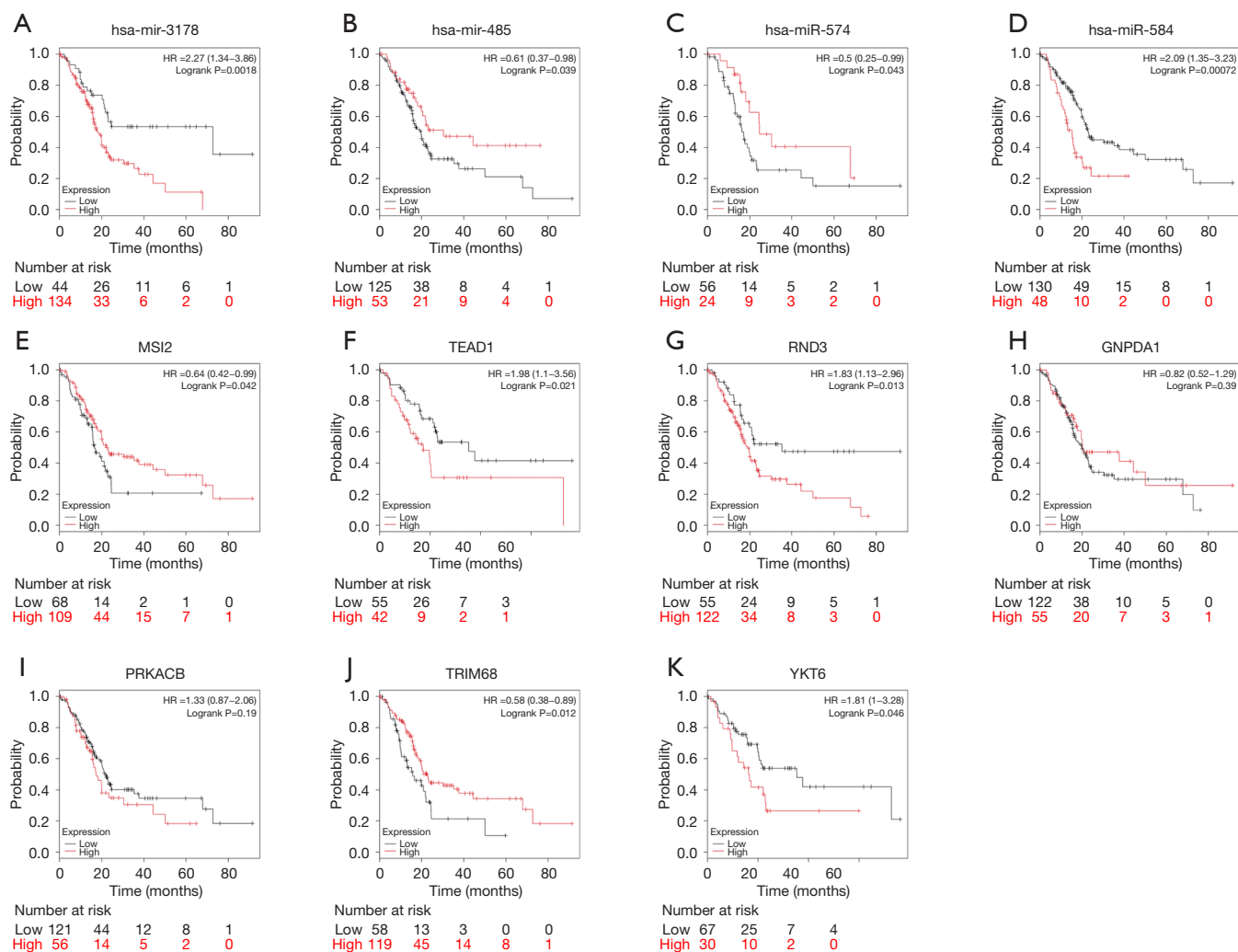


Figure 5 Kaplan-Meier survival curves of PAAD patients from Kaplan-Meier plotter for the four DE miRNAs (A-D) and seven targeted DE mRNAs (F-K). P value <0.05 was considered statistically significant. Three DE miRNAs, hsa-miR-3178 (A), hsa-miR-485-3p (B), and hsa-miR-584-5p (D), were significantly associated with the OS of PAAD patients. hsa-miR-574-5p (C) was significantly linked to OS of female PDAC patients. Three DE mRNAs, MSI2 (E), RND3 (G), and TRIM68 (J), were significantly associated with the OS of PDAC patients. Two DE mRNAs, TEAD1 (F) and YKT6 (K), were significantly linked to OS of male PAAD patients. In addition, GNPDA1 (H) and PRKACB (I) were not qualified predictors for PAAD patient survival.

and seven targeted DE mRNAs (MSI2, TEAD1, RND3, GNPDA1, PRKACB, TRIM68, and YKT6), all upregulated, which was consistent with novel hypothesis of miRNAa (4,6,17), in gemcitabine resistant PC cells compared with their parental cells. Second, we discovered that these seven targeted DE mRNAs in chemotherapy-resistant cells were associated with immune infiltration status, which was analyzed using CIBERSORT algorithm according to gene expression profiles of PAAD from TCGA

database. Finally, we found that the gemcitabine therapeutic effect was closely associated with effector memory CD4⁺ T cells in PC patients.

It is generally accepted that miRNAs inhibit translation by mRNA degradation or destabilization and translational repression (18). Moreover, researches over the past decade have shown that miRNAs can not only suppress but also activate the expression of target genes (4,6,17), namely, miRNAa phenomena. In the present study, we identified

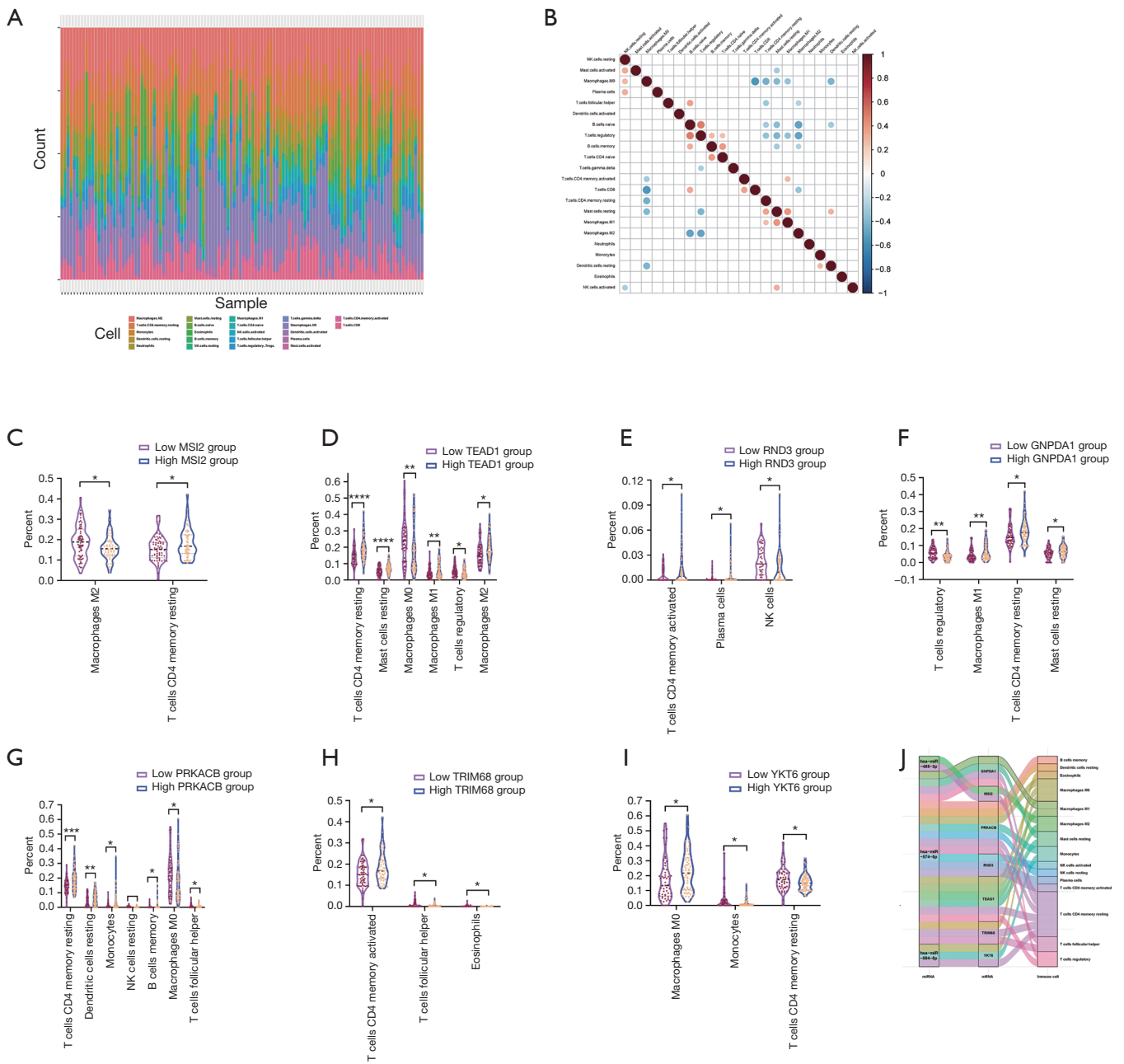


Figure 6 TCGA PAAD patients' immune cell fractions were altered by seven targeted DEmRNAs. (A) TCGA PAAD patients' immune infiltration status was analyzed by CIBERSORT using RNA-seq data; (B) some immune cell types showed high correlation with others. T cells CD4⁺ memory activated showed positive correlation with T cells CD8, whereas T cells CD4⁺ memory resting negatively correlated with macrophages M0, T cells follicular helper, B cells naïve, and T cells regulatory, indicating totally different roles of resting and activating CD4⁺ memory T cells; (C-I) regrouping PAAD dataset by level of seven DEmRNAs expression, the fraction of CD4⁺ memory T cells significantly changed. T cells CD4⁺ memory resting was significantly altered by MSI2, TEAD1, GNPDA1, PRKACB, TRIM68, and YKT6, whereas T cells CD4⁺ memory activated were significantly altered by RND3; (J) alteration of infiltrating immune cells through seven DEmRNAs targeted by hsa-miR-485-3p, hsa-miR-574-5p, and hsa-miR-584-5p.

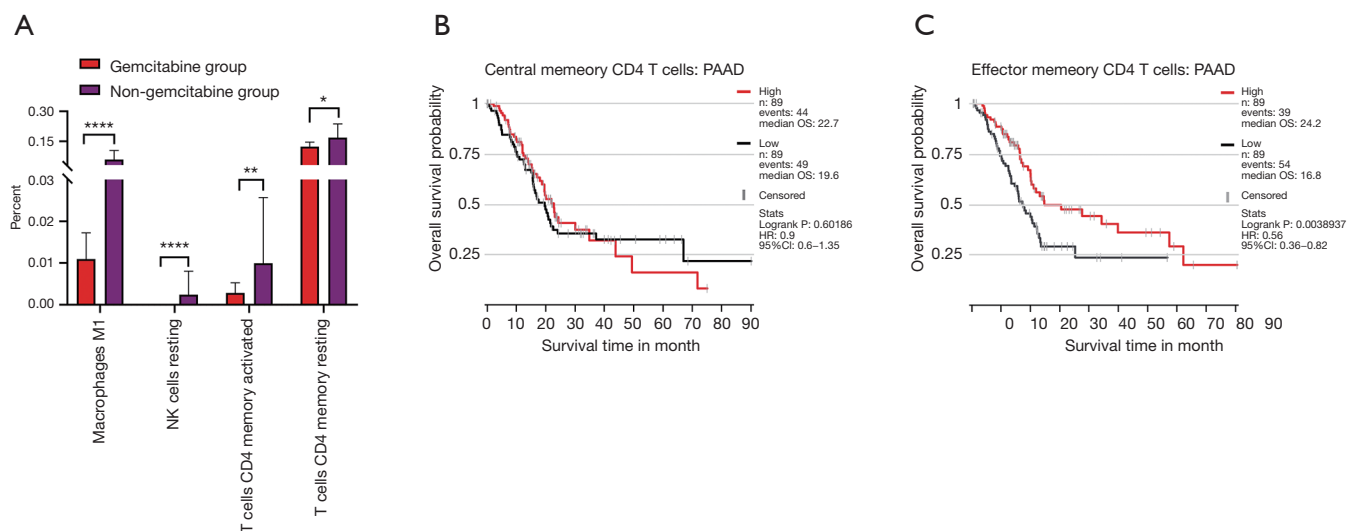


Figure 7 Gemcitabine therapeutic effect influenced fractions of immune cell fractions. (A) Fractions of macrophages M1, NK cells resting, T cells CD4⁺ memory activated, and T cells CD4⁺ memory resting were altered in the presence of gemcitabine according to TCGA PAAD patients' RNA-seq data who took gemcitabine or not; (B) central memory CD4⁺ T cells had no influence on prognosis of PC patients; (C) effector memory CD4⁺ T cells had a positive effect on prognosis of PC patients.

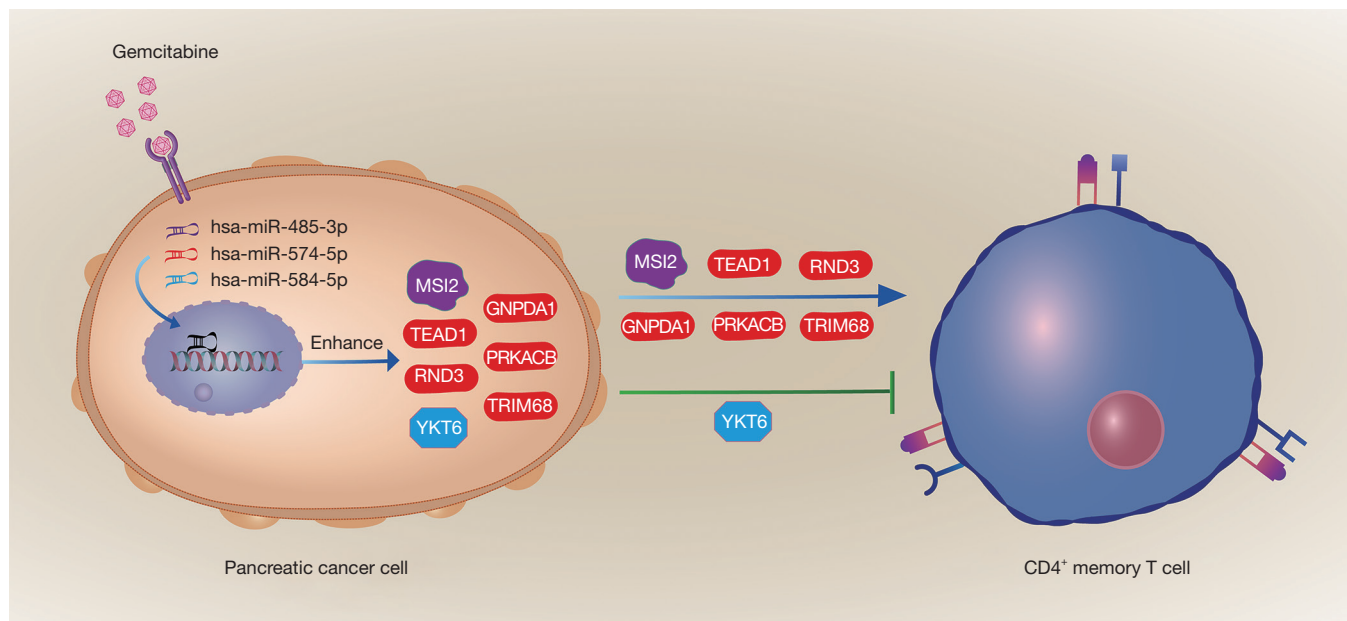


Figure 8 Schematic illustration of a CD4⁺ memory T cell immune-related miRNA regulatory network associated with PC gemcitabine resistance. In gemcitabine-resistant pancreatic cancer cells, hsa-miR-485-3p, hsa-miR-574-5p, and hsa-miR-584-5p were up-regulated, served as activators of gene expression according to miRNA hypothesis, promoted the expression of seven targeted mRNAs, including MSI2, TEAD1, GNPDA1, RND3, PRKACB, TRIM68, and YKT6. Complicatedly, MSI2, TEAD1, GNPDA1, RND3, PRKACB, and TRIM68 increased the fractions of CD4⁺ memory T cells, while YKT6 suppressed them.

four DEmiRNAs (hsa-miR-3178, hsa-miR-485-3p, hsa-miR-574-5p, and hsa-miR-584-5p) and their targeted DEmRNAs (MSI2, TEAD1, RND3, GNPDA1, PRKACB, TRIM68, and YKT6) from GEO database and found, unexpectedly, their expression tendency increased in the gemcitabine treatment group, which was consistent with miRNAa phenomena.

Several studies have testified that above gemcitabine-resistant-related DEmiRNAs and DEmRNAs are associated with chemotherapy resistance in different cancers. hsa-miR-3178 is significantly up-regulated after pre-neoadjuvant chemotherapy, and its overexpression is linked to drug resistance in breast cancer (19,20). Similarly, Lucotti *et al.* showed that hsa-miR-485-3p was highly expressed in prostate cancer cells and enabled the cancer cells to survive the treatment with fludarabine (21). MSI2 promotes the resistance to gemcitabine and cisplatin and the development of PC (22). TEAD1 is associated with chemotherapy resistance in patients with advanced bladder cancer (23). Dysregulation of RND3 is related to chemotherapy resistance in gastric cancer, nasopharyngeal carcinoma, and lung cancer cell human gastric carcinoma (24). PRKACB plays key roles in the development of multidrug resistance of Hodgkin's lymphoma (25). YKT6 is significantly up-regulated in p53-mutated breast tumors and associated with a low response rate to docetaxel (26).

Gemcitabine therapeutic effect was proven to decrease memory T cells and promote naive T cell activation (27), consistent with our results. However, the intrinsic mechanism is totally unclear. In our study, we propose a completely fresh approach that gemcitabine therapeutic effect may alter fraction of CD4⁺ memory T cell through miRNA-induced RNA activation-targeted mRNA network in PC. None of the four DEmiRNAs (hsa-miR-3178, hsa-miR-485-3p, hsa-miR-574-5p, and hsa-miR-584-5p) had been reported to have an impact on CD4⁺ memory T cell. However, hsa-miR-3178 played a role in cancer immune surveillance by regulating DUSP1, PD-L1, and MUC1 (28). miR-485 restricts influenza virus infection by priming antiviral immune response (29,30) and is associated with the immune system in Alzheimer's disease (31). miR-584 is significantly up-regulated in inflamed pulps and plays an intricate and specific role in inflammation and immunity (32). All seven DEmRNAs (MSI2, TEAD1, RND3, GNPDA1, PRKACB, TRIM68, and YKT6) did not show any direct effects on CD4⁺ memory T cell. Nevertheless, TEAD1/p65 complex could regulate innate

immune response (33). PRKACB-encoding Cβ2 regulates innate as well as adaptive immune sensitivity *in vivo* (34). TRIM68 could regulate IFN production by targeting TFG (35). In addition, DEmRNAs can have an indirect effect on CD4⁺ memory T cell through PPI network. Most of DEmiRNAs and DEmRNAs influence immune system through a variety of ways.

DEmRNAs can influence fraction of CD4⁺ memory T cells through several pathways, including "IL-6-JAK-STAT3 signaling pathway" (NES =1.58, P=0.001), "IL-27 pathway" (NES =1.54, P=0.016), and "cytokine signaling in immune system" (NES =1.55, P=0). STAT3 transcription factor signaling is critical in human memory T cell formation (36). IL-27R signaling plays a role in altering fraction of CD4⁺ memory T cells and regulating the magnitude and quality of secondary immune responses during rechallenge infections (37). These data indicate that transformation of CD4⁺ memory T cell number can be relevant with these signal pathways. Besides, gemcitabine-resistant PC cell group showed a CD4⁺ memory T cell signature while "memory CD4 T cell *vs.* B cell up" (NES =1.43, P=0.010) were significantly enriched in the gemcitabine-resistant group, and "naive *vs.* memory CD4 T cell up" (NES =-1.32, P=0.009) was well enriched in the parental group. These findings confirm alteration of CD4⁺ memory T cells and potential mechanism under gemcitabine resistance.

Finally, we found that effector memory CD4⁺ T cells and not central memory CD4⁺ T cells had a positive effect on the prognosis of PC patients, indicating a substantial role of effector memory CD4⁺ T cells. IFN-γ priming is regarded as a mechanism affecting effector memory CD4⁺ T cells (37). Our results also showed that "go response to interferon gamma" (NES =1.85, P=0) and "go cellular response to interferon gamma" (NES =1.81, P=0) were significantly enriched in the gemcitabine-resistant group. Together, these findings suggested that gemcitabine therapeutic effect altered effector memory CD4⁺ T cells by IFN-γ priming and responding to related pathways.

Memory CD4⁺ T cells has been reported to be an important part in tumor microenvironment. Memory CD4⁺ T cells has been identified in more infiltrated in colorectal cancer than normal tissue, particularly in T1-2 tumor stage (38). Central memory CD4⁺ T cells enrichment score seem higher in invasive tumors that were not ductal/lobular carcinoma in triple-negative breast cancer (39). Central memory CD4⁺ T cells seem relative hypometabolism and favorable prognosis in lung adenocarcinoma (40). These

reports suggest the clinical relevance of memory CD4⁺ T cells in various tumors and important role in tumor microenvironment. In patients with advanced PC treated with gemcitabine, a slight increase in the absolute numbers of effector memory CD4⁺ T cells is noted during treatment, suggesting the potential key role of memory CD4⁺ T cells in treatment with gemcitabine (41). Gemcitabine treatment is regard to promote immunosuppressive microenvironment in PC (42-44). However, no researches about memory CD4⁺ T cells alteration in gemcitabine resistant PC patients and their clinical relevance are reported, which has been discussed in our study.

Conclusions

Our study provides a splendid insight on gemcitabine resistance and association between chemotherapy resistance and tumor immune infiltration status in PC. However, further experiments are required, as we did not completely prove our conclusion.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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