

Development of a 12-biomarkers-based prognostic model for pancreatic cancer using multi-omics integrated analysis

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Pancreatic cancer is one of the most malignant tumors of the digestive system, with insidious, rapid onset and high mortality. The 5-year survival rate is only 10%. Therefore, in-depth exploration of the potential mechanism affecting the prognosis of pancreatic cancer, and search for biomarkers that can effectively predict the prognosis of pancreatic cancer are of practical clinical importance. The mRNA sequencing data, miRNA sequencing data, methylation data and SNP data of pancreatic cancer patients available in The Cancer Genome Atlas (TCGA) were used for analysis to identify biomarkers that significantly affect the prognosis for the patients. Finally, a prognostic prediction model was developed using principal component analysis (PCA) method. The genes that significantly affected the prognosis of pancreatic cancer were as follows: 5 DmiRNAs (*hsa-mir-1179*, *hsa-mir-1224*, *hsa-mir-1251*, *hsa-mir-129-1* and *hsa-mir-129-2*), 6 DmRNAs&DMs&MethyCor database entries (*MAPK8IP2*, *CPE*, *DPP6*, *MSI1*, *IL20RB* and *S100A2*), and *FMN2* gene from differential expressed mRNAs and differential single-nucleotide polymorphism (DmRNAs&DSNPs) database. Prognostic index (PI) = $\sum w_i x_i - 0.717716$. A patient was predicted as high/low risk if the PI was larger/smaller than 0.034045. Our study resulted in a comprehensive prognostic model for pancreatic cancer patients based on multi-omics analysis, which could offer better guidance for the clinical management of patients with early-stage pancreatic cancer.

Key words: pancreatic cancer, TCGA, survival analysis, functional enrichment analysis, PPI network, prognostic model

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Abbreviations: AUC, area under curve; CCG, Center for Cancer Genomics; GO, gene ontology; HR, hazard ratio; KEGG, Kyoto encyclopedia gene and genome; PCA principle, component analysis; PI, prognostic index; ROC, receiver operating characteristic; SNP, single-nucleotide polymorphism; TCGA, The Cancer Genome Atlas

INTRODUCTION

Pancreatic cancer is one of the most malignant digestive system tumors with insidious and rapid onset and high mortality. Due to the lack of specific clinical manifestations and clinical biomarkers at the early stage, most pancreatic cancer patients are diagnosed at an advanced stage, accompanied by distal metastasis and extensive local invasion, and the 5-year survival rate is only 10% (Siegel & Miller, 2018). Therefore, in-depth exploration of the potential disease mechanisms and identification of biomarkers that can effectively

predict the prognosis of pancreatic cancer is of practical clinical significance.

Numerous research proved that the biological behavior of pancreatic cancer is complex and involves various mechanisms, such as epigenetics, mutations, non-coding RNA regulation etc. Increasing evidence shows that methylation plays an important role in the tumorigenesis of pancreatic cancer. Arginine methylation of *MDH1* inhibits the malignant progression of pancreatic cancer by inhibiting glutamate metabolism (Wang *et al.*, 2016). Moreover, one study reported that the MLL1-H3K4me3 pathway mediates pancreatic cancer immune evasion by regulating PD-L1 expression (Minassian *et al.*, 2017). It is well-known that mutations of KRAS and P53 play an important role in the tumorigenesis of pancreatic cancer. In addition, mutation of PRSS1 was found to be involved in the malignancy of pancreatic cancer (Yi *et al.*, 2016). The crucial role of miRNA in the tumorigenesis of pancreatic cancer has been gradually discovered, as a result of the continuous development of sequencing technology. The *mir-135b-5p* promotes migration and invasion of pancreatic cancer cells by targeting *NR3C2* (Zhang *et al.*, 2017), and *mir-138-5p* inhibits autophagy of pancreatic cancer cells by regulating *SIRT1* (Tian *et al.* 2017).

Therefore, it is a relatively limited approach to search for biomarkers that affect the prognosis of pancreatic cancer based on single-omics. In this study, multiple sets of data (mRNA, miRNA, methylation and single-nucleotide polymorphism (SNP)) for pancreatic cancer available in The Cancer Genome Atlas (TCGA) were downloaded for integrated analysis to identify more solid biomarkers.

MATERIALS AND METHODS

Patients' datasets. Center for Cancer Genomics (CCG) collected tumor tissues and normal tissues (frozen or paraffin-embedded) from patients who choose to participate. Raw data were obtained by high-throughput sequencing and normalized, with the following analysis of molecular and pathology data. Multi-omics data of pancreatic cancer were downloaded from TCGA database (<https://www.cancer.gov/>) and included 183 cases of mRNA data (RNASeq V2), 182 cases of miRNA data (IlluminaHiSeq miRNAseq), 195 cases of Illumina Human Methylation450 BeadArray, and 170 cases of SNP data. Samples were selected for the study according to the criteria: 1) pancreatic cancer patients were pathologically diagnosed, 2) patients records contained all four kinds of data and 3) the follow-up data were complete. Finally, 150 patients were selected for our study. All data analyzed in this study was obtained from pancreatic adenocarcinoma tissues. The median of 150 patients' overall

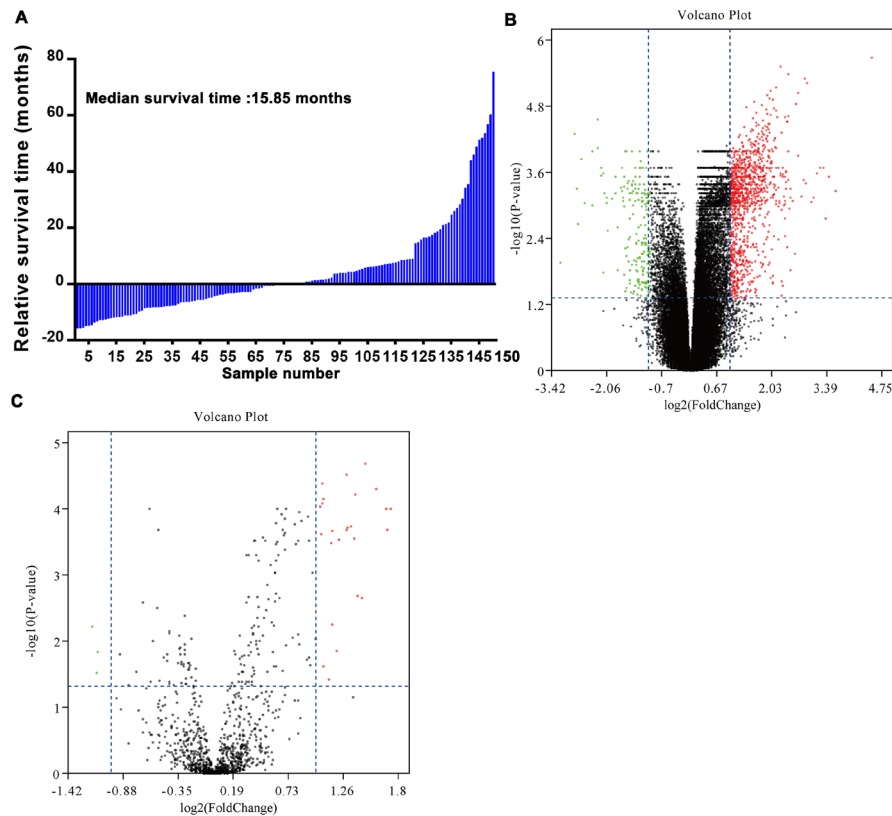


Figure 1. Differential expression analysis of mRNA and miRNA data.

(A) Patients were divided into L/S cohort according to survival time (median survival time: 15.85 months). The volcano plots of DmRNAs (B) and DmiRNAs (C).

survival (OS) time was 15.81 months. The patients were divided into L/S cohorts with long/short survival time, according to the median OS, and each cohort contained 75 samples. We also analyzed the correlation between the survival time and additional radiation, pharmaceutical therapy and histological type using the chi-square test. To further illustrate the underlying mechanism affecting the prognosis of pancreatic cancer patients we compared the multi-omics differences between the two cohorts.

Data processing. Differential mRNA and miRNA expression was analyzed using DESeq2 (Anders & Huber, 2010) function ($P < 0.05$, $|\log_{2}FC| > 1$). Each probe value for the methylation data was expressed as the β value ($\beta = U / (M + U + 1)$), where M is the methylated probe signal strength and U is the unmethylated probe signal value. The limma package (Ritchie *et al.* 2015) was used for differential expression analysis ($P < 0.05$, $|\log_{2}FC| > 1$), and MethylMix package (Gevaert 2015) was used to analyze the correlation between gene methylation level and mRNA expression value (Pearson correlation coefficient test, $R > 0.5$, $P < 0.05$). Chi-square test ($P < 0.05$) was used to test the significance of differences in the frequency of gene mutation between the L/S cohorts.

Functional enrichment analysis. We used Database for Annotation, Visualization and Integrated Discovery (DAVID) website (<https://david.ncifcrf.gov/>; Reczko *et al.*, 2012; Paraskevopoulou *et al.*, 2013) for gene ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) for pathway enrichment analysis ($P < 0.05$).

Protein-protein interaction network construction. The mRNA interaction data came from the STRING database (<https://string-db.org/>; Szklarczyk *et al.*, 2019) and the mRNA-miRNA interaction data was derived

from DIANA tools (Reczko *et al.*, 2012; Paraskevopoulou *et al.*, 2013). This interaction information was imported into Cytoscape software (Shannon *et al.*, 2003) to build an integrated interaction network.

Kaplan-Meier survival analysis and prognostic model construction. Kaplan-Meier survival analysis was performed for all significantly differentially expressed mRNA/miRNA to further screen for biomarkers which significantly correlated with OS of pancreatic cancer patients. Principal component analysis (PCA) method was used to construct the prediction model based on 12 genes, and the receiver operating characteristic (ROC) curve and area under the curve (AUC) were used to test the performance of the classifier.

RESULTS

Differential expression analysis of mRNA and miRNA

Patients, 150, were divided into L/S cohorts according to the median survival time which was 15.85 months. DESeq2 package was used to identify the differentially expressed mRNA and miRNA between L and S cohort ($P < 0.05$, $|\log_{2}FC| > 1$). 1,255 significantly differentially expressed mRNAs (DEmRNAs) and 33 miRNAs (DEmiRNAs) were found (Fig. 1).

Methylation analysis

The limma package was used to identify significantly differentially methylated genes in the methylation data, and the results revealed that there were 9,601 significantly differentially methylated genes (DMethGs) be-

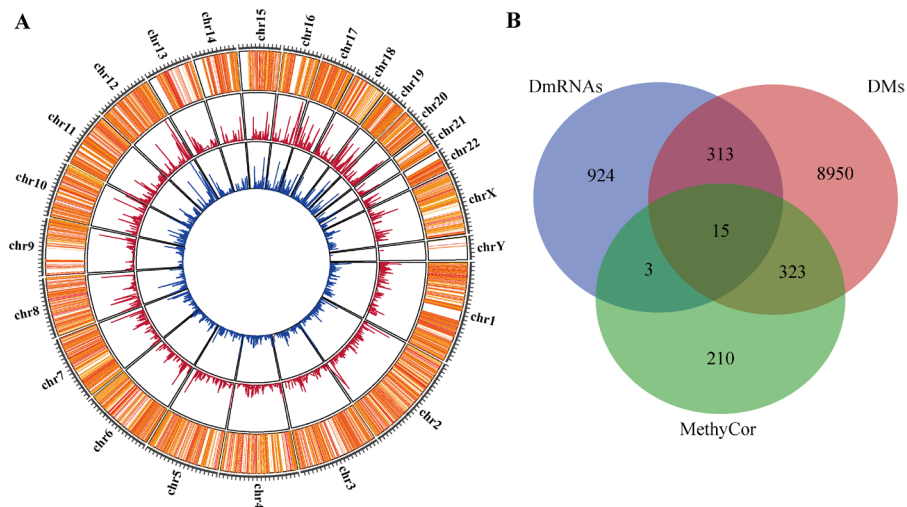


Figure 2. Methylation analysis.

(A) Differentially methylated genes between L/S cohorts. The outmost circle represents the heatmap of DMs. The middle/inner circle represents the median β value of L/S cohorts. (B) Venn plot of DmRNAs, DMs, and MethyCor genes.

tween L and S cohort ($P < 0.05$, $|\log_{2}FC| > 1$) (Fig. 2A). Further, MethylMix package was used to analyze the relationship between methylation data and mRNA expression data, and revealed that the methylation degree of 551 genes was significantly correlated with their mRNA expression level (MethyCor) ($P < 0.05$, $|\tau|$

> 0.5 ; Fig. S1). Based on the intersection of DEmRNAs, DMethGs and MethyCor datasets, we identified 15 common genes (Fig. 2B) (Table 1). Therefore, we could infer that the significantly differential mRNA expression of these 15 genes is caused by their significantly differential methylation.

Table 1. 15 genes which significantly differentially expressed and methylated between L/S cohorts, moreover, the mRNA expression level significantly correlated with methylation level.

Symbol	DmRNAs		DMs		Cor	
	log2FC	p_val	log2FC	p_val	R	p_val
AGBL4	1.682	0.000048	-1.345	0.0399131	-0.507	0
CALML3	1.6327	0.0009	-1.382	0.0081808	-0.607	0
CPE	1.4863	0.000071	-1.676	0.0007348	-0.567	0
DPP6	1.1228	0.0002	-1.448	0.0245278	-0.557	0
IL20RB	-1.5246	0.00036	1.447	0.0103424	-0.592	0
MAPK8IP2	1.4143	0.000127	-1.259	0.0393195	-0.507	0
MSI1	1.5301	0.00004	-1.386	0.0196918	-0.66	0
PCSK1	1.7477	0.000218	-1.455	0.0430081	-0.573	0
PIWIL1	1.0241	0.009	-1.405	0.0404999	-0.609	0
PODXL2	1.0138	0.000354	-1.261	0.0052812	-0.517	0
RAB26	1.2709	0.000306	-1.256	0.0049592	-0.551	0
S100A2	-1.1196	0.0034	1.18	0.0274155	-0.606	0
SOX15	-1.224	0.000158	1.194	0.0031299	-0.641	0
TDRD1	1.467	0.000732	-1.436	0.0100102	-0.633	0
TFF3	1.1042	0.0013	-1.273	0.0055974	-0.72	0

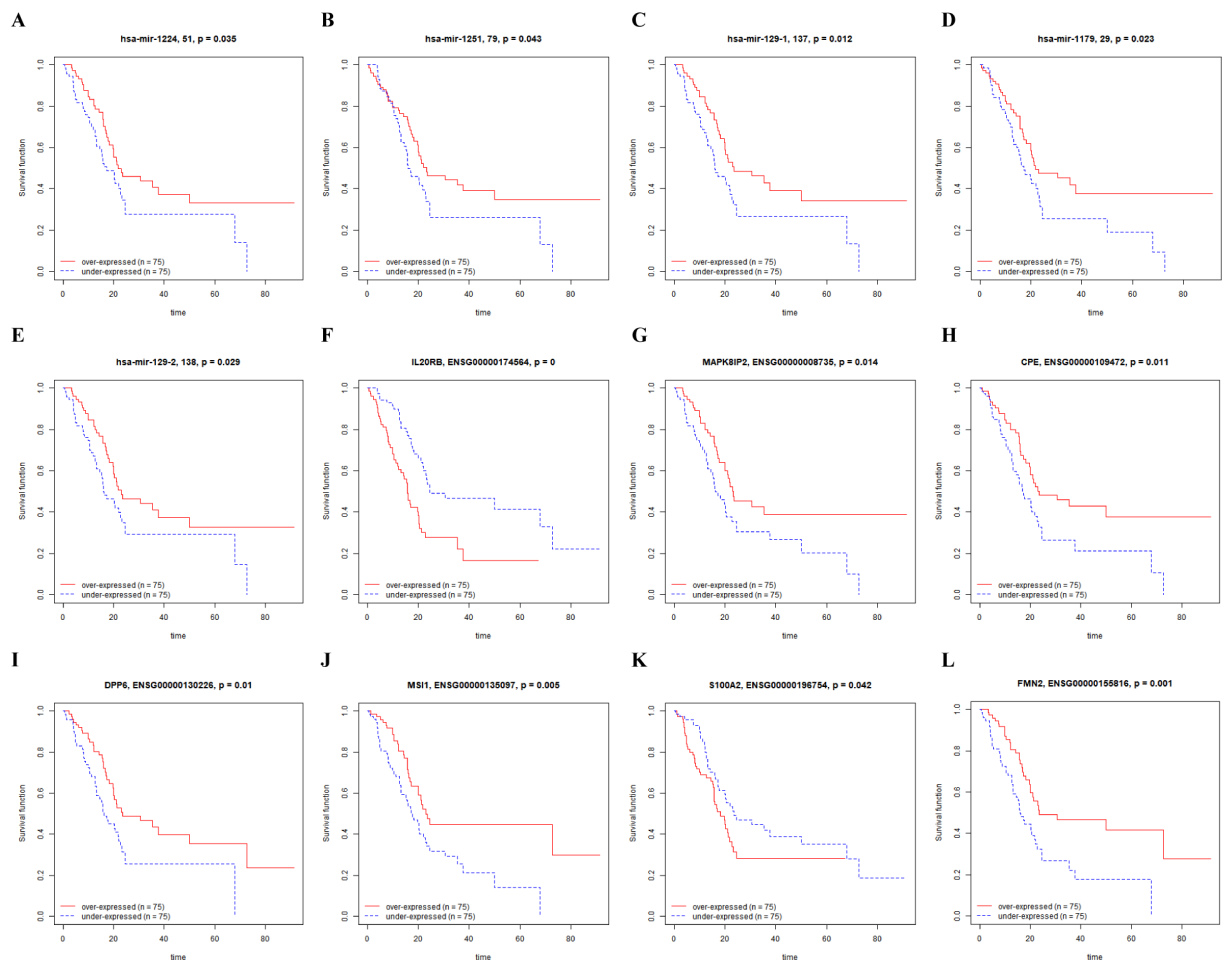


Figure 4. Survival plots of the 12 biomarkers which are strongly associated with the OS of pancreatic cancer patients. (A–E) Survival plots of 5 DmiRNAs. (F–K) Survival plots of 6 genes from DmRNAs & DMs & MethCor dataset. (L) Survival plot of FMN2 in DmRNAs & DSNPs dataset. All biomarkers plotted with p -value for Kaplan–Meier plot (log-rank test).

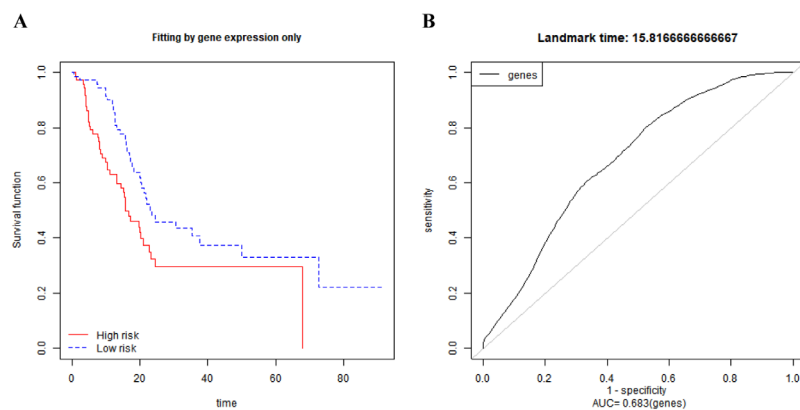


Figure 5. Survival plot for the prognostic model. (A) Survival plot for high- vs low-risk cohort. (B) ROC plot for the prognostic model.

value). A patient was predicted as high/low risk if the PI was larger/smaller than 0.034045.

High AUC for the prognostic model (AUC=0.683) and low P -value with the high hazard ratio (HR) in fitted Cox proportional hazards model ($P=0.035$) suggest that this can be a better prognostic model for pancreatic cancer patients (Fig. 5).

Functional enrichment analysis

The 0 target genes of 5 miRNAs that significantly affected the prognosis of pancreatic cancer patients were used for querying DIANA tools (Reczko *et al.*, 2012; Paraskevopoulou *et al.*, 2013). 1,437 target genes were identified with combined score >0.7 as the criterion, and 46 of them were DmRNAs. Then, the interaction rela-

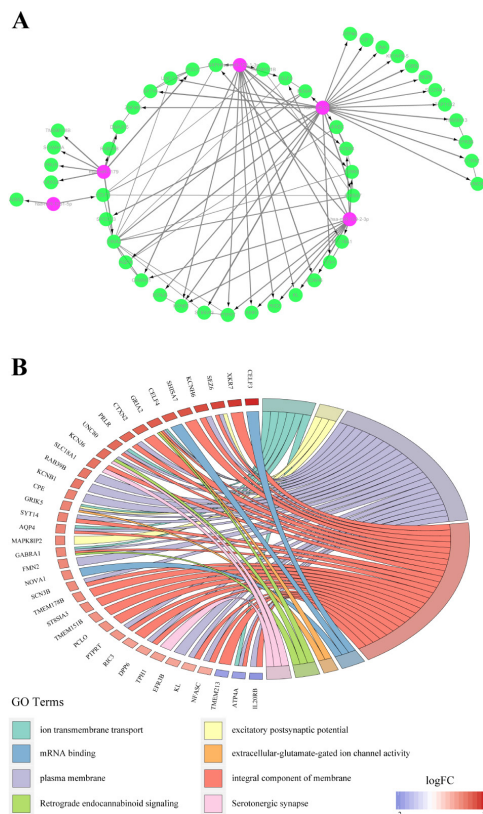


Figure 6. PPI network and functional enrichment plot.

(A) PPI network of 5 DmiRNAs and their target genes. The red dots represent miRs and green dots represent the target genes. The thickness of interaction lines represents the combined score. (B) The circular plot represents the functional enrichment of 46 target genes and 7 biomarkers.

relationship of these 46 target genes was obtained using the STRING database (Szklarczyk *et al.*, 2019). The results from DIANA and STRING were integrated to construct the PPI network graph using Cytoscape software (Shannon *et al.*, 2003), providing a better visual understanding of the relationship between these miRs and target genes (Fig. 6A).

To further understand the function of the 12 biomarkers, GO function and KEGG pathway enrichment analysis was performed on 53 genes (46 target genes and 6 DmRNAs & DMs & MethyCor dataset entries and 1 entry from DmRNAs & DSNPs dataset) using DAVID website. The enrichment results were as follow: biologi-

cal process (BP) contained ion transmembrane transport and excitatory postsynaptic potential; cellular component (CC) contained plasma membrane and integral component of membrane; molecular function (MF) contained mRNA binding and extracellular-glutamate-gated ion channel activity. The most significantly enriched KEGG pathways were Retrograde (Fig. 6B) endocannabinoid signaling and Serotonergic synapse.

Patients' treatment

150 patients selected for our study included 145 pancreas adenocarcinoma ductal and 5 pancreas colloid carcinoma. Patients had undergone additional radiation, pharmaceutical therapy or not, and the survival time of the patients was not correlated to the treatment (Table 4).

DISCUSSION

Pancreatic cancer is one of the most malignant tumors that seriously threaten human health. Despite the continuous medical progress, and the invention of new treatment methods and agents, the 5-year survival rate of pancreatic cancer patients is still inferior. Therefore, there is an urgent need to find new biomarkers that can effectively predict the prognosis and overall survival for pancreatic cancer patients.

Previous studies identified many genes involved in pancreatic cancer tumorigenesis through various mechanisms and significantly affecting the prognosis of pancreatic cancer. *ZNF281* promotes growth and invasion of pancreatic cancer cells by activating Wnt/ β -catenin signaling (Qian *et al.*, 2017). *SNX6* predicts poor prognosis and contributes to the metastasis of pancreatic cancer cells via activating epithelial-mesenchymal transition (Hu *et al.*, 2018). It was reported that methylation of *SULT1E1*, *IGF2BP3* and *MAP4K4* is significantly correlated with prognosis of pancreatic cancer patients (Chen *et al.*, 2019). Hideyuki Hayashi and others (Hayashi *et al.*, 2017) found that the number of mutations in *KRAS*, *CDKN2A*, *TP53*, and *SMAD4* can be used to predict the prognosis of pancreatic cancer (Hayashi *et al.*, 2017). Furthermore, their study revealed that miRNA is also significantly correlated with the prognosis of pancreatic cancer (Shi *et al.*, 2018).

However, all the above traditional studies based on single omics failed to substantially benefit and significantly improve OS for pancreatic cancer patients. Therefore, our study was different from the traditional approach, being an integrated multi-omics analysis and re-

Table 4. The correlation between relative overall survival and patient treatments was analyzed using chi-square test.

Variable	n	Relative overall survival		χ^2	p value
		Long	Short		
Additional pharmaceutical therapy					
Yes	76	36	40	0.43	0.62
No	74	39	35		
Additional radiation therapy					
Yes	141	71	70	0.12	1.00
No	9	4	5		
Histological type					
Pancreas adenocarcinoma ductal	145	72	73	0.21	1.00
Pancreas colloid carcinoma	5	3	2		

sulted in identifying 12 effective biomarkers and building a prognostic model based on these 12 biomarkers.

Mitogen-Activated Protein Kinase 8 Interacting Protein 2 (MAPK8IP2), also known as JNK-Interacting Protein 2 (JIP2), is thought to be involved in the regulation of the c-Jun amino-terminal kinase (JNK) signaling pathway (Yasuda *et al.*, 1999). JNK signaling pathway plays an important role in tumorigenesis (Ma *et al.*, 2017).

Liu and others (Liu *et al.*, 2014) reported that Carboxypeptidase E (CPE), a member of the M14 family of metalloproteases, can promote tumor proliferation in pancreatic cancer cells and mouse models (Liu *et al.*, 2014), and the mRNA expression value of CPE in pancreatic cancer patients was higher than that in adjacent tissues according to TCGA database. Interestingly, our study found that higher CPE level predicts longer survival time of pancreatic cancer patients. Therefore, we speculate that the function of CPE in pancreatic cancer patients is not only pro-cancer or anti-cancer, and its complex mechanism of action needs further study to be fully elucidated. Classically, Dipeptidyl Peptidase Like 6 (DPP6) is described as a membrane protein thought to be involved in voltage-gated potassium channels function. It was found that DPP6 also participates in malignancy of esophageal adenocarcinoma (Xi & Zhang, 2017). MSI1 (Musashi RNA Binding Protein 1) was long used as a marker of stem cells (Okano *et al.*, 2002), and was reported to be involved in tumorigenesis of several cancers (Kharas & Lengner, 2017). The function of Interleukin 20 Receptor Subunit Beta (IL20RB) is to form the heterodimeric receptor for Interleukin 20 (IL20) with Interleukin 20 Receptor Subunit Alpha (IL20RA). Besides, it was reported to be involved in the JAK-STAT pathway (Blumberg *et al.*, 2001), which plays an important role in various tumors (Waldmann & Chen, 2017; Tiacci *et al.*, 2018). Syed Haider *et al.* reported that IL20RB is correlated with shorter survival of pancreatic patients, which was highly consistent with our findings, indicating that IL20RB is an indicator of pancreatic cancer prognosis (Haider *et al.*, 2014). S100 calcium-binding protein A2 (S100A2) is a member of the S100 family of proteins containing 2 EF-hand calcium-binding motifs. Many studies suggested that S100A2 plays an oncogenic role in pancreatic cancer (Ohuchida *et al.*, 2007; Bachet *et al.*, 2013; Ji *et al.*, 2014). Furthermore, our study confirmed these earlier results and used it as an essential element in the prognostic model. Formin-2 (FMN2) is a member of the formin family, and it is mainly involved in the organization of actin cytoskeleton (Peng & Liou, 2012). Studies reported that FMN2 is involved in the malignancy of various tumors (Jin *et al.*, 2017; Li *et al.*, 2018).

The discovery of miRNA opened a brand-new area in the bioscience research. More and more functions of miRs have been discovered, especially in the various biological behaviors of tumors. miRNAs are detectable in biofluids including urine, cerebrospinal fluid and blood, which make them highly available and accurate biomarkers for the prediction of prognosis of cancer patients (Hayes *et al.*, 2014). In this study, therefore, miRNAs were included in the exploration of biomarkers of pancreatic cancer. The results showed that 5 miRNAs (*hsa-mir-1179*, *hsa-mir-1224*, *hsa-mir-1251*, *hsa-mir-129-1* and *hsa-mir-129-2*) significantly affected the prognosis of pancreatic cancer patients. The functional enrichment analysis showed that target genes were significantly enriched in ion transport and mRNA binding function.

With an increasing number of neoplasm research, ion transport was found to play an important role at every

stage of the cancer disease. Moreover, ion transport is involved in every step of tumorigenesis, proliferation, invasion and metastasis (Djamgoz *et al.*, 2014), and even plays an indispensable role in the process of destroying the cancer cells by the immune cells (Panyi *et al.*, 2014).

Furthermore, we found that the target genes of these miRs are significantly enriched in mRNA binding, which means these target genes are heavily involved in each step of mRNA metabolism. mRNA metabolism is in turn vital for tumorigenesis.

In summary, our study found that gene expression patterns were significantly discriminated in pancreatic cancer patients with different survival time. We used multi-omics analysis to identify 7 mRNAs and 5 miRNAs as biomarkers, all of which were significantly correlated with the prognosis of pancreatic cancer patients. In addition, the previous studies confirmed that these biomarkers are involved in various malignant processes, such as tumorigenesis, proliferation or metastasis through different mechanisms, but their specific mechanism of action in pancreatic cancer still needs to be elucidated. Finally, the PCA method was used to construct the prognostic prediction model based on the 12 biomarkers. This model may provide guidance for the clinical management of patients with early-stage pancreatic cancer.

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Competing interests

The authors state that there are no conflicts of interest to disclose.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HPL conceived and designed the experiments; YHJ analyzed and interpreted the results of the experiments, MYS and YZ performed the experiments.

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