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Regular paper

Identification of significant prognostic risk markers for pancreatic ductal adenocarcinoma: a bioinformatic analysis

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Objective: This study aimed to identify novel prognostic biomarkers of pancreatic ductal adenocarcinoma (PDAC) using bioinformatics analyzes. Methods: Clinical information, microRNAs (miRNAs), and genes expression profile data from PDAC cases were downloaded from the Cancer Genome Atlas (TCGA) database. The potential prognostic risk miRNAs and genes were screened using the Elastic Net Cox proportional risk regression hazards (EN-COX) model. The receiver operating characteristic (ROC) curve and the Kaplan-Meier (KM) curve were used to identify miRNAs and genes of significant prognostic risk. Furthermore, significant prognostic risk miRNAs were functional enrichment analyses based on their target genes. Furthermore, the survival analyzes of the hub genes were validated through OncoLnc. Results: Complete clinical records and expression data of 797 miRNAs and 19969 genes from 137 PDAC cases were obtained, of which 59 potential prognostic risk factors, including 54 genes and 5 miRNAs, were selected by EN-COX analyzes. A total of 17 significant prognostic risk markers were identified (all $P<0.05$), including 16 genes and 1 miRNA (miRNA-125a). The miRNA-125a target genes were found in the MiRWalk database and the function enrichment analyzes were performed in the the DAVID website. Furthermore, according to data from the Oncomine and Human Protein Atlas (HPA) databases, the mRNA and protein level of frizzled class receptor 8 (FZD8) were overexpressed in pancreatic cancer tissues compared to the corresponding noncancer normal tissues ($P<0.001$). However, both glutathione S-transferase mu 4 (GSTM4) and inducible T cell costimulator ligand (ICOSLG) were negatively regulated in tissues of pancreatic cancer tissues ($P<0.001$). Finally, survival analysis was used to validate these factors by the OncoLnc database, and the results revealed that overexpression of ICOSLG was associated with a better prognosis ($P=0.025$). Conclusions: This study showed that the expression levels of FZD8, GSTM4 and ICOSLG were significantly different between PDAC and non-tumor tissues, especially ICOSLG, which could be a prognostic indicator and therapeutic target for PDAC.

Keywords: Pancreatic ductal adenocarcinoma, Bioinformatics, Prognostic markers, Cancer Genome Atlas

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Abbreviations: BRCA, breast cancer susceptibility gene ;KRAS, Kirsten rat sarcoma; PAAD, pancreatic adenocarcinoma; PDAC, pancreatic ductal adenocarcinoma; SMAD4, decapentaplegic homolog 4

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC), which represents more than 90% of pancreatic cancer and is the most common pathological type of pancreatic adenocarcinoma (PAAD), is one of the most concerned malignancies due to poor diagnosis and limited treatment (Parrasia *et al.*, 2021). According to the statistics of America, 5700 cases were newly diagnosed with pancreatic cancer in 2020 and 447050 cases died from it (Siegel *et al.*, 2020). Lacking obvious early symptom and specific tumor screening, PDAC is not diagnosed until the disease is at an advanced stage presenting local nerve and vascular invasion and metastasis to distant sites (Yang *et al.*, 2021). In most patients, PAAD is not resectable after diagnosis. According to the National Institutes of Health statistics (NIH), the 5-year survival rate for patients with PAAD is only 10.8% based on cases of PAAD-associated mortality between 2011 and 2017 (https://seer.cancer.gov/statfacts/html/pancreas.html). Therefore, the exploration of effective prognostic markers may provide new insights into the predictive outcome of PDAC.

It is well known that most malignancies are generated by random accumulation of massive genetic and epigenetic aberrations, and the prognostic and therapeutic implications associated with these aberrations are becoming more and more crucial (Hatziapostolou and Iliopoulos, 2011). Many previous studies have confirmed the involvement of abnormal gene expression in PDAC, and alteration of these gene expressions can act as indicators for diagnosis, treatment, and prognosis evaluation (Peng *et al.*, 2019). For example, the mutation of the viral oncogene homolog gene of Kirsten rat sarcoma (KRAS) occurred in more than 90% of cases of PDAC (Jonckheere *et al.*, 2017). Some studies revealed that the KRAS gene mutation was significantly associated with tumor stage, liver metastasis, and median survival time, and therefore it may be used for the detection of PDAC (Waters & Der, 2018, Fan *et al.*, 2018). Pathogenic mutations in the breast cancer susceptibility gene (BRCA) were identified in 4.6% of a large cohort of clinic patients (Holter *et al.*, 2015). Furthermore, mothers against decapentaplegic homolog 4 (SMAD4) deficiency also accelerated the development of PDAC, which works to block the progression of KRAS-initiated neoplasms (Yokose *et al.*, 2020). According to a research, 78 % of pancreatic tumors have abnormalities at the

G1/S checkpoint of the cell cycle (TP53, CNKN2A, TP53BP2 mutations) (Bailey *et al.*, 2016). Chung *et al.* found an obvious correlation between the extracellular high mobility group 1 (HMGB1) and the tumor stage and prognosis of PDAC, therefore HMGB1 may serve as a marker in the diagnosis and evaluation of the prognosis (Chung *et al.*, 2012). Although several prognostic risk factors have been identified, sensitivity and specificity are not satisfactory. It is still urgent to identify the best prognostic biomarkers of PDAC.

Recently, on the basis of the analysis of large integrated data and bioinformatics, key genes related to tumor development and prognosis could be identified with the widespread popularity of gene chips and the rapid development of high-throughput sequencing technology. Potential key genes related to the pathogenesis of PDAC were selected by bioinformatics meta-analysis based on the Cancer Genome Atlas (TCGA) database (Ma *et al.*, 2019). In addition, there are more and more online tools favored by researchers because of the powerful function, like easy-to-use, no need to install, to analyze the specific disease with someone else's official server.

This study aimed to investigate and identify significant prognostic risk markers for the diagnosis and treatment of PDAC by bioinformatic analyzes.

MATERIALS AND METHODS

Data sources and preprocessing

In the present study, clinical data from 197 PAAD cases were downloaded from the TCGA database (https://cancergenome.nih.gov), which included clinical characteristics, genomic characterization, and high-throughput sequence data from cancer patients. Of these cases, 150 were PDAC and the rest were other pathological types. Among the 150 PDAC cases, 2 cases were excluded for the lack of accurate data of overall survival time. Finally, there were 148 PDAC cases that contained clinical data and survival time data. In addition, 183 cases of pancreatic cancer with microRNAs (miRNA) and gene expression profile data were downloaded. After data integration, there were a total of 137 cases that contained both clinical data and genes expression profile data.

The expression values of miRNAs and genes were estimated by reads per kilobase of exon per million mapped reads (RPKM) (Wagner *et al.*, 2012). Data from different samples were normalized by median.

Identification of potential prognostic risk miRNAs and genes

Elastic net (EN) is an ideal variable selection method that is used to deal with collinearity and effectively reduce dimension. Cox proportional regression hazards analysis is a semiparametric model designed to analyze survival data. Elastic Net Cox's proportional risk regression hazards (EN-COX) presents the advantages of the above method (Wu, 2012). In this study, EN-COX was performed to screen potential prognostic risk genes and miRNAs using the glmnet package in R studio (version: R 3.63.) (Engebretsen and Bohlin, 2019). The parameter was the minimum of λ.

Identification of prognostic risk markers

The receiver operating characteristic (ROC) curve was used to evaluate classifiers in bioinformatical applications. The cases were divided into two groups according to the cut-off values. The cases with miRNAs or genes expression values below the cut-off value were ranked as low-expression groups, while the cases with expression values above the cut-off value were ranked as high-expression groups. Based on the data obtained from potential prognostic risk genes, miRNAs and the corresponding expression profile data, the ROC curves were generated using the pROC package in R studio to determine the cut-off values for grouping the cases (Robin *et al.*, 2011).

The Kaplan-Meier (KM) curve was used for univariate analysis of survival data. In this study, the KM curves were generated using the survival package in R studio (Therneau, 2012). Later, the log-rank test was used to determine whether there were significant differences between two groups ($P<0.05$ was considered a significant difference).

Function prediction of prognostic risk miRNAs

The target genes for the prognostic risk miRNAs obtained above were searched in the MiRWalk portal database (Sticht *et al.*, 2018). The regulatory networks were then constructed between selected prognostic risk miRNAs and their target genes using Cytoscape (http://www.cytoscape.org/), which is an open source software used to visualize biological networks and integrate data (Doncheva *et al.*, 2019). After that, the target genes mentioned above were performed functional enrichment analyses in DAVID Bioinformatics Resources database (Huang *et al.*, 2009). The results were plotted as bubble diagrams using the ggplot2 package in R Studio. $P<0.05$ was set as the threshold.

Expression of prognostic risk markers in human pancreatic adenocarcinoma

Prognostic risk markers were assessed at the RNA level of human pancreatic adenocarcinoma samples by comparing with paracancer tissues through the Oncomine database (www.oncomine.org) (Rhodes *et al.*, 2004), and their protein expression was detected in the HPA database (www.proteinatlas.org) (Uhlén *et al.*, 2015). $P<0.05$ was considered to be significant difference.

Survival analyses of the prognostic risk markers

OncoLnc is a tool to interactively explore survival correlations and download clinical data coupled to expression data for mRNAs, miRNAs, or long noncoding RNAs (lncRNAs). OncoLnc contains survival data for 8,647 patients from 21 cancer studies performed by the TCGA database, together with RNA-SEQ expression for mRNAs and miRNAs from TCGA, and lncRNA expression from MiTranscriptome (Anaya, 2016). By means of OncoLnc, survival difference of the prognostic risk markers was validated once more.

RESULTS

Data preprocessing

After normalizing the original TCGA data, the expression values of 797 miRNAs and 19969 genes were obtained in 137 PDAC cases.

Table 1. The result of identified prognostic risk markers

Name	Optimum cut-off value	*P*-value of the KM curve	AUC of the ROC curve
ADRB3	2.412	3.14E-02	0.725
AIPL1	0.580	1.04E-06	0.626
EMR3	4.066	1.01E-03	0.789
FAM196B	1.792	2.72E-02	0.662
FZD8	8.062	3.12E-05	0.604
GATA1	1.070	4.41E-02	0.732
GSTM4	9.103	4.72E-03	0.692
ICOSLG	8.987	3.53E-03	0.837
KRT39	1.060	3.26E-02	0.619
RHO	0.578	2.52E-03	0.606
SCN11A	2.515	9.05E-04	0.768
SLC25A44	9.510	2.81E-03	0.633
SPATA2	8.549	1.35E-02	0.674
TRIM67	3.510	3.08E-02	0.843
TTLL2	1.771	2.38E-03	0.630
ZWILCH	8.186	5.85E-03	0.640
has-mir-125a	0.183	3.23E-02	0.645

KM, Kaplan-Meier; ROC, receiver operating characteristic; AUC, the area under the curve of ROC.

Identification of potential prognostic risk genes and miRNAs

According to the parameter of λ=0.107, 59 potential prognostic risk factors, including 5 miRNAs and 54 genes, were screened through EN-COX.

Identification of prognostic risk markers

According to the threshold of P<0.05 of the KM curve and AUC>0.6 of the ROC curves, 17 prognostic risk markers including 16 genes and 1 miRNA were considered significantly correlated with the prognostic risk of PDAC patients (Table 1).

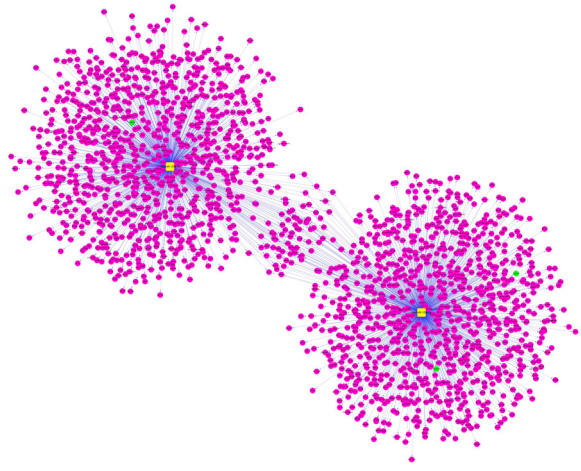

Figure 1. The regulatory network of miRNA-125a and target genes.
The yellow rectangle nodes represented miRNA-125a-3p and miRNA-125a-5p, and the circular nodes represented the target genes, while the green circular nodes represented the prognostic risk genes identified in this study.

Function prediction of prognostic risk miRNAs

miRNA-125a contained two forms: miRNA-125a-3p and miRNA-125a-5p. There were 1982 target genes for miRNA-125a recovered from the MiRWalk database, while miRNA-125a-3p had 1030 target genes, and miRNA-125a-5p had 1021 target genes. (Fig. 1). There were 69 common target genes between them. Besides, ICOSLG, SPATA2, and GSTM4 were prognostic risk genes and miRNA-125a target genes, while ICOSLG and SPATA2 belonged to miRNA-125a-3p target genes, and GSTM4 was the miRNA-125a-5p target gene. The target genes of miRNA-125a-3p were mainly enriched in the positive regulation of phosphatidylinositol 3-kinase signaling (BP), DNA-directed RNA polymerase II (CC), RNA polymerase II activity (MF) and metabolic pathways (KEGG) (Fig. 2 and Supplementary Table I~IV at https://ojs.ptbioch.edu.pl/index.php/abp/). MiRNA-125a-5p target genes were mainly enriched in phospholipid transport (BP), an integral component of the Golgi membrane (CC), protein binding (MF) and the calcium signaling pathway (KEGG) (Fig. 3 and Supplementary Table V~VIII at https://ojs.ptbioch.edu.pl/index.php/abp/).

The mRNA and protein levels of FZD8, GSTM4 and ICOSLG

In view of the Badea pancreas data set in the Oncomine database, the mRNA level of frizzled class receptor 8 (FZD8) was significantly higher in human PDAC specimens compared to paracancer specimens (P<0.001), and the para-cancer specimens (P<0.001), and inducible T-cell costimulator ligand (ICOSLG) was significantly down-regulated in pancreatic cancer tissues (P<0.001). In view of the logsdon pancreas data set, glutathione S-transferase mu 4 (GSTM4) revealed significantly lower expression in tumor tissues compared to the corresponding non-tumor tissues in mRNA level of mRNA (P<0.001).

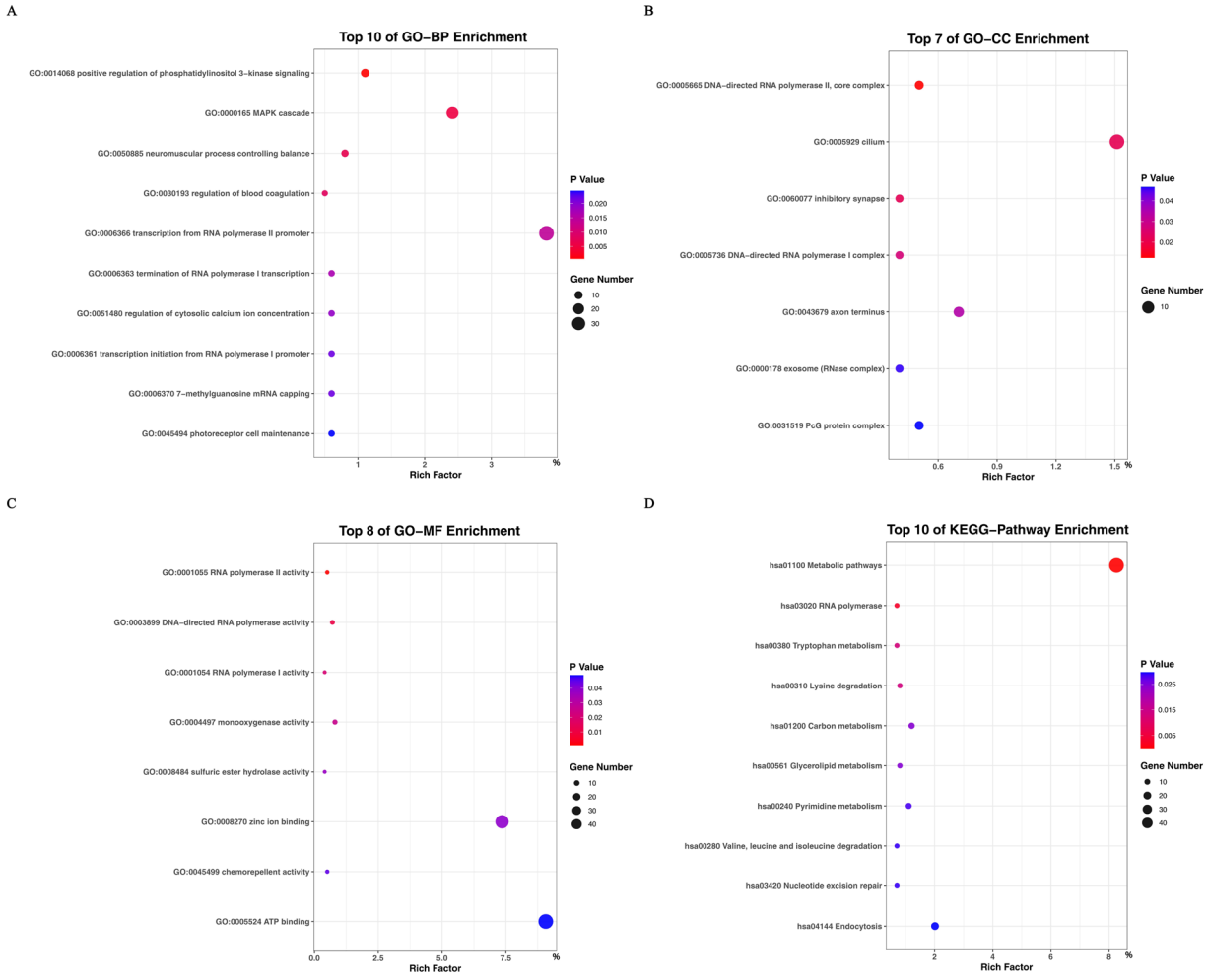

Figure 2. The top functional enrichment results of the target genes of miRNA-125a-3p
The Y-axis on the left revealed the top several functional enrichment results of the target genes. The X-axis indicated the percentage of gene involved in (**A**) Biological Process (BP), (**B**) Cellular Components (CC), (**C**) Molecular Function (MF) and (**D**) KEGG pathway. The color represented *P* Value, and a range from red to blue indicated P Value (lowest to highest, respectively) The size of bubble showed genes number involved in BP, CC, MF and KEGG pathway. If the items that meet the criteria were more than 10, only the top 10 were displayed.

Moreover, the protein expressions of FZD8, GSTM4 and ICOSLG demonstrated similar results in the immunohistochemical images from HPA database (Fig. 4).

Survival analyzes of prognostic risk markers

With the aid of the OncoLnc web server, the overexpression of ICOSLG was obviously associated with a better prognosis (P=0.0252). However, the expression of FZD8 and GSTM4 was significantly not related to the survival time of PDAC (P=0.0652 and 0.573, respectively) (Fig. 5).

DISCUSSION

As one of the most lethal cancers worldwide, the 5-year survival rate of PDAC patients is only 10.8% in the US. Due to poor diagnosis and high malignancy, the outcome and prognosis of PDAC still remain poor in recent decades. PDAC is usually diagnosed in an advanced stage with common symptoms including jaundice, pain, and weight loss. Most patients with PDAC cannot have surgery when diagnosed. As a consequence, it is necessary to explore new biomarkers to predict the outcome of PDAC patients. In this study, 17 prognostic risk markers were found, including 1 miRNA and 16 genes. The expressions of them were evaluated in pancreatic cancer at both the RNA and protein levels, respectively. FZD8, GSTM4, and ICOSLG revealed significant differential expression in tumor tissues compared to the corresponding non-tumor tissues. Moreover, ICOSLG was obviously associated with the prognosis of PDAC in the OncoLnc database.

FZD8 is one of the Frizzled receptors that belong to the Wnt ligand family. FZD8 activates the canonical Wnt/β-catenin signaling pathway that plays a vital role in the development and progression of multiple carcinomas. Li and others (Li *et al.*, 2017) reported that FZD8 was robustly up-regulated in bone-metastatic prostate cancer cell lines and tissues, and a high expression level of FZD8 was significantly and positively associated with progression and bone metastasis. Chen's findings showed that FZD8 promotes gastric cancer invasion and metastasis *via* the β-catenin pathway (Chen *et al.*, 2020). Wang and others (Wang *et al.*, 2012) suggested that FZD8 may be used as a potential therapeutic target in

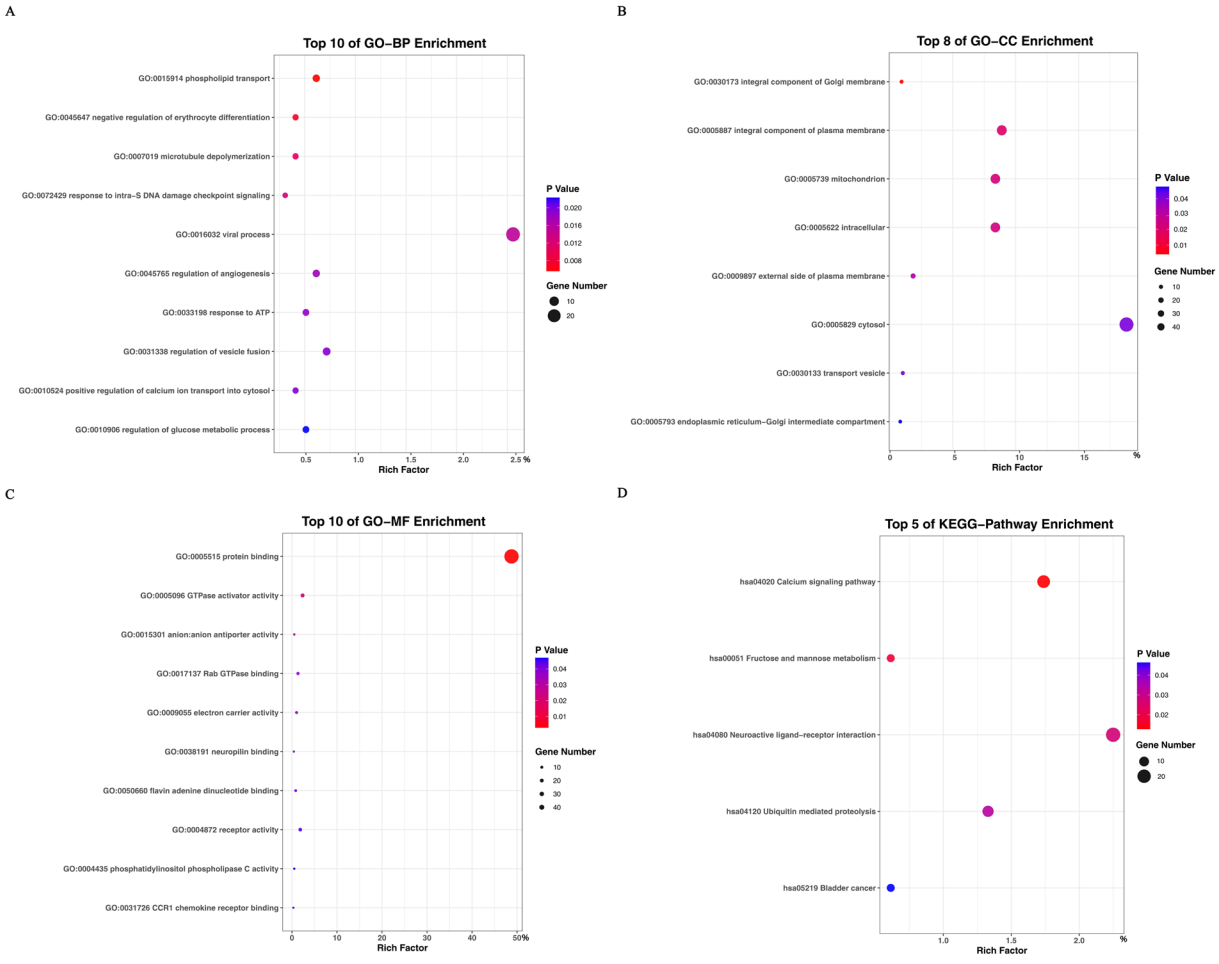

Figure 3. The top functional enrichment results of the target genes of miRNA-125a-5p.
The Y-axis on the left revealed the top several functional enrichment results of the target genes. The X-axis indicated the percentage of gene involved in (**A**) Biological Process (BP), (**B**) Cellular Components (CC), (**C**) Molecular Function (MF) and (**D**) KEGG pathway. The color represented P Value, and a range from red to blue indicated P Value (lowest to highest, respectively) The size of bubble showed genes number involved in BP, CC, MF and KEGG pathway. If the items that meet the criteria were more than 10, only the top 10 were displayed.

lung carcinoma due to its overexpressed expression in human lung cancer tissue and cell lines and suppression in cancer cell proliferation. This in turn reduced the Wnt signaling activity and enhanced the sensibility of lung cancer to chemotherapeutics. Additionally, some studies revealed a higher expression of FZD8 in breast cancer and colorectal cancer compared to their corresponding adjacent tissues (Jiang *et al.*, 2015; Xu *et al.*, 2016). It is well known that K-ras mutation occurs in 90% of pancreatic cancer cases. Wang *et al.* revealed that FZD8 was inhibited in K-ras mutant pancreatic cells. This further suppressed the non- canonical Wnt/Ca^{2+} signaling, contributing strongly to its tumorigenic properties. Restoration of FZD8 expression in K-ras mutant pancreatic cells demonstrated a decrease in malignant transformation (Wang *et al.*, 2015). Our study found that FZD8 was upregulated in pancreatic cancer and was associated with a better prognosis, which was consistent with most previous studies, implying that FZD8 mainly activated Wnt/β-catenin pathway in pancreatic cancer.

GSTM4 is a member of μ subfamily in Glutathione S-transferases (GSTs) family. GSTs are a group of detoxifying enzymes that catalyze glutathione in conjunction with oncogenes, drugs, toxic substances, and products of oxidative stress, with the aim of reducing their toxic combination with cell components. GSTs were divided into eight subfamilies which include α (GSTAs), κ (GSTKs), μ (GSTMs), ω (GSTOs), π (GSTPs), θ (GSTTs), ζ (GSTZs) and membrane-bound GSTs (MGSTs). It was reported that GSTM4 was implicated in tumorigenesis and resistant to chemotherapy. Zhuo *et al.* showed that GSTM4 was overexpressed in Ewing's sarcoma and promoted tumor development and chemoresistance by inhibiting cell apoptosis (Zhuo *et al.*, 2014). Barros *et al.* discovered a higher level of GSTM4 expression in human MCF-1 breast cancer cells, which helped to maintain the reduction status of cytochrome C and suppressed cell apoptosis, resulting in the chemoresistance of MCF-1 cells (Barros *et al.*, 2013). However, a few studies demonstrated that downregulation of GSTM4 in breast cancer cell lines was related to their chemotherapeutic resistance (Watson *et al.*, 2007). Furthermore, compared to well differentiated laryngeal cancer tissues, poor laryngeal cancer differentiation demonstrated reduced GSTM4 expression (Sedat *et al.*, 2010). Unfortunately, there have been no reports in the literature on GSTM4 expression in pancreatic adenocarcinoma yet.

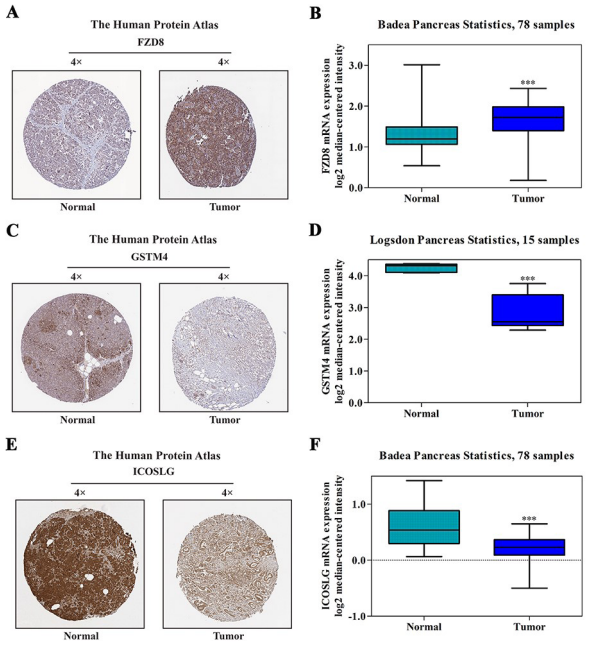

Figure 4. Prognostic risk markers expression in human pancreatic carcinoma specimens.
(**A, C and E**) FZD8-GSTM4 and ICOSLG were expressed in normal pancreatic tissues and pancreatic carcinoma specimens. Images were taken from the Human Protein Atlas online database. (**B, D, and F**) Oncomine data showed that the FZD8, GSTM4 and ICOSLG mRNAs were differentially expressed in the normal pancreas compared to the pancreatic tumor (P<0.001), respectively.

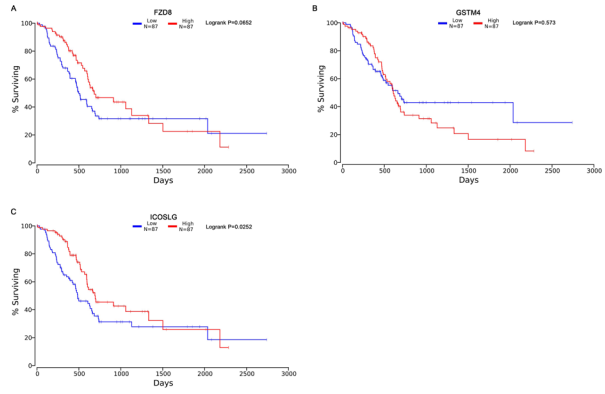

Figure 5. Survival analyzes of prognostic risk markers.
Survival curves of FZD8 (**A**), GSTM4 (**B**), and ICOSLG (**C**) Using the median expression of genes as the cutoff point, high expression of ICOSLG was obviously associated with a better prognosis (P=0.025) However, the expression of FZD8 and GSTM4 was not related to the survival time of PDAC (P=0.065 and 0.573, respectively)

ICOSLG encodes inducible T-cell co-stimulator ligand (ICOSLG). This belongs to the B7 family and is identified as a new kind of molecule on the cell surface. It is usually expressed in antigen-presenting cells such as B lymphocytes, dendritic cells, and macrophagocytes. ICOSLG is the only ligand of the inducible co-stimulator (ICOS) that belonged to the CD28 superfamily. Physiologically, ICOS is highly expressed in activated T lymphocytes and regulatory T cells, and ICOS: The ICOSLG interaction promoted the activation and proliferation of T cells and the secretion of cytokines. Recent studies revealed that the ICOS/ICOSLG signaling pathway was involved in inflammatory responses, autoimmune diseases, and cancers (Merrill *et al.*, 2013). Many studies reported that ICOSLG was highly expressed in several tumors such as breast cancer, melanoma, and acute myeloid leukemia. Its high expression was correlated with progression, immune escape, chemoresistance, and poor prognosis of cancers (Nam *et al.*, 2015; Martin-Orozco *et al.*, 2010). Scott *et al.* found that ICOSLG transcription was reduced in myeloma, leading to inhibition of its antitumor immunity (Scott *et al.*, 2015). In this study, we found that ICOSLG is downregulated in PDAC at both RNA and protein levels, and high expression of ICOSLG was significantly associated with a better prognosis. Nevertheless, there is still a lack of other research on the functions of ICOSLG in pancreatic cancer.

When validated in the oncomine and HPA databases, miRNA-125a expression was not significantly different in PDAC or there was no information on the gene.

Bioinformatics is the field of science developed by the combination of biology and information technology. It is the computational techniques used for solving biological problems. Data problems such as representation (graphics), storage and retrieval (databases), analysis (statistics, artificial intelligence, optimization, etc.) and biology problems such as sequence analysis, structure or function prediction, data mining, etc. Along with the rapid development of the field, there are some issues proposed (Sethi & Behera, 2016). First, the bioinformation algorithm is too far from maturity: for example, whole exome sequencing combined with diseases is used for disease diagnosis or prediction, and the false positive rate is too high. Algorithms are messy, bioinformatics researchers disagree, and there are more than a dozen types of sequencing software alone. There is no unified standard for difference analysis, and the data forms in GEO chips are all different, so the results cannot represent accurately predicted phenotypes. Second, there are too many uncontrollable factors: from sampling to bioinformatic operation, each step in the process may result in different results if another person operates it. There are even dozens of specifications of sequencing instruments, without unified standards. Since this was a purely bioinformatic study that lacked clinical samples and data to verify our results, our study has certain limitations. Therefore, we will collect clinical data and conduct laboratory experiments in vivo and in vitro to testify to this result. Although their differential expressions were confirmed in the Oncomine and HPA databases, the specific mechanisms and pathways of FZD8, GSTM4 and ICOSLG in pancreatic cancer involved in tumor prognosis should be explored.

CONCLUSION

This study discovered that FZD8, GSTM4, and ICOSLG expression levels were significantly associated with PDAC by using bioinformatic analyses. Moreover, according to the data from the TCGA dataset, these 3 genes were related to the survival of PDAC patients. Furthermore, it revealed that ICOSLG was obviously associated with the prognosis of PDAC in the OncoLnc database. Therefore, ICOSLG was probably used as a prognostic indicator for PDAC.

Conflict of interest

The authors have no potential conflicts of interest in this work.

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