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Original Paper

Integrating MicroRNA Expression Profiling Studies to Systematically Evaluate the Diagnostic Value of MicroRNAs in Pancreatic Cancer and Validate Their Prognostic Significance with the Cancer Genome Atlas Data

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Key Words
MicroRNA • Pancreatic cancer • TCGA • Meta-analysis

Abstract
Background/Aims: MicroRNAs (miRNAs) are promising biomarkers for pancreatic cancer (PaCa). However, systemic and unified evaluations of the diagnostic value of miRNAs are lacking. Therefore, we performed a systematic evaluation based on miRNA expression profiling studies. ***Methods:*** We obtained miRNA expression profiling studies from Gene Expression Omnibus (GEO) and ArrayExpress (AE) databases and calculated the pooled sensitivity, specificity, and summary area under a receiver operating characteristic (ROC) curve for every miRNA. According to the area under the curve (AUC), we identified the miRNAs with diagnostic potentiality and validated their prognostic role in The Cancer Genome Atlas (TCGA) data. Gene Ontology (GO) annotations and pathway enrichments of the target genes of the miRNAs were evaluated using bioinformatics tools. ***Results:*** Ten miRNA expression profiling studies including 958 patients were used in this diagnostic meta-analysis. A total of 693 miRNAs were measured in more than 9 studies. The top 50 miRNAs with high predictive values for PaCa were identified. Among them, miR-130b had the best predictive value for PaCa (pooled sensitivity: 0.73 [95% confidence intervals (CI) 0.44-0.91], specificity: 0.81 [95% CI 0.59–0.93], and AUC: 0.84 [95% CI 0.73-0.95]). We identified nine miRNAs (miR-23a, miR-30a, miR-125a, miR-129-1, miR-181b-1, miR-203, miR-221, miR-222, and miR-1301) associated with overall

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survival in PaCa patients by combining our results with TCGA data. The results of a Cox model revealed that two miRNAs (miR-30a [hazard ratio (HR)=2.43, 95% CI 1.05-5.59; p=0.037] and miR-203 [HR=3.14, 95% CI 1.28-7.71; p=0.012]) were independent risk factors for prognosis in PaCa patients. In total, 405 target genes of the nine miRNAs were enriched with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and cancer-associated pathways such as Ras signaling pathways, phospholipase D signaling pathway, and AMP-activated protein kinase (AMPK) signaling pathway were revealed among the top 20 enriched pathways. There were significant negative correlations between miR-181b-1 and miR-125a expression levels and the methylation status of their promoter region. ***Conclusion:*** Our study performed a systematic evaluation of the diagnostic value of miRNAs based on miRNA expression profiling studies. We identified that miR-23a, miR-30a, miR-125a, miR-129-1, miR-181b-1, miR-203, miR-221, miR-222, and miR-1301 had moderate diagnostic value for PaCa and predicted overall survival in PaCa patients.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers in which the overall 5-year survival rate is as low as 5%-6% [1, 2]. Surgical procedures are the only curative method; however, most patients are diagnosed in the progressive stage and have missed the opportunity to have an operation. Early detection of pancreatic cancer (PaCa) is challenging, which is perhaps the main factor associated with its low survival. Thus, finding reliable biomarkers is a beneficial strategy for reducing mortality in PDAC patients. Carbohydrate antigen 19-9 (CA 19-9) has been extensively used as a standard biomarker in PaCa screening over the past thirty years. However, CA 19-9 is not a pancreatic-specific biomarker [3], and its diagnostic value was undesirable, especially for asymptomatic patients [4-6]. Therefore, an increasing number of studies combined other biomarkers with CA 19-9 for improved integrated diagnostic accuracy [7]. Currently, identification of the molecular alterations that occur in benign pancreatic diseases and are aggravated in PaCa could be another promising strategy for early diagnosis [8].

MicroRNAs (miRNAs) are endogenous small noncoding RNA molecules comprised of approximately 22 nucleotides that can suppress the translation of target messenger RNAs (mRNAs) and play an important role in cell differentiation by mapping to 3' untranslated regions (UTRs) [9]. They play key roles in posttranscriptional regulation [10, 11]. Classically, after RNA-induced silencing complex (RISC) loading, mature miRNA directs the complex to target mRNAs, leading to expressive suppression. Many target genes of tumor-associated miRNAs have been found to regulate cancer development pathways [12-15]. Hence, emerging studies have discovered that the differential expression of miRNA, such as miR-21, miR-99a, and miR-34a, could predict the prognosis of PaCa patients [16-18]. In pancreatic tumor tissue, aberrant expression of miRNAs, such as miR-21, miR-221, miR-34a, and miR-217, were reported, which suggested that these molecular regulators had the potential to serve as biomarkers [17, 19-21]. Meta-analyses were conducted to evaluate the diagnostic value of miRNAs, and they showed moderate predictive values for PaCa diagnosis [22, 23]. In each trial included in the two meta-analyses, the sensitivity and specificity of miRNAs were assessed, and one or several miRNAs with the highest diagnostic value were screened out. Notably, these miRNAs were different. Then, after pooling these data together and integrating them in a meta-analysis, the comprehensive results told us only the summary diagnostic value of miRNAs overall and not that of specific miRNAs. In clinical applications, the guiding significance of these conclusions is limited. In the past years, a number of miRNA expression profiling studies in PaCa patients have been proposed; however, most of these studies lack large-scale verification. Therefore, a systematic and unified evaluation of miRNAs for PaCa diagnosis is needed, and it will have clear clinical significance and applications.

In this study, we performed a systematic evaluation of miRNAs for the diagnosis of PaCa by integrating the multiple miRNA expression profiling studies. We pooled the sensitivity and specificity of all miRNAs in the files and constructed a summary receiver operating characteristic (ROC) curve. We ranked and screened out the miRNAs with high diagnostic accuracy based on the area under the curve (AUC) values. Furthermore, we validated the prognostic significance of these miRNAs and explored their target genes with The Cancer Genome Atlas (TCGA) data.

Materials and Methods

Search strategy and inclusion criteria

Two authors (X. Zhao and SC. Lv) independently performed study retrieval from Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo) and ArrayExpress (AE, https://www.ebi.ac.uk/arrayexpress) databases for miRNA expression profiling studies in PaCa patients. Our medical subject heading terms were "(miR OR microRNA) AND ("pancreatic neoplasms" OR "PaCa") AND (Homo sapiens)". The publication time was not limited. A miRNA profiling assay was included if it systematically presented the value of miRNA between patients with PaCa and those with benign pancreatic disease or healthy controls. We excluded the profiles with less than 20 samples to enhance the credibility of the results. Two authors (X. Zhao and SC. Lv) screened the title, summary, general design and description according to search terms. Next, a full-text dataset examination was carried out to reconfirm admission in the meta-analysis. If any disagreements arose, the reviewers conducted a discussion with a third author (Q. Wu).

Data extraction and processing

Data extraction and processing were carried out by two investigators (X. Zhao and SC. Lv). A consensus meeting was held to resolve any discrepancies between the two authors. If the disagreement continued, a third investigator (Q. Wu) drew the final conclusion. Series matrix files of each profiling study were extracted to assess miRNA expression; the raw microarray data were preprocessed by quantile normalization or log2 transformation. According to the platform file, we translated the miRNA IDs into symbol names and thus we obtained the expression values of the matrix data. Then, we divided the patients into a PaCa group and a healthy or benign disease group based on the sample annotation. The best threshold was determined by Youden's J statistic [24] and calculated using the "coords" function of the package "pROC" [25]. The optimal cut-off point value was the threshold that maximized the distance to the identity (diagonal) line. We constructed the 2×2 contingency table, including the numbers of true positive (TP), false negative (FN), false positive (FP), and true negative (TN) results for every miRNA in each profile.

Diagnostic value assessment for miRNA

Based on a bivariate random effects regression model, we calculated the pooled sensitivity (SEN) (TP/[TP+FN]) and specificity (SPE) (TN/ [TN+FP]) with their standard error and 95% confidence intervals (CIs). The diagnostic odds ratio (DOR), positive likelihood ratio (PLR), and negative likelihood ratio (NLR) were also included. Then, the SEN and SPE were determined using a bivariate summary ROC (SROC) curve [26], and the AUC and 95% CIs were calculated [27]. We thus obtained the pooled sensitivity, specificity and the summary AUC of each miRNA and its frequency in all included profiles. We included only the miRNAs that overlapped in more than 9 profiling studies to improve the diagnostic credibility of miRNA. Then, we ranked the miRNAs according to the summary AUC value and filtered out the top 50 miRNAs with high predictive value.

We computed Higgin's I-squared statistic [28] to evaluate heterogeneity, and if I^2 was greater than 50%, heterogeneity was suggested. Metaregression was applied to investigate the source of heterogeneity. We employed study-specific covariates, such as population and sample source.

Validating the prognostic significance of miRNAs with TCGA data

We extracted the miRNA expression and corresponding clinical data of PDAC patients in the TCGA dataset from the Firehose website (https://gdac.broadinstitute.org/) [29]. The expression level of miRNA was measured by the Illumina HiSeq platform. We used a binary logarithm to assess the expression values because the levels of most miRNAs were rather high. We examined the prognostic value of the top 50 miRNAs by trisecting the patients into three parts (high, moderate, and low expression groups) based on miRNA expression levels. Then, a Kaplan-Meier survival curve was used to compare the differences in overall survival (OS) among the three groups. We considered that a miRNA had prognostic significance if the overall survival was statistically different between the high expression group and the low expression group. A multivariate Cox regression model was applied to verify which miRNAs were independent risk factors for OS, and the hazard ratio (HR) with 95% CI was presented.

Pathways analysis of miRNAs

We screened out the possible target genes of the aforementioned miRNAs by using the online resource miRWalk2.0 (http://zmf.umm.uni-heidelberg.de/mirwalk2), which integrated multiple miRNAs prediction databases to document miRNA binding sites within the complete sequence of a gene [30]. We predicted putative target genes with the criterion of p-value < 0.01 using this tool. Then, we extracted the mRNA expression profiles of PDAC patients in the TCGA dataset and carried out a Pearson correlation analysis between the miRNA and mRNA expression values with the criterion of p-value < 0.05. As miRNA tended to inhibit the expression of target genes, we selected the genes reversely correlated with the expression of relevant miRNAs. A regulatory network between the identified miRNAs and target genes was constructed and visualized with Cytoscape [31]. Gene Ontology (GO) classifications were employed to gain insights into the biological functions of these target genes [32], and the Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to detect potential pathways [33].

Statistical analysis

R statistical programming language version 3.4.3 (R Core Team, 2017) was used to perform all computations and basic data visualization. In diagnostic meta-analysis, the "General Package for Meta-Analysis" (Version 4.9-0) was used to calculate pooled sensitivity, specificity, and area under the SROC curve.

Results

Characteristics of the included miRNA expression profiling studies

Our database retrieved 62 and 561 relevant miRNA expression profiling studies in the AE and GEO datasets, respectively. After removing duplicates, we reviewed the title, summary, overall design and description of the remaining 574 studies. According to the inclusion criteria, 14 studies were enrolled for sample number assessment (GSE59856, GSE24279, E-TABM-664, GSE31568, GSE85589, GSE60980, GSE71533, GSE29352, E-MTAB-753, GSE32688, GSE41372, GSE25820, GSE43797, and GSE28862). Four profiles

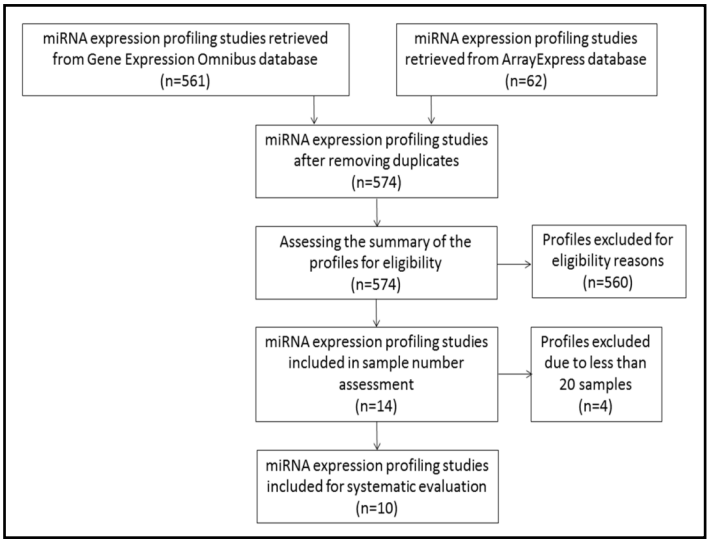


Fig. 1. Flow chart of study identification and selection for the diagnostic meta-analysis.

Table 1. The characteristics of the ten miRNA expression profiling studies

No	Profile accession number	Author	Publication date	Publication title	Country	Sample source	Control sample number	Pancreatic cancer sample number
1	GSE59856	Kojima M	1-Mar-15	MicroRNA markers for the diagnosis of pancreatic and biliary tract cancers	Japan	Serum	150	100
2	GSE24279	Andreas Keller	1-Jan-12	Pancreatic cancer and pancreatitis miRNA profiles	Germany	Tissue	22	136
3	E-TABM-664	Cristian Taccioli	11-Mar-10	MicroRNA profiling of human pancreas to identify expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis	USA	Tissue	57	61
4	GSE31568	Andreas Keller	5-Sep-11	The human Whole miRNOme project version 1	Germany	Serum	70	45
5	GSE85589	Jaehoon Lee	30-Oct-16	Expression data from pancreatic cancers patients and healthy controls	South Korea	Serum	19	88
6	GSE60980	Vandana Sandhu	6-Nov-15	mRNA and miRNA expression profiles for fresh frozen periampullary adenocarcinomas (pancreatobiliary and intestinal type) and adjacent normal tissues	Norway	Tissue	6	52
7	GSE71533	Vandana Sandhu	29-Jul-17	Microenvironmental regulation by miRNAs in pancreatic cancer	Norway	Tissue	16	36
8	GSE29352	Adam Enver Frampton	18-May-11	miRNA expression profiles in pancreatic cystic tumours and pancreatic cancer	United Kingdom	Tissue	20	14
9	E-MTAB-753	Massimo Carella	1-Aug-11	Pancreatic cancer miRNA expression profiles	Italy	Tissue	17	17
10	GSE32688	Linh My Tran	8-Oct-11	Integrative survival-based molecular profiling of human pancreatic cancer	USA	Tissue	7	25

with less than 20 samples (GSE41372, GSE25820, GSE43797, and GSE28862) were excluded, and ultimately, ten miRNA profiling assays were enrolled for diagnostic meta-analysis (Fig. 1).

The characteristics of the eligible profiles are presented and include the GEO accession number, corresponding author, publication time, profiling title, country, sample source, number of tumors and controls (Table 1). In total, 958 samples were included in our study. Among them, 384 (40%) were from patients with benign pancreatic diseases or normal controls and 574 (60%) were from PaCa patients. The miRNA expression values were measured with serum samples in three profiles (GSE59856, GSE31568, and GSE85589), whereas they were measured with tissue samples in the other seven profiles.

Systematic evaluation of the diagnostic value of miRNAs for PaCa

The numbers of miRNAs among the included profiles ranged from 364 to 2575 (Fig. 2A). As a whole there were 2575 miRNAs measured in the 10 studies. One hundred and forty-eight miRNAs overlapped in the ten files and 545 were measured in nine studies. There were 343, 678, and 678 miRNAs detected in 4, 3, and 2 files, respectively. The frequency diagram of miRNAs was illustrated in Fig. 2B.

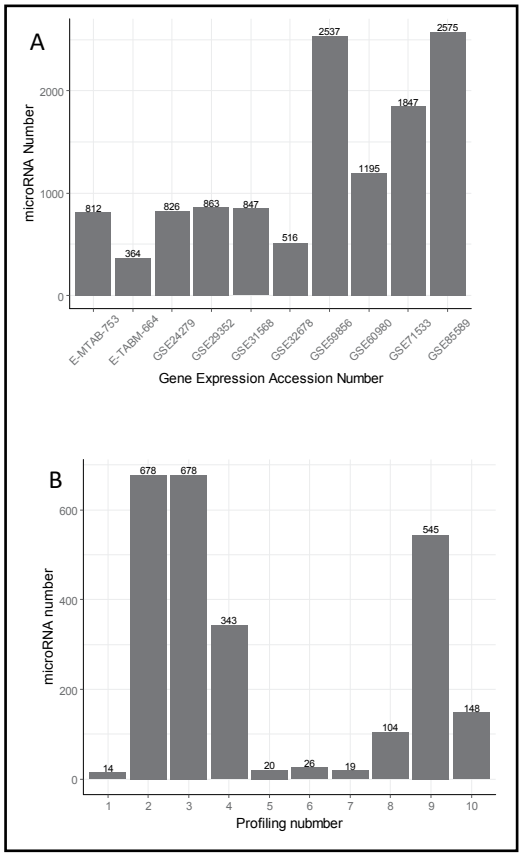


Fig. 2. The numbers and frequency distribution of miRNAs in the ten profiles. A. The number of miRNAs measured in each study. B. The distribution of miRNAs. The abscissa indicates the number of profiles, and the ordinate represents the number of miRNAs.

As more frequently measured miRNA would be more reliable for diagnosing PaCa, we focused on the ones monitored in at least 9 studies. In total, 693 miRNAs were measured in more than 9 profiles. Hence, we calculated the pooled sensitivity, specificity, and summary AUC for all miRNAs (Supplementary Table 1). For all supplemental material see www.karger.com/10.1159/000493033. We ranked these miRNAs based on AUC values and presented the top 50 with the highest diagnostic value

Table 2. The top 50 miRNAs with high diagnostic accuracy in pancreatic cancer. SN-sample number, SEN-sensitivity, SPE-specificity, PLR-positive likelihood ratio, NLR-negative likelihood ratio, DOR-diagnostic odds ratio, AUC-area under the curve, PN-profiling number

miRNAs ID	Gene Expression Omnibus and ArrayExpress accession number	SN	SEN	SPE	PLR	NLR	DOR	AUC	PN
miR-130b-3p	GSE24279, GSE71533, GSE60980, GSE31568, GSE85589, E-MTAB-753, GSE29352, E-TABM-664, GSE59856	925	0.73	0.80	3.87	0.35	13.80	0.84	9
miR-148a-3p	GSE71533, GSE24279, E-TABM-664, GSE60980, GSE31568, GSE29352, GSE59856, E-MTAB-753, GSE85589	924	0.75	0.79	3.74	0.35	13.30	0.84	9
miR-200c-3p	GSE24279, GSE71533, GSE60980, GSE85589, GSE31568, GSE29352, E-TABM-664, GSE59856, E-MTAB-753	925	0.56	0.85	3.77	0.53	7.78	0.83	9
miR-217	GSE24279, GSE71533, GSE60980, E-MTAB-753, E-TABM-664, GSE31568, GSE85589, GSE59856, GSE29352	925	0.86	0.52	2.00	0.30	8.50	0.83	9
miR-30c-5p	GSE24279, GSE71533, GSE60980, GSE29352, E-MTAB-753, E-TABM-664, GSE31568, GSE85589, GSE59856	925	0.83	0.65	2.74	0.30	11.30	0.82	9
miR-129-1-3p	GSE71533, GSE32678, GSE85589, E-MTAB-753, GSE60980, GSE24279, GSE31568, GSE59856, GSE29352	839	0.57	0.85	3.70	0.51	7.68	0.82	9
miR-23b-3p	GSE24279, GSE29352, GSE60980, GSE31568, GSE71533, E-TABM-664, GSE32678, E-MTAB-753, GSE85589, GSE59856	957	0.66	0.81	3.46	0.43	8.34	0.82	10
miR-30b-5p	GSE71533, GSE24279, GSE60980, GSE29352, E-MTAB-753, GSE31568, GSE85589, E-TABM-664, GSE59856	925	0.84	0.61	2.40	0.29	9.71	0.82	9
miR-222-3p	GSE71533, GSE24279, E-TABM-664, E-MTAB-753, GSE29352, GSE60980, GSE85589, GSE59856, GSE31568	925	0.76	0.74	3.09	0.34	11.10	0.81	9
miR-155-5p	GSE24279, GSE85589, GSE32678, E-TABM-664, E-MTAB-753, GSE29352, GSE71533, GSE60980, GSE31568, GSE59856	957	0.72	0.77	3.08	0.37	8.87	0.81	10
miR-575	GSE59856, GSE71533, GSE60980, GSE32678, E-MTAB-753, GSE85589, GSE29352, GSE31568, GSE24279	839	0.81	0.69	2.74	0.29	10.80	0.81	9
miR-1290	GSE71533, GSE59856, GSE24279, GSE85589, GSE29352, E-MTAB-753, GSE60980, GSE31568, GSE32678	839	0.67	0.80	3.52	0.41	8.97	0.81	9
miR-30e-5p	GSE71533, GSE24279, GSE60980, GSE29352, E-MTAB-753, GSE31568, E-TABM-664, GSE85589, GSE59856	925	0.56	0.84	3.44	0.52	7.52	0.81	9
miR-107	GSE71533, GSE24279, GSE60980, E-MTAB-753, E-TABM-664, GSE29352, GSE31568, GSE59856, GSE32678, GSE85589	957	0.74	0.74	3.01	0.36	9.18	0.81	10
miR-181b-5p	GSE24279, GSE71533, E-TABM-664, GSE60980, E-MTAB-753, GSE85589, GSE31568, GSE32678, GSE59856, GSE29352	957	0.62	0.81	3.33	0.48	7.84	0.81	10
miR-663a	GSE59856, GSE32678, GSE71533, GSE60980, GSE31568, E-MTAB-753, GSE85589, GSE29352, GSE24279	839	0.64	0.81	3.37	0.45	8.07	0.80	9
miR-93-5p	GSE24279, GSE59856, GSE71533, GSE29352, E-TABM-664, GSE85589, GSE60980, E-MTAB-753, GSE31568	925	0.74	0.75	3.01	0.36	8.84	0.80	9
miR-628-5p	GSE85589, GSE60980, GSE29352, GSE24279, GSE32678, GSE31568, GSE59856, GSE71533, E-MTAB-753	839	0.58	0.82	3.22	0.53	6.40	0.80	9
miR-27b-3p	GSE24279, GSE71533, GSE29352, GSE60980, E-TABM-664, E-MTAB-753, GSE31568, GSE59856, GSE85589	925	0.73	0.74	2.90	0.38	8.51	0.80	9
miR-132-5p	GSE71533, GSE60980, GSE31568, GSE32678, GSE29352, E-MTAB-753, GSE85589, E-TABM-664, GSE24279, GSE59856	957	0.47	0.84	2.91	0.63	4.72	0.80	10
miR-15b-5p	GSE24279, GSE71533, E-MTAB-753, GSE29352, GSE60980, GSE31568, E-TABM-664, GSE85589, GSE59856	925	0.66	0.78	3.00	0.44	7.36	0.80	9
miR-30a-5p	GSE24279, GSE71533, GSE60980, E-MTAB-753, GSE29352, GSE59856, GSE31568, GSE85589, E-TABM-664	925	0.77	0.72	2.82	0.33	9.64	0.80	9
miR-423-5p	GSE59856, GSE71533, GSE31568, GSE60980, GSE29352, E-TABM-664, GSE85589, GSE24279, E-MTAB-753	925	0.73	0.73	2.81	0.37	7.83	0.79	9
miR-1301-3p	GSE71533, GSE24279, GSE60980, GSE31568, GSE32678, GSE29352, E-MTAB-753, GSE85589, GSE59856	839	0.57	0.81	2.94	0.54	5.84	0.79	9
miR-647	GSE71533, GSE31568, E-MTAB-753, GSE29352, GSE24279, GSE32678, GSE85589, GSE60980, GSE59856	839	0.56	0.82	3.00	0.55	5.81	0.79	9
miR-29c-3p	GSE71533, GSE24279, GSE60980, GSE29352, GSE31568, E-MTAB-753, GSE85589, E-TABM-664, GSE59856	925	0.56	0.81	2.90	0.56	6.00	0.79	9
miR-221-5p	GSE71533, GSE60980, GSE85589, GSE32678, GSE31568, GSE29352, E-MTAB-753, GSE24279, GSE59856	839	0.57	0.81	2.91	0.55	5.91	0.79	9
miR-455-3p	GSE71533, GSE85589, GSE31568, E-MTAB-753, GSE29352, GSE60980, GSE32678, GSE59856, GSE24279, E-TABM-664	957	0.76	0.70	2.57	0.36	8.13	0.79	10
miR-26a-5p	GSE71533, GSE59856, GSE29352, GSE60980, GSE24279, GSE31568, E-MTAB-753, GSE85589, E-TABM-664	925	0.69	0.76	2.89	0.41	7.12	0.79	9
miR-335-5p	GSE71533, GSE24279, GSE85589, GSE60980, E-MTAB-753, GSE29352, GSE31568, E-TABM-664, GSE59856	924	0.50	0.87	3.87	0.58	7.02	0.79	9
miR-1225-5p	GSE60980, GSE59856, GSE85589, E-MTAB-753, GSE32678, GSE24279, GSE29352, GSE31568, GSE71533	839	0.47	0.81	2.44	0.66	3.81	0.78	9
miR-103a-3p	GSE71533, GSE24279, GSE29352, E-MTAB-753, E-TABM-664, GSE60980, GSE59856, GSE31568, GSE85589	925	0.70	0.75	2.96	0.40	7.82	0.78	9
miR-125b-5p	GSE24279, GSE71533, GSE60980, GSE29352, E-MTAB-753, E-TABM-664, GSE85589, GSE31568, GSE59856	925	0.57	0.81	3.05	0.55	5.85	0.78	9
miR-92a-3p	GSE71533, GSE24279, GSE29352, E-MTAB-753, E-TABM-664, GSE85589, GSE60980, GSE59856, GSE31568	925	0.65	0.78	3.04	0.46	6.82	0.78	9
miR-320a	GSE71533, GSE31568, E-TABM-664, GSE24279, GSE29352, GSE59856, GSE60980, E-MTAB-753, GSE85589	925	0.73	0.71	2.56	0.39	6.73	0.78	9
miR-30a-3p	GSE24279, GSE71533, GSE60980, E-MTAB-753, GSE29352, GSE31568, GSE59856, E-TABM-664, GSE85589	925	0.60	0.80	3.08	0.52	6.66	0.78	9
miR-10a-5p	GSE71533, GSE24279, E-TABM-664, GSE60980, GSE31568, GSE85589, E-MTAB-753, GSE59856, GSE29352	925	0.61	0.79	2.99	0.49	6.16	0.78	9
miR-203a-3p	E-TABM-664, GSE29352, E-MTAB-753, GSE71533, GSE31568, GSE60980, GSE59856, GSE24279, GSE85589	925	0.52	0.85	3.46	0.57	6.27	0.78	9
miR-32-5p	GSE71533, GSE85589, GSE29352, GSE60980, E-TABM-664, GSE24279, E-MTAB-753, GSE31568, GSE59856	925	0.48	0.84	3.11	0.60	5.49	0.78	9
miR-125a-3p	GSE59856, GSE71533, GSE60980, E-MTAB-753, GSE85589, E-TABM-664, GSE29352, GSE31568, GSE24279	925	0.71	0.73	2.94	0.41	8.52	0.77	9
miR-23a-3p	GSE24279, GSE60980, GSE71533, E-MTAB-753, GSE29352, E-TABM-664, GSE85589, GSE59856, GSE31568	925	0.63	0.80	3.32	0.46	7.68	0.77	9
miR-16-5p	GSE60980, GSE31568, GSE24279, GSE59856, GSE29352, GSE71533, E-MTAB-753, E-TABM-664, GSE85589	925	0.72	0.71	2.54	0.41	6.34	0.77	9
miR-891a-5p	GSE71533, GSE29352, GSE32678, E-MTAB-753, GSE31568, GSE24279, GSE60980, GSE59856, GSE85589	839	0.77	0.66	2.33	0.37	6.76	0.77	9
miR-17-5p	GSE59856, GSE29352, GSE71533, GSE31568, E-MTAB-753, E-TABM-664, GSE60980, GSE85589, GSE24279, GSE32678	957	0.75	0.66	2.26	0.39	6.07	0.77	10
miR-1246	GSE59856, GSE60980, GSE24279, E-MTAB-753, GSE85589, GSE32678, GSE71533, GSE29352, GSE31568	839	0.75	0.67	2.36	0.38	6.42	0.77	9
miR-320c	GSE71533, GSE31568, GSE24279, GSE59856, GSE32678, GSE60980, GSE85589, GSE29352, E-MTAB-753	839	0.72	0.70	2.43	0.40	6.13	0.77	9
miR-1275	GSE71533, GSE32678, GSE60980, GSE85589, GSE24279, GSE29352, GSE31568, GSE59856, E-MTAB-753	839	0.63	0.76	2.69	0.48	5.92	0.77	9
miR-375	GSE71533, GSE24279, GSE60980, E-TABM-664, GSE85589, GSE59856, E-MTAB-753, GSE29352, GSE31568	925	0.69	0.73	2.68	0.44	6.63	0.77	9
miR-1307-3p	GSE71533, GSE32678, GSE24279, GSE59856, GSE85589, GSE29352, E-MTAB-753, GSE60980, GSE31568	839	0.63	0.78	2.93	0.49	6.36	0.77	9
miR-106a-5p	GSE59856, GSE71533, GSE29352, GSE32678, GSE85589, GSE31568, E-TABM-664, E-MTAB-753, GSE24279	900	0.70	0.72	2.52	0.42	6.24	0.77	9

(AUC ranged from 0.77 to 0.84) in Table 2. In this way, we identified the possible miRNAs with diagnostic potential using the ten miRNA profiling assays. Among the top fifty miRNAs, miR-130b was measured in 9 profiles and displayed significant diagnostic value, so we used it as an example to explain the statistical results.

The pooled sensitivity and specificity of miR-130b were 0.73 (95% CI 0.44-0.91) and 0.81 (95% CI 0.59–0.93; Fig. 3), respectively. The summary AUC of miR-130b was 0.84 (95% CI 0.73-0.95; Fig. 4A). Substantial heterogeneity existed among the datasets (overall I^2 for bivariate model 99%, 95% CI 99-100). Then, we performed metaregression analysis to identify the source of the heterogeneity. We stratified the profiles into a serum source group (GSE59856, GSE31568, and GSE85589) and a tissue source group (GSE24279, GSE29352, GSE60980, GSE71533, E-TABM-664, GSE32678, and E-MTAB-753) to evaluate the influence of sample source on diagnostic efficiency. Sensitivity was significantly higher in the tissue source group than in the serum source group (0.86 [95% CI 0.71-1.00] vs 0.41 [95% CI 0.00-0.82]; p=0.03). We also compared patient ethnicities and found that sensitivity was higher in Western patients than in Asian patients (0.85 [95% CI 0.72-0.98] vs 0.24 [95% CI 0.10-0.58]; p=0.01). These two covariates yielded no significant influence on specificity. Thus, the heterogeneity could be explained by different ethnicities and sample sources based on metaregression analysis.

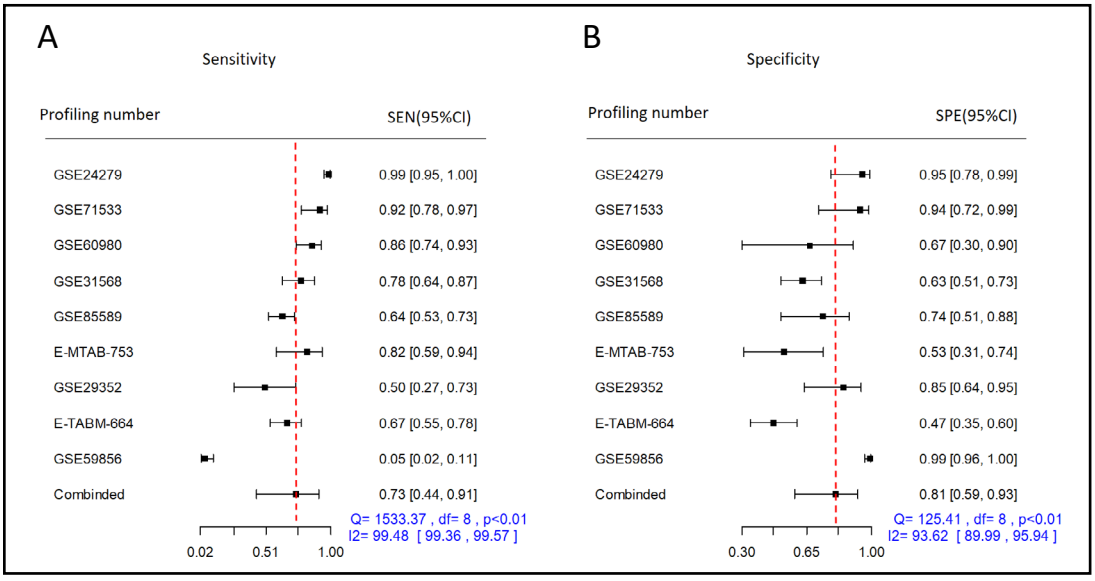
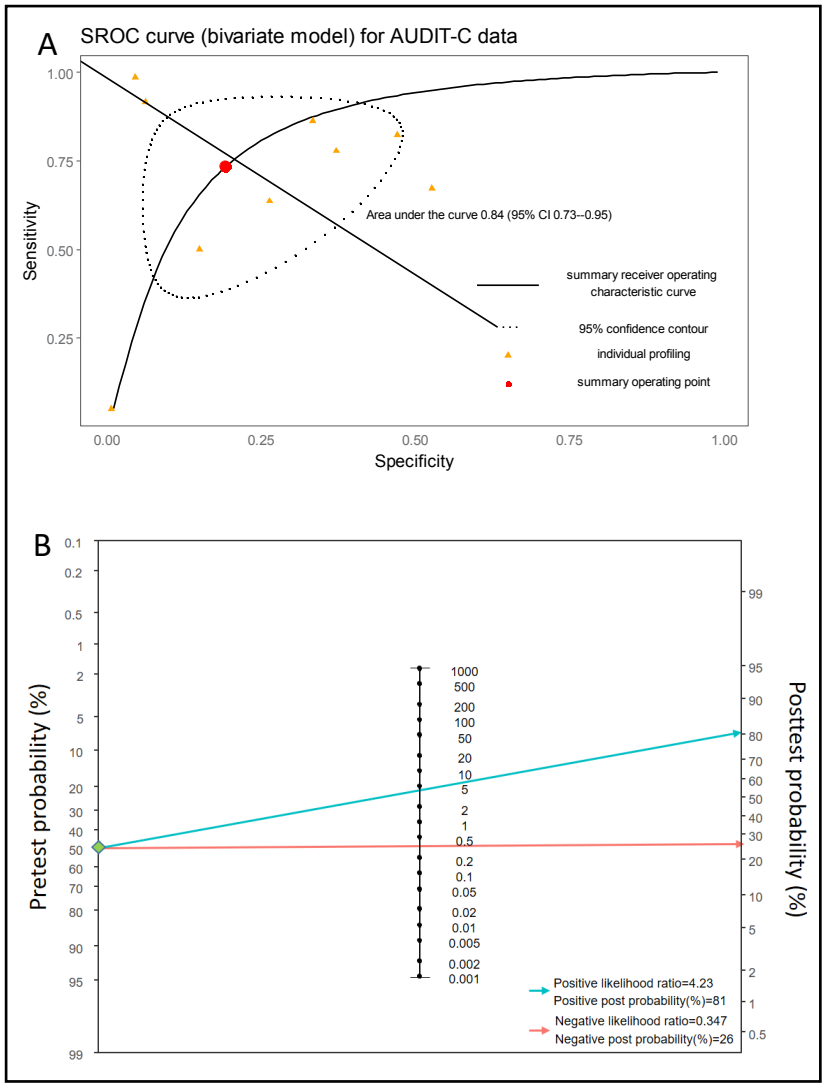


Fig. 3. A and B. The pooled sensitivity and specificity of miR-130b for pancreatic cancer diagnosis.

Fig. 4. The evaluation of miR-130b for pancreatic cancer diagnosis. A. The summary receiver operating characteristic curve revealed that the area under the curve was 0.84 (95% confidence intervals 0.73-0.95). B. The fagan nomogram of miR-130b for pancreatic cancer diagnosis.



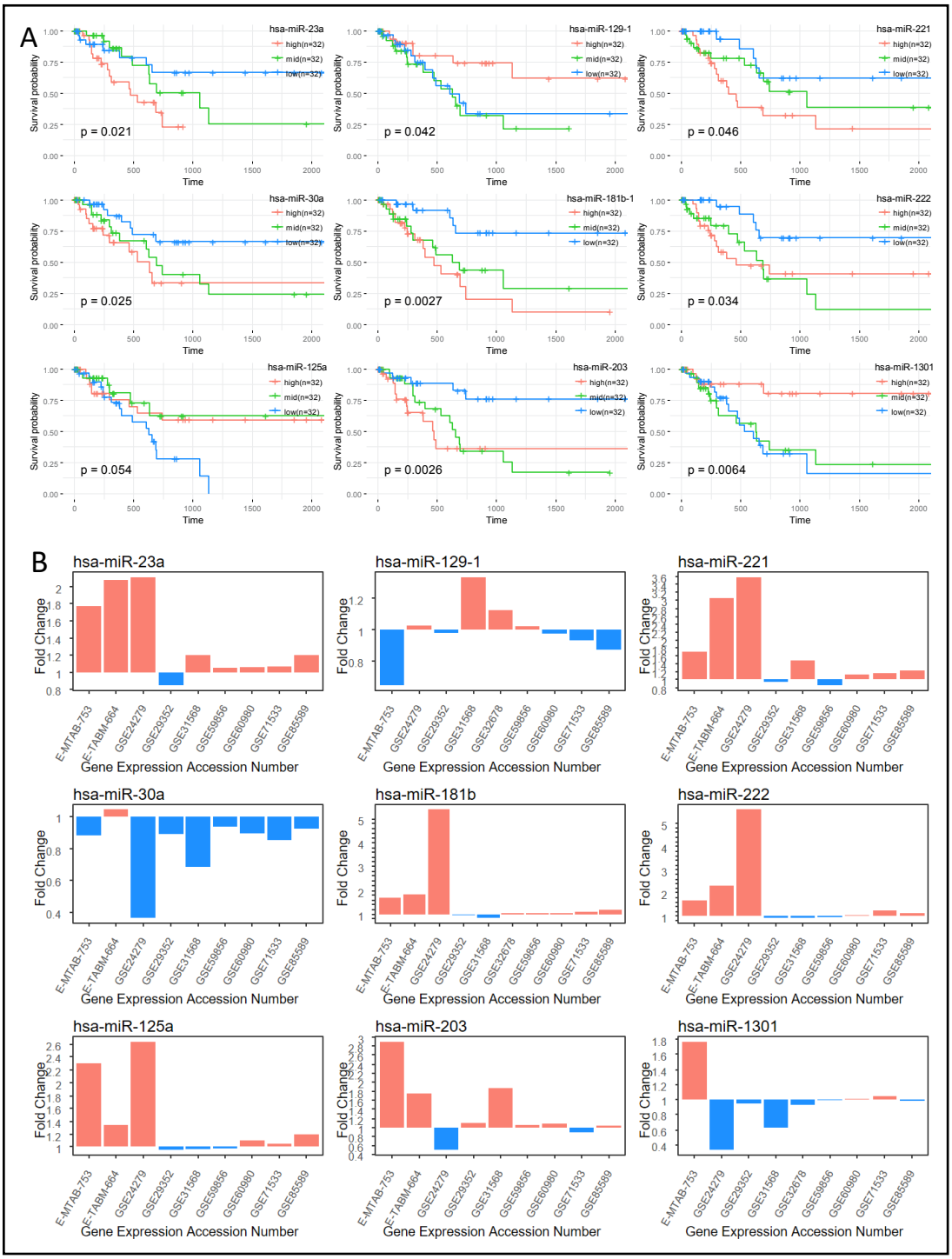


Fig. 5. The correlation between the nine miRNAs (miR-23a, miR-129-1, miR-221, miR-30a, miR-181b-1, miR-222, miR-125a, miR-203, miR-1301) and pancreatic ductal adenocarcinoma patient survival and overall expression in profiling studies. A. Kaplan-Meier survival curves for pancreatic cancer patients. The PDAC patients were equally divided into three groups according to miRNA expression level. The red line indicated one third of high expression group, the blue line indicated one third of low expression group and the green line indicated the moderate expression group. B. The fold change of nine miRNAs (tumor divided by normal). The red bar indicated overexpression of miRNA compared with controls and the blue bar represented low expression.

We applied Fagan's nomogram to evaluate the clinical diagnostic role of miR-130b for PaCa (Fig. 4B). The result displayed that the posttest probability rose to 81% if the pretest probability was 50%. The possibility of positive diagnosis in PaCa patients was 4 times more than that of healthy controls. On the other hand, the possibility decreased to 26% and the NLR was 0.35. Thus, this result indicated that miR-130b could be a potential molecular biomarker in clinical applications.

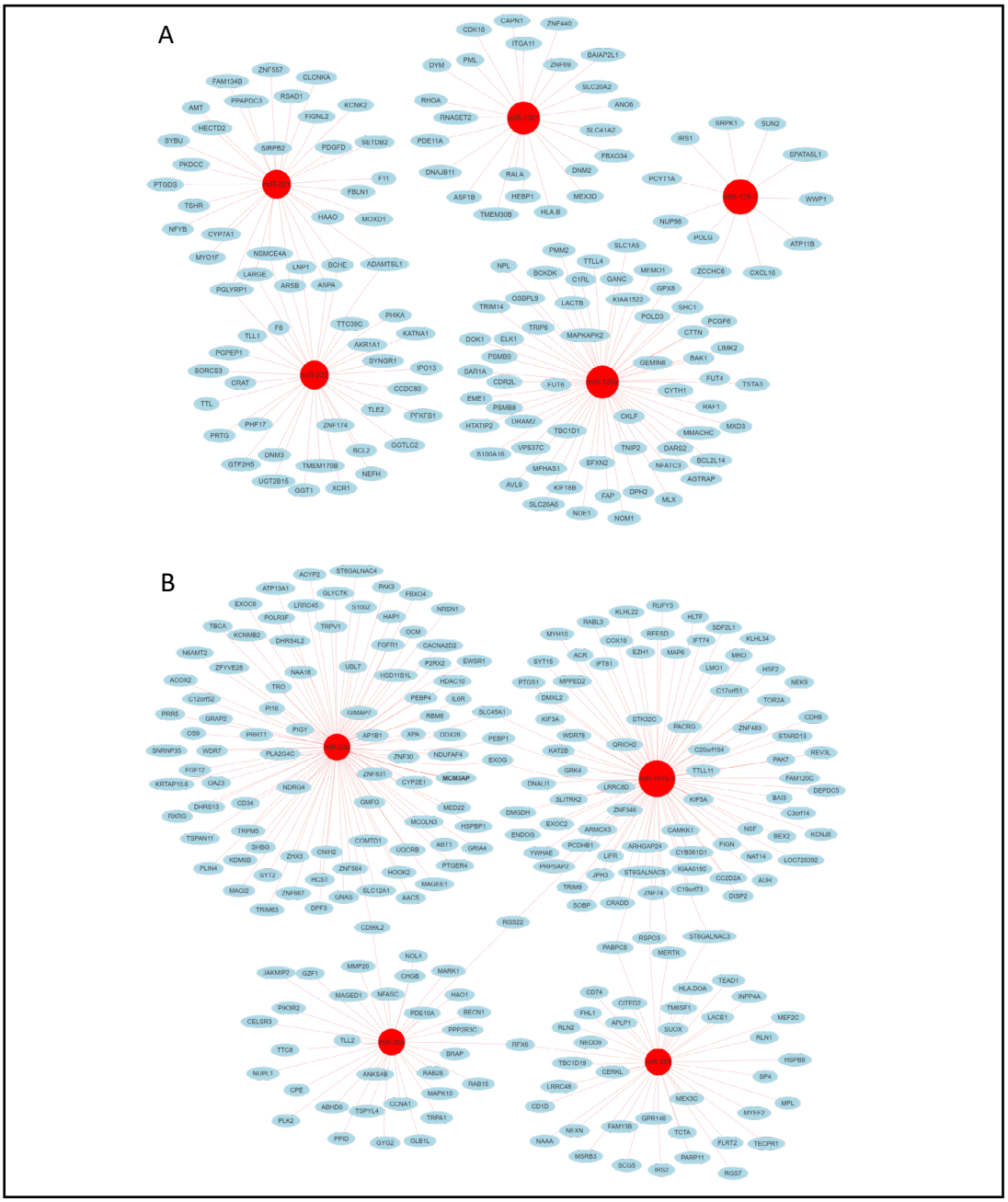


Fig. 6. A and B. The interaction network between nine miRNAs (miR-23a, miR-30a, miR-181b-1, miR-203 miR-125a, miR-129-1, miR-221, miR-222, and miR-1301) and target genes. These miRNAs could match 3' untranslated region of predicted genes on the basis of miRWalk 2.0 database. In addition, a statistically negative correlation between mRNA of these genes with miRNA expression was validated with pancreatic ductal adenocarcinoma data in The Cancer Genome Atlas. The red circles and blue ellipses represent miRNAs and genes, respectively.

Identification of prognostic miRNAs for PDAC

We obtained clinical and miRNA expression data from TCGA to evaluate whether the top 50 miRNAs predicted the prognosis of PDAC patients. We examined the top 50 miRNAs using Kaplan-Meier survival analysis and found that the expression level of 9 miRNAs (miR-23a, miR-30a, miR-125a, miR-129-1, miR-181b-1, miR-203, miR-221, miR-222, and miR-1301) had significant associations with the OS of patients (Fig. 5A). The fold change (tumor divided by normal) of the expression value of the nine miRNAs is presented in Fig. 5B. miR-23a, miR-221, miR-181b-1, miR-222, and miR-203 were likely to be overexpressed in PaCa samples, and the high level of these five miRNAs were associated with poor survival, suggesting that their function was probably related to tumor promoting activity. Conversely, miR-129-1 and miR-1301 tended to be lowly expressed in tumor samples, and the high levels of the two miRNAs were linked with better survival, which indicated that they were likely to be associated with tumor inhibition. In the acquired data, it appeared that miR-30a was lowly expressed in tumor samples; however, in PDAC patients, the low level of this miRNA was related to good survival. Similarly, miR-125a appeared as a protective factor in PDAC patients; however, in most of the nine profiling studies, it was overexpressed in tumor samples.

Using PDAC data of TCGA, we divided the patients into high and low expression groups according to mean miRNA expression values to determine which miRNA was the independent factor in prognosis prediction. Then, we combined the nine miRNAs expression levels with clinical pathological factors (age, sex, tumor dimension, histological grade, the number of positive lymph nodes, radiation, resection status, pathological stage, TNM classification and molecular target drug therapies) to perform multivariate analysis using a Cox hazard regression model. We demonstrated that miR-30a (HR=2.43, 95% CI 1.05-5.59; p=0.037) and miR-203 (HR=3.14, 95% CI 1.28-7.71; p=0.012) were significant independent risk factors for poor survival (Supplementary Table 2 and 3). Our results highlighted that miR-30a and miR-203 had potential diagnostic and prognostic significance for PDAC patients.

Pathways and GO enrichment of miRNA target genes

We revealed the potential function of the aforementioned nine miRNAs (miR-23a, miR-30a, miR-125a, miR-129-1, miR-181b-1, miR-203, miR-221, miR-222, and miR-1301) by using the miRWalk2.0 database to screen out putative genes in which the 3' UTR matched the miRNA seeds (p<0.01). Then, by combining the mRNA and miRNA profiling assays of PDAC patients in TCGA, we determined that 405 genes were significantly negatively correlated with these miRNAs (p<0.01). The miRNA target gene regulatory network was constructed and visualized with Cytoscape software (Fig. 6). In this network, it appeared that the EXOG, SLC45A1, and PEBP1 genes were targeted by miR-23a and miR-181b-1. RSPO3, MERTK, and PABPC5 were regulated by miR-181b-1 and miR-203. KEGG pathway

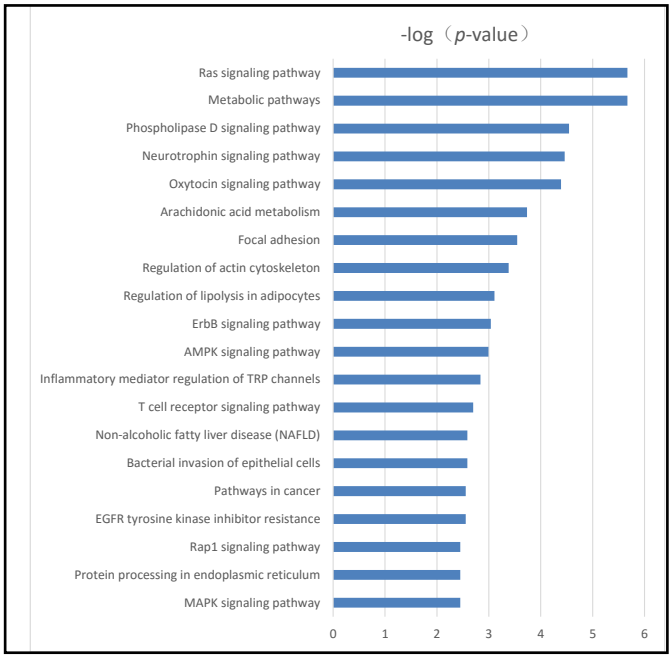


Fig. 7. The top twenty Kyoto Encyclopedia of Genes and Genomes pathway enrichments for the target genes of miR-23a, miR-30a, miR-125a, miR-129-1, miR-181b-1, miR-203, miR-221, miR-222, and miR-1301.

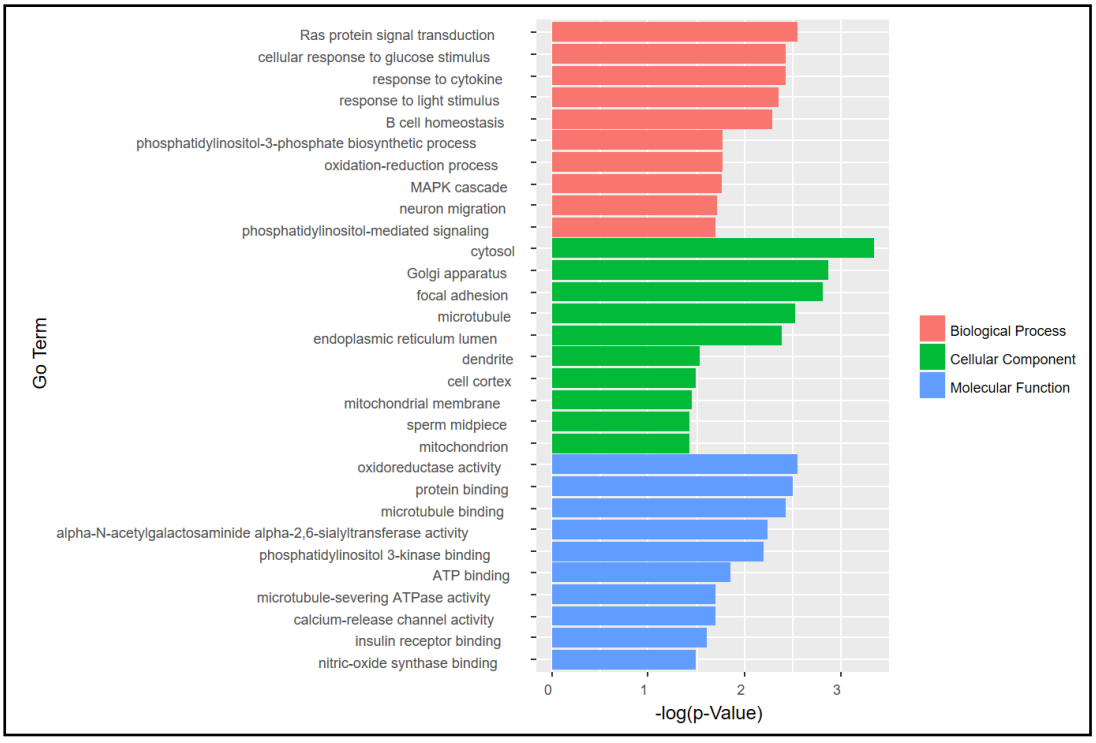
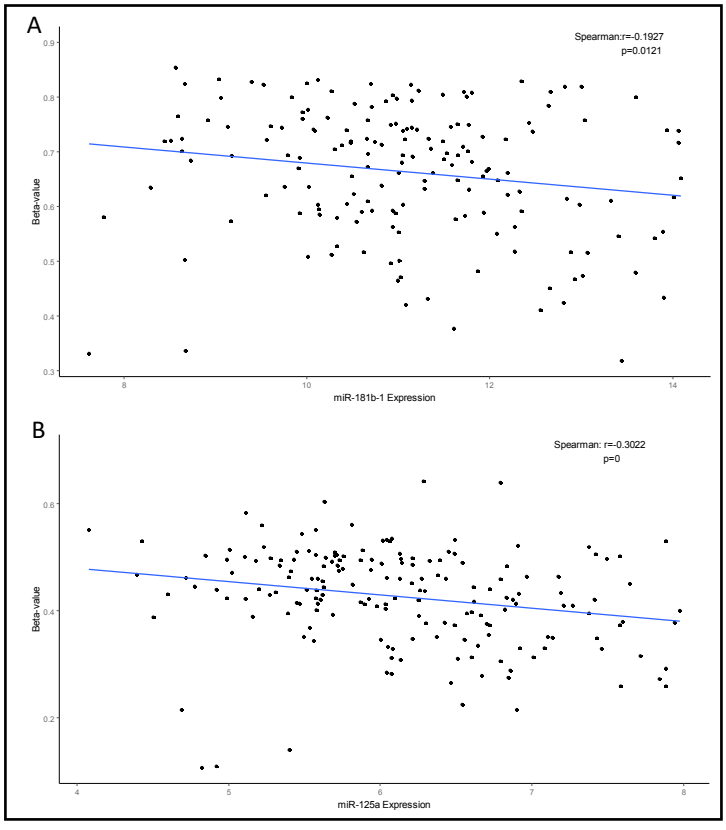


Fig. 8. The top ten Gene Ontology functional annotation terms for the target genes of miR-23a, miR-30a, miR-125a, miR-129-1, miR-181b-1, miR-203, miR-221, miR-222, and miR-1301.

Fig. 9. A and B. The expression of miR-181b-1 and miR-125a negatively correlated with the methylation status of the 3.5-kb genomic region upstream of the transcription start sites.



enrichment was performed with these target genes, and the top 20 pathways are presented in Fig. 7. The Ras signaling pathway, phospholipase D signaling pathway, and AMP-activated protein kinase signaling pathway were highly enriched, all of which play important roles in tumorigenesis.

We carried out GO classification enrichment analysis and presented the top ten terms in Fig. 8. to understand the biological function of the target genes. It was noted that Ras protein signal transduction was highly enriched in biological processes (GO: 0007265, $p<0.01$). Cytosol (GO: 0005829, $p<0.01$) and oxidoreductase activity (GO: 0016491, $p<0.01$) were highly enriched in cellular components and molecular functions, respectively.

Hypermethylation of the promoter region was part of the epigenetic regulatory mechanism for miRNA expression. We extracted the methylation data of PDAC patients from TCGA, which contained 450K probes marked with beta values indicating the whole genome sequence methylation status. We used miRBase data (http://www.mirbase.org/) to obtain the gene sequence sites encoding the nine miRNAs. In miR-181b-1 (chr1: 198858873-198858982 [-]) and miR-125a (chr19: 51693254-51693339 [+]), the CpG islands (CpGIs) within 3.5 kb upstream of miRNAs were detected, and the negative correlations between methylation status and miRNAs expression were significant (Fig. 9).

Discussion

We conducted a meta-analysis using ten miRNA expression profiling studies to systematically evaluate the diagnostic value of miRNAs in PaCa. We pooled the sensitivity and specificity of 693 miRNA measured in more than 9 profiles. Then, a summary ROC curve was constructed, and the AUC was calculated for each of the 693 miRNAs. According to the AUC value, we screened out the top 50 miRNAs as candidates for PaCa diagnosis. Among them, 9 miRNAs were associated with the OS of PDAC patients according to the results of Kaplan-Meier survival analysis based on TCGA data. We identified that miR-30a and miR-203 were independent risk factors for the poor survival of PaCa patients based on a multivariate Cox regression model. Bioinformatics analysis was conducted and included target gene prediction and pathway and GO enrichments.

Differential miRNA expression screening between PaCa and normal samples has been widely used to identify biomarkers for PaCa diagnosis [34, 35]. However, the heterogeneity among different microarray datasets and platforms makes it difficult to compare the results of various miRNA expression profiling studies. Meta-analysis based on summary ROC was prevalent in medical diagnostic tests. Data were pooled from multiple sources, and biases were reduced by weighting the results [36]. Enlightened by this method, we pooled the sensitivity and specificity of miRNA profiles and constructed a summary ROC curve. Thus, we could assess the predictive value of miRNA based on AUC values. Our work provided a method for the systematic evaluation of miRNA in PaCa diagnosis and found several potential target miRNAs (miR-23a, miR-30a, miR-125a, miR-129-1, miR-181b-1, miR-203, miR-221, miR-222, and miR-1301). Among them, miR-129-1, miR-181b-1, and miR-1301 were not reported in the PaCa studies that we retrieved from PubMed. Thus, our work, which was based on ten miRNA profiling assays, provided convincing evidence for intensive exploration of the molecular mechanisms of the nine miRNAs.

miR-203 is known as a skin-specific miRNA; it is differentially expressed in some cancers and plays different roles in various tumors. It was reported that miR-203 was significantly overexpressed in PaCa tissue relative to normal samples [37]. Ren's work suggested that miR-203 downregulated SIK1 expression and promoted cell proliferation, migration, and invasion in PaCa cells [38]. A recent meta-analysis revealed that an increased level of miR-203 was associated with poor OS in PaCa patients [39]. Our study demonstrated that miR-203 was measured in nine profiling studies in 925 samples and that it tended to be upregulated in PaCa; it had a pooled SEN of 0.52, SPE of 0.85, and AUC of 0.78, which distinguished PaCa from controls. Based on TCGA data, miR-203 was an independent risk

factor for PDAC patients (HR=3.14, 95% CI 1.28-7.71; p=0.012). Consequently, miR-203 is a potential biomarker for PaCa diagnosis and prognosis, and it is worthwhile to investigate its regulatory mechanism.

The function of miR-30a has been inconsistently reported in different cancers. For example, miR-30a was found to target heterochromatin protein (HP1γ) and suppress colorectal cancer growth [40]. Similar results were revealed in hepatocellular carcinoma (HCC), breast cancer, and gastric cancer [41-43], which suggested that miR-30a served as a tumor inhibitor. However, in non-small cell lung cancer (NSCLC) and glioma patients, plasma miR-30a was significantly overexpressed relative to the benign control group [44, 45]. Our study utilized acquired data to demonstrate that miR-30a was expressed at a low level in PaCa with a pooled SEN of 0.6, SPE of 0.80 and AUC of 0.78, which could distinguish PaCa from controls. In PDAC patients in TCGA, a high level of miR-30a in tumor tissues was associated with poor survival (HR=2.43). Further studies are needed to reveal the function and regulatory mechanisms of miR-30a.

It was reported that miR-181b-1 contributed to tumor initiation and progression in colon, lung, and HCC [46-48]. In mouse xenografts, miR-181b-1 was activated by the signal transducer and activator of transcription 3 (STAT3) and had a pronounced effect on tumor growth [49]. miR-181b-1 was complementary to the cylindromatosis tumor suppressor gene (CYLD) 3'UTR, and the expression of miR-181b-1 was inversely correlated with CYLD levels. Luciferase reporter assay revealed that miR-181b-1 bound to the 3'UTR and inhibited CYLD mRNA and protein expression [49]. In human colon adenocarcinomas, a positive correlation between STAT3 and miR-181b-1 was observed, suggesting that the miR-181b-1/CYLD pathway was relevant in human cancers [49]. In the current study, we identified that miR-181b-1 was likely to be overexpressed in most miRNA profiling studies and had moderate predictive value for PaCa (SEN of 0.62, SPE of 0.81, and AUC of 0.81). During cancer formation, miRNAs are regulated by epigenetic modifications, such as methylation and histone modifications [50, 51]. Interestingly, the negative correlation between miR-181b-1 expression and methylation status within the 3.5-kb genomic region upstream of the transcription start site was significant (r=-0.19; p=0.01). A similar result was found for miR-125a (r=-0.3; p<0.01), which supported the idea that epigenetic alteration may downregulate miRNA expression [52].

In the current study, miR-221 and miR-222 were mainly overexpressed in tumor samples and had moderate predictive value (AUC of 0.79 and 0.81 respectively). In the TCGA data, PDAC patients with high levels of miR-221 and miR-222 had worse OS than patients in the low expression group. Previous studies showed that miR-221 was overexpressed in PaCa tissues and associated with distant metastasis [53, 54], which suggested that this miRNA might serve as a biomarker for the diagnosis of PaCa [55]. In addition, it was reported that miR-221/222 induced the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9 [56], targeting the phosphatase and tensin homolog-protein kinase B (PTEN-Akt) pathway [57]. In pancreatic cells, miR-221 was essential for the PDGF-mediated EMT phenotype [58], and overexpression of mir-221-3p promoted 5-FU resistance [59]. Altogether miR-221 and miR-222 are generally considered to be oncogenic miRNAs.

It is interesting that the role of miR-1301 in cancer is disputed. It has been shown that miR-1301 expression was downregulated in HepG2 cells and that this miRNA mediated cell apoptosis via Wnt/β-catenin signaling by targeting B-cell CLL/lymphoma 9 protein (BCL9) [60, 61]. However, Liang's study revealed that miR-1301 negatively regulated tumor suppressor full-length Kruppel-like factor 6 (KLF6-FL) and induced cellular migration and angiogenesis [62]. In prostate cancer cells, this miRNA promoted proliferation by inhibiting PPP2R2C and facilitated the expansion of cancer stem cells by suppressing GSK3β and SFRP1 [63, 64]. In contrast, another study reported that miR-1301 downregulated prostate cancer migration and invasion by regulating the UBE4B-p53 pathway [65]. Similarly, miR-1301-3p played a prohibitive role in glioma cells by directly targeting N-Ras [36]. Thus, the role of miR-1301 in tumorigenesis was discordant in different cancers and even within the same cancer. In PaCa, we found that miR-1301 was lowly expressed in most miRNA expression

profiling studies. In the PDAC patients in TCGA, the OS of the high expression group was significantly better than that of the low expression group, which suggested that miR-1301 was related to tumor suppressive activity. Consequently, molecular biology experiments are needed to elucidate the regulatory mechanisms of miR-1301 in PaCa.

The results of metaregression for miR-130b revealed that the diagnostic sensitivity of tumor tissue was superior to that of serum samples. The main method to obtain pathological detection preoperatively was the fine needle aspiration biopsy; however, it is not routinely used in clinical practice because it induces trauma. Exosomes are small microvesicle secreted by parent cells and contain miRNA, protein, lipids and nucleic acids. It was reported that miR-21 and miR-1246 were enriched in human breast cancer [66]. Sohn W's studies revealed that the levels of circulating exosomal miR-181, miR-221, and miR-224 were significant higher in HCC patients than in chronic hepatitis B patients; however, there were little differences in the level of circulating miRNAs between these two groups [67]. The level of circulating exosomal miR-10b and miR-30c was significantly elevated in 29 PDAC patients relative to chronic pancreatitis patients; however, the level of CA 19-9 was normal or slightly increased in 8 cases [68]. Goto T's study showed that circulating exosomal miR-191, miR-21, and miR-451a levels were superior to circulating miRNA levels for differentiating PDAC patients from intraductal papillary mucinous neoplasm (IPMN) patients [69]. Above all, it appears that circulating exosomal miRNA has a potential advantage over circulating miRNAs in PDAC diagnosis, whereas more work is required to compare its diagnostic value to that of tumor tissue.

Several pathways associated with tumorigenesis were enriched with target genes of the nine miRNAs. For example, in the Ras pathway, GF, RTK, MAGI, PAK, PLA, and Gs were putative target genes for miR-23a that were negatively correlated with the expression of miR-23a according to TCGA data. miR-23a was reported as an oncogenic miRNA in many cancers [70]. The results of our study encourage us to design an in-depth experiment to discover the regulatory function of miR-23a in the Ras pathway. We found that the mRNA expression of Rho was inversely correlated with the value of miR-1301 in PDAC tissue samples in TCGA data and that miR-1301 could match the 3'-UTR of this gene sequence based on an algorithm. Activation of the Rho/ROCK pathway was reported to be related to tumor invasion in colorectal, gastric, and ovarian cancers [71-73]. Our study provided the insight that miR-1301 may limit activation of the Rho/ROCK pathway by reducing the expression of Rho, but further experimental validation is needed. One gene can be regulated by multiple miRNAs, and we noted that the PI3K pathway may be regulated by miR-23a, miR-30a, miR-129, and miR-222; similarly, the Ras signaling pathway may be targeted by miR-1301, miR-30a, and miR-23a according to our correlation analysis and algorithm. GO term enrichment of biological processes demonstrated that putative miRNA target was the MAPK signaling pathway. Several miRNAs suppressed the MAPK pathway, prohibiting cancer cell proliferation [74, 75].

To the best of our knowledge, this study was the first to systematically explore the diagnostic role of miRNAs with extensive miRNA profiling data. It was helpful to screen out promising miRNAs that could be used to potentially diagnose PaCa. Furthermore, we used survival analysis to narrow-down the miRNAs and identified that miR-23a, miR-30a, miR-125a, miR-129-1, miR-181b-1, miR-203, miR-221, miR-222, and miR-1301 had diagnostic and prognostic potential for PaCa patients. Importantly, miR-181b-1 and miR-1301 have not been reported in PaCa studies. Thus, intensive clinical validation and pathway exploration of these two miRNAs are worthwhile to discover novel biomarkers for PaCa. There were some limitations to the current study: first, 1370 miRNAs were measured in less than 4 profiles and were not included in diagnostic assessment because of their low reliability. Some candidates with predictive value for PaCa might be found in these miRNAs. Second, miRNA is a recently discovered posttranscriptional regulatory factor, and the profiling assay data for PaCa are limited. Until now, it has been difficult to extensively evaluate all miRNAs in PaCa diagnosis. In addition, publication bias was uncertain since some miRNA studies did not publish their expression data.

Conclusion

We systematically evaluated the predictive values of 693 miRNAs for PaCa based on ten miRNA expression profiling studies. We noted nine miRNAs (miR-23a, miR-30a, miR-125a, miR-129-1, miR-181b-1, miR-203, miR-221, miR-222, and miR-1301) with diagnostic and prognostic practicability for PaCa. The Ras signaling pathway and hypermethylation of the promoter region were probably associated with the downstream target and the upstream epigenetic regulatory mechanism of these miRNAs, respectively.

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Disclosure Statement

The authors disclose no potential conflict of interests.

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