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Systematic review: exosomal microRNAs associated with pancreatic cancer for early detection and prognosis

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Abstract. – OBJECTIVE: Pancreatic cancer (PC) is one of the most common malignant tumors of the digestive system with a high degree of malignancy. Currently, there have been many studies on exosomal microRNAs (miRNAs) discovery in pancreatic cancer. This systematic review aimed to give an overview about known exosomal miRNAs and discuss their diagnostic performance, as well as prognostic value in PC.

MATERIALS AND METHODS: PubMed and Web of Science were used for systematic literature research for this review. This literature research was mainly to identify studies that performed plasmatic and serological testing for exosomal miRNAs in pancreatic cancer patients and controls. Two independent reviewers separately extracted data on study characteristics and results.

RESULTS: In total, nine prior studies were included in this review. Of which, eleven different single exosomal miRNAs and three exosomal miRNA panels were reported.

CONCLUSIONS: When single exosomal miRNA was used as a diagnostic tool, the specificity is generally high, but the sensitivity is commonly low. When multiple of exosomal miRNAs were used simultaneously, higher sensitivities can be obtained at relatively reasonable specificity levels with certain miRNA combinations. Developing a combination of miRNA markers may be a promising approach for early detection of pancreatic cancer.

Key Words:
Exosome, Biomarker, Pancreatic cancer, MicroRNA, Early cancer detection, Prognosis.

Introduction

Pancreatic cancer (PC) is the seventh leading cause of cancer death from malignant tumors worldwide, although the incidence only accounts for 2.5% of cancers[1,2]. The five-year cause-specific survival of PC is only 8.5%, which is the lowest among all major cancer types[3,4]. It is estimated that, by 2025, death from PC may become the third leading cause of death from cancer overall in the EU after lung and colorectal cancers[5]. In 2030, it will be the second leading cancer-related cause of death, after lung in the US[6]. Currently, computed tomography (CT), magnetic resonance imaging (MRI), and endoscopic retrograde cholangiopancreatography (ERCP) technology are the primary diagnostic tools for PC detection[7,8]. In addition to extremely limited capability for early detection, those tools are associated with significant limitations: affordability, lack of comfortability, and radiation exposure. Patients with suspected pancreatic cancer are typically in advanced stage when they can be diagnosed with current technology[9]. Although some circulating tumor markers, such as CA19-9, CA-50, CEA, have been used clinically, their sensitivity still is relatively limited[10-12]. Therefore, finding efficient and sensitive biomarkers would be the critical step in reducing the mortality rate of pancreatic cancer[7,13]. Exosomes are secreted extracellular vesicles (EV) with sizes ranging between 30 to 100 nm[14,15]. Although exosomes

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are made by most cell types, some tumor cells can secrete 10 times as much exosomes as normal cells[16-18]. Exosomes were known to be important messengers for long-range cell-to-cell communication[19-21]. Within the lipid bilayer membrane, various proteins and nucleic acids are contained in exosomes[14,22,23]. These "cargoes" can be harvested at high concentrations for subsequent analysis, after easy exosome isolation. In addition, exosomal miRNAs are protected from plasma and cellular RNases[24-27]. Thus, exosomal miRNAs can be readily profiled either from serum or from other body fluids (such as saliva and urine)[28,29]. All of these indicate that exosomal miRNAs have potential in tumor screening, diagnosing, and following-up various cancers[29,30].

Materials and Methods

Search Strategy

A comprehensive systematic literature search was conducted on PubMed and Web of Science up to May 25, 2019. The preferred reporting items for the Systematic Review and Meta-analysis (PRISMA) statement were used as the reference standard[31]. The search strategy was as follows: (exosome OR exosomal OR extracellular vesicles OR membrane vesicles OR intracellular multivesicular endosomes) AND (pancreatic OR pancreas) AND (microRNAs OR miRNAs OR micro RNA OR miRNA) AND (neoplasm OR neoplasms OR cancer OR cancers OR adenocarcinoma).

Eligibility Criteria

We excluded duplicate and non-English publications at first. The remaining eligible studies must meet the following criteria: [1] the articles must be about pancreatic cancer; [2] the miRNA must be present in the exosomes; [3] the article must explore the relationship between exosomal miRNAs and pancreatic cancer; [4] sensitivity, specificity, and area under the receiver operator characteristic curve (AUC) were provided. Articles that meet one of the following criteria were excluded: [1] reviews; [2] mechanistic studies; [3] studies without controls; [4] the article without sensitivity, specificity, or AUC.

Data Extraction

Two curators retrieved the data and another supervised the process independently. The information extracted from the selected studies shows as follows: first author, publication years, country, study design, sample size, study population characteristics (gender, age), exosomal miRNAs that were differentially expressed between cases and healthy controls (or benign disease), and indicators that used to assess the diagnostic performance (sensitivity, specificity, AUC, and *p*-value).

Quality Assessment

To minimize the biases caused by various reasons, we evaluated the quality of screened articles. Two independent curators applied the Diagnostic Accuracy Research Tool Quality Assessment Tool (QUADAS-2) to assess the quality of the included articles[32]. The QUADAS-2 consists of four key domains: *Patient Selection, Index test(s), Reference Standard, and Flow and Timing*. Each domain contains four major aspects of risk assessment and applicability assessment.

Results

There are 63 papers found through PubMed query and 93 from Web of Science. 27 articles were duplicated or non-English publications (Figure 1). 67 articles were excluded for thematic irrelevance after manual screening titles and abstracts. By reading the full text, 62 articles were selected, but only 13 studies among those included qualitative syntheses. The articles were excluded based on the following reasons: mechanistic research (n=14); without exosomal miRNAs (n=20); reviews (n=13); without full-text access (n=3). In the remaining 12 studies, two studies lack sensitivity and specificity and one is without suitable control. Finally, only nine articles included for synthesis in this review[33-40]. QUADAS-2 analysis indicates that the nine selected articles had a low risk and higher applicability in four parts **(Supplementary Table I)**.

Characteristics of the Included Studies

The main characteristics of the included studies are shown in Table I. 11 single exosomal miRNAs were investigated in the studies included in this review. Three extinct exosomal miRNA panels were also reported (Table I, Table II). Some biomarkers were evaluated in more than three articles and some were only in one article. The studies contained in this review were mainly conducted in Asia and America. Patients with PC were on average more than 60 years old in case-cohort. The number of females was more than fifty percent, either in the case or control cohort. In each study included, patients with PC were staged by TNM classification.

Table I. Characteristics of the studies included in this review.

Exosomal miRNAs	First author[ref]	Country	Study population characteristics		Platform		p-value	Biofluids	Difference (case vs. control)
			Case (stage I, IIa, IIb III, IV); mean/median age (range)	Controls; mean/median age (range)	Exosome isolate	Identify miRNA expression			
miR-21	Que et al[38]	China	22 PC, 65.3±9.99	6 AC, (40.7 ± 8.99); 7 BPTs, (67 ± 6.19) 6 CP, (63.8 ±12.67) 8 HP, (60.3 ± 8.08)	Ultra-centrifugation	QRT-PCR	0.215	Serum	↑
	Goto et al[36]	Japan	32 PC, 62.0 ±10.1 (2/7/4/5/14)	29 IPMN, (73.8±7.8); (14/11/4)	Exo-quick	QRT-PCR	<0.001	Serum	↑
	Nakamura et al[27]	Japan	27 PDAC, 71 (47–79) (2/3/13/2/7)	8 CP 59.5 (38–79)	Ultra-centrifugation	QRT-PCR	<0.001	Pan-Juice	↑
	Kawamura et al[40]	Japan	55PDAC 67 (66±11) (4/27/24--)	20 HP	Ultra-centrifugation	QRT-PCR	<0.05	Serum	↑
miR-451a	Goto et al[36]	Japan	32 PC (64.0±10.1) (2/7/4/5/14)	29 IPMN (73.8±7.8); (14/11/4)	Exo-quick	QRT-PCR	<0.001	Serum	↑
	Takahasi et al[37]	Japan	50 PDAC	20 HP	Ultra-centrifugation	QRT-PCR	0.001	Serum	↑
	Kawamura et al[40]	Japan	55 PDAC 67 (66±11) (4/27/24--)	20 HP	Ultra-centrifugation	QRT-PCR	<0.05	Serum	↑
miR-17-5P	Que et al[38]	China	22PC; (65.3±9.99)	6 AC, (40.7 ± 8.99); 7 BPTs, (67 ± 6.19) 6 CP, (63.8 ±12.67) 8 HP, (60.3 ± 8.08)	Ultra-centrifugation	QRT-PCR	<0.001	Serum	↑
miR-196a	Xu et al[35]	USA	15 PC, 66.66 (7/8/---);	15 HP	Exo-quick	QRT-PCR	<0.001	Serum	↑
miR-1246	Xu et al[35]	USA	15 PC, 66.66 (7/8/---)	15 HP	Exo-quick	QRT-PCR	<0.001	Serum	↑
	Machida et al[34]	Japan	9 PC, 66 (53-83)	13 HP, 65 (45-84)	Reagent	QRT-PCR	<0.001	Salivary	↑
miR-4644	Machida et al[34]	Japan	9 PC, 66 (53-83);	13 HP, 65 (45-84)	Reagent	QRT-PCR	<0.001	Salivary	↑
miR-191	Goto et al[36]	Japan	32 PC, 62.0 ±10.1 (2/7/4/5/14)	29 IPMN, (73.8±7.8)	Exo-quick	QRT-PCR	<0.001	Serum	↑
miR-122-5p	Zhou et al[39]	China	31PC	37 NC	Exo-quick	QRT-PCR	<0.001	Plasma	↑
miR-193b-3p	Zhou et al[39]	China	31PC	37 NC	Exo-quick	QRT-PCR	<0.001	Plasma	↑
miR-155	Nakamura et al[27]	Japan	27 PDAC, 71 (47–79) (2/3/13/2/7)	8 CP 59.5 (38–79)	Ultra-centrifugation	QRT-PCR	0.008	Pan-Juice	↑
miR-4525	Kawamura et al[40]	Japan	55 PDAC, 67 (66±11) (4/27/24--)	20 HP	Ultra-centrifugation	QRT-PCR	<0.05	Serum	↑

AC: Ampullary cancer; BPTs: Benign pancreatic tumor; CP: Chronic pancreatitis; HP: Healthy participants; NC: Normal controls; NET: pancreatic neuroendocrine tumors; Pan-juice: pancreatic juice; ↓: lower expressed in case group; ↑: overexpressed in case group; Intraductal papillary mucinous neoplasm (IPMN).

Table II. The diagnostic performance of single exosomal miRNAs (PC vs. non-PC).

Exosomal miRNAs	First author (Ref)	Country	Roles	Cases (PC)	Controls (non-PC)	Se (%)	Sp (%)	*p*-value	AUC
miR-17-5p miR-21	Que et al[38]	China	Diagnostic Diagnostic	22 22	27 27	92.6 81.5	72.7 95.5	<0.001 <0.001	0.887 0.897
miR-196a, miR-1246	Xu et al[35]	USA	Diagnostic Diagnostic	7 7	15 15			0.010 0.021	0.81 0.73
miR-191, miR-21, miR-451a	Goto et al[36]	Japan	Diagnostic Diagnostic Prognostic	32 32 32	29 29 29	71.9 80.7 65.6	84.2 81.0 85.7	<0.001 <0.001 <0.001	0.788 0.826 0.769
miR-1246, miR-4644,	Machida et al[34]	Japan	Diagnostic Diagnostic	9 9	13 13	66.7 75.6	100 76.9	0.008 0.020	0.814 0.763
miR-122-5p, miR-193b-3p,	Zhou et al[39]	China	Diagnostic Diagnostic	31 31	37 37	/	/	0.002 0.032	0.722 0.651
miR-21 miR-155	Nakamura et al[27]	Japan	Diagnostic Diagnostic	27 27	8 8	81 89	88 88	0.001 0.008	0.90 0.89
miR-21 miR-451a miR-4525	Kawamura et al[40]	Japan	Prognostic Prognostic Prognostic	55 55 55	20 20 20	72.7 72.7 81.8	72.7 77.3 86.4	<0.05 <0.05 <0.05	
miR-451a	Takahasi et al[37]	Japan	Prognosis	50	20	69.2	70.8	<0.001	

Abbreviations: *p*-value, (PC *vs.* non-PC); Se=sensitivity; Sp=specificity; AUC: receiver operator characteristic curve (AUC); PC: pancreatic cancer patients. non-PC: non pancreatic cancer patients.

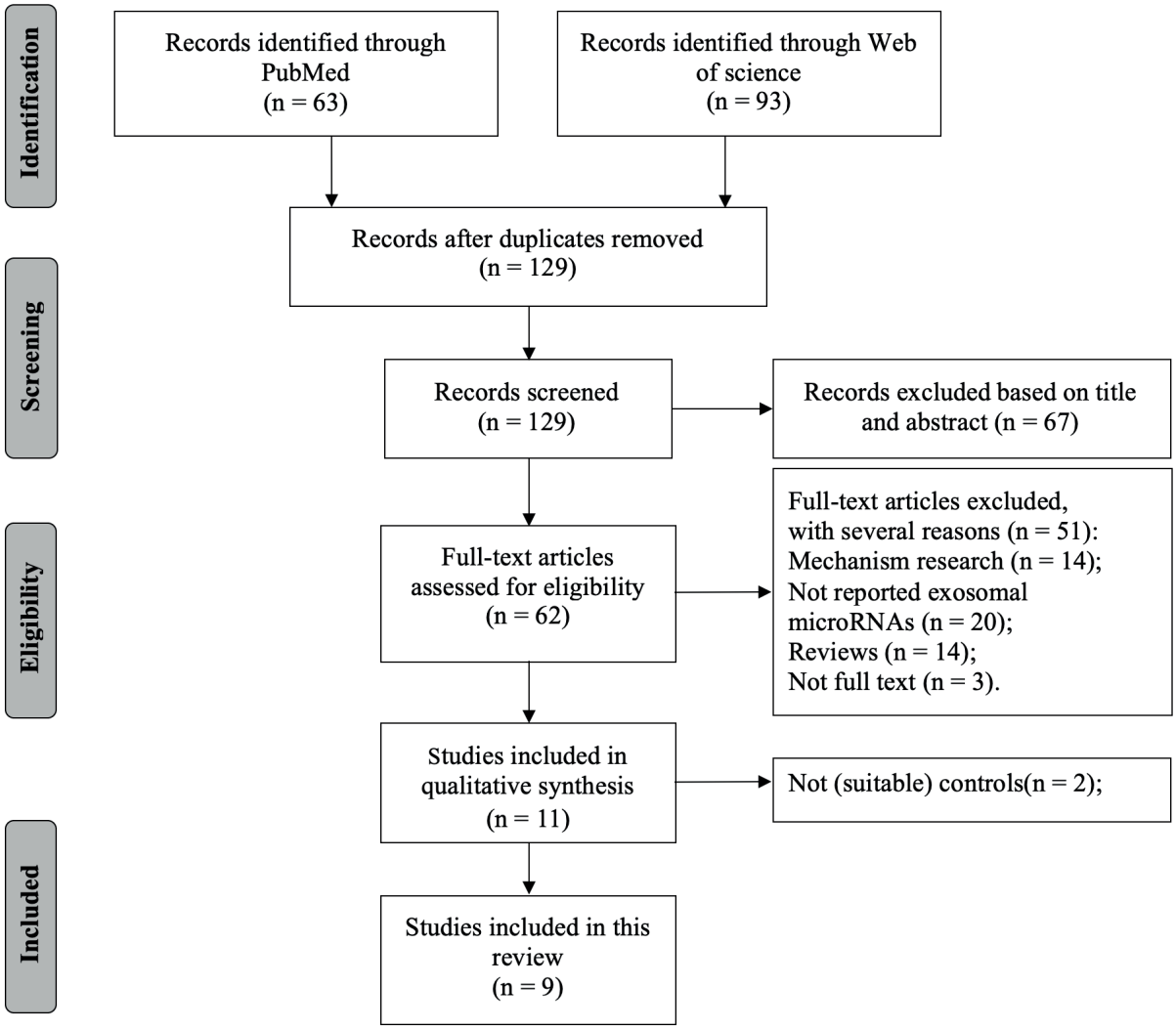

Figure 1. Flow chart of literature selection process.

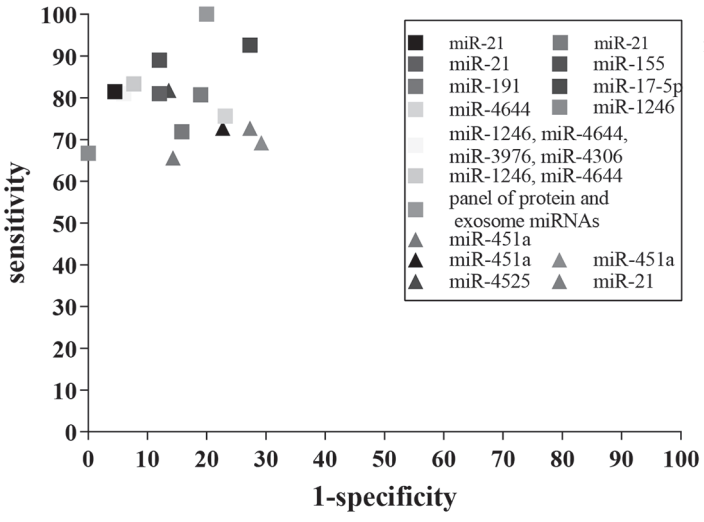

Figure 2. The diagnostic (or prognostic) performance of the candidate biomarkers.
▲ The performance of the diagnostic biomarke;
■ The performance of the prognostic biomarker

Table III. The diagnostic performance of the exosomal miRNAs panels in this review.

Panels	First author[ref]	Cases	Controls	Biofluid	Diagnostic performance		
					Se (%)	Sp (%)	AUC
miR-122-5P, miR-193b-3P	Zhou et al[39]	31	37	Plasma	/	/	0.767
miR-1246, miR-4664	Machida et al[34]	9	13	Salivary	0.833	0.923	0.833
miR-1246, miR-4644, miR-3976, miR-4306	Madhavan et al[33]	131	89	Serum	0.81	0.94	0.994
miR-1246, miR-4644, miR-3976, miR-4306, CD44v6, tspan8, EPCAM, MET, CD104	Madhavan et al[33]	131	89	Serum	1.0	0.80	/

Abbreviations: *p*-value: (PC *vs.* non-PC); Se=sensitivity; Sp=specificity; AUC: receiver operator characteristic curve (AUC); Case: pancreatic cancer (PC); Controls: non-PC (included healthy participants, chronic pancreatitis).

Detection Methods of Exosomal MiRNAs

In this review, five articles investigated exosomal miRNAs harvested from plasma, and others were collected either from serum or from salivary (two studies were from pancreatic juice; Table I). In three studies[27,34,38], the candidate exosomal miRNAs were selected from previous studies. Two articles selected the candidate miRNAs using microarrays miRNAs analysis and next-generation sequencing analysis[33,36]. In the remaining articles, one study validated their expression in plasma exosomes after screening candidate circulating miRNAs in relevant cell lines and another was to screen plasma circulating miRNAs through the mirVana PARIS kits and detected their expression levels in exosomes[35,39]. The methods of exosome extraction preparation were mainly Exoquick Exosome Precipitation Solution (System Biosciences, Mountain View, CA, USA). Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was the common validation method for miRNAs expression in exosomes.

Diagnostic Value of Single Exosomal MiRNAs

Table II and Figure 2 display the diagnostic value of single exosomal miRNAs for PC detection. Exosomal miR-21 (exo-miR-21), as diagnostic biomarkers, were investigated in four studies and exo-miR-451a were investigated in three investigations[27,36-38,40]. For exo-miR-21, the sensitivity ranged from 72.7% to 81.5% and the specificity ranged from 72% to 95.5%. For exo-miR-451a, the sensitivity and specificity are shown in Table II (sensitivity: 65.6% to 72.7%, specificity: 70.8% to 77.3%). Although exo-miR-1246 was also evaluated in two studies[34,35], its diagnostic value could only be obtained directly from one study (specificity: 66%, specificity: 100%). For exo-miR-17-5p, the sensitivity and specificity were 92.6%, 72.7% separately. For exo-miR-191, the sensitivity was 71.9% when the specificity up to 84.2%[36,38]. For exo-miR-4644, the sensitivity was 75.6%, and the specificity was 76.9%[33]. The sensitivity and specificity, for exo-miR-155, were 89% and 88%, respectively[27]. Exo-miR-1246 has the highest specificity (100%) and the sensitivity was only 66.7%[33]. For exo-miR-4525, both the sensitivity (81.8%) and specificity (86.4%) were relatively reasonable[40].

Diagnostic Value of Exosomal miRNAs Panels

Three combinatorial exosomal miRNA panels were devised (Table III). Panel 1 (isolated from plasma) consists of exo-miR-122-5p and exo-miR-193b-3p. The AUC of panel 1 was 0.767[39]. Panel 2 (isolated from serum) was made of four exo-miRNAs: exo-miR-1246, exo-miR-4644, exo-miR-3976, exo-miR-4306. The diagnostic performance for this panel was relatively reasonable (sensitivity: 83%, specificity: 94%)[33]. Panel 3 (exo-miR-1246, exo-miR-4644) were isolated from salivary exosomes (sensitivity: 83.3%, specificity: 92.3%)[34]. The diagnostic performance of combining exosomal miRNAs and PC-initiating (PACIC) markers are also shown in Table III. It is notable that the sensitivity can reach up to 100% with relatively satisfactory specificity (80%)[33].

Exosomal MiRNAs Associated With Specific Tumor Stage

Two reports[36,37] evaluated exosomal miRNAs associated with specific stages of PC (Table IV).

Compared with early-stage pancreatic cancer patients, significant differences for exo-miR-21, exo-miR-451a, exo-miR-4525, exo-miR-17-5p have been found in advanced-stage[36,40]. For exo-miR-21, Kawamura et al[40] reported higher expression levels in patients with advanced-stage than in early stage. Nevertheless, Que et al[38] showed that there were no correlations for exo-miR-21 among tumor stages. For exo-mir-4525 and exo-mir-451a, both studies[38,40] found that their expression levels increased in late stage. The prognostic performance of these miRNAs was shown in Table V. They were also compared with traditional biomarkers (CEA, CA19-9).

Discussion

Since exosomal miRNAs are stably present in several body fluids, cancer-associated exosomal miRNAs have been evaluated in many studies[30,41-43] as emerging biomarkers. Therefore, cancer-associated exosomal miRNAs from current researches were summarized to provide an updated and comprehensive perspective.

Three panels and eleven single exosomal miRNAs were reported in nine studies served as PC detection (Table II and Figure 2). Exo-miR-21, exo-miR-451a, exo-miR-1246 were the most frequently evaluated biomarkers. Exo-quick kits were widely used to precipitate exosomes in reports included in this review. Both speed and intuitive nature were advantages to the use of these kits[14]. To ensure the quality of the extracted miRNAs, the exosomes were purified after they were separated from the samples. Similarly, the selection of exosomal detection kits was also important for the capture and identification of exosome[44].

In patients with cancer, there is often the aberrant expression of tumor biomarkers, including tumor-associated antigens (TAAs)[45]. In parallel with TAAs, exosomal miRNAs are differentially expressed between PC and non-PC. Among the selected investigations, exo-miR-21, exo-miR-17-5b, exo-miR-155, exo-miR-191, exo-miR-451a, exo-miR-122-3P, exo-miR-193b-3p, exo-miR-196a, exo-miR-1246, and exo-miR-4644 have substantial evidence of differential expression in pancreatic cancer. To a given extent, they have demonstrated some potential in PC detection. For exo-miR-451a, exo-miR-4542, and exo-miR-21, they may have potential as prognostic biomarkers (Figure 2). However, the clinic prospect of a single biomarker is limited, due to the lack of specificity and suboptimal diagnostic performance. For example, exo-miR-21 has been found to be associated with various cancers[46-48], making it less ideal as a specific pancreatic cancer biomarker.

Fortunately, the combination biomarkers have been explored and may overcome the current limitations of single exosomal miRNA biomarkers. For example, exo-miR-122-5p or exo-miR-193b-3p alone has a low sensitivity and specificity for PC detection, but the sensitivity of the combinatory panel has significantly improved and the specificity at a reasonable level[39] (Table III, Table V). However, the specificity of panels that only consist of two biomarkers is still clinically unsatisfactory. For instance, with a combinatory panel of exo-miR-1246 and exo-miR-4644, both the sensitivity and AUC are significantly improved. Nevertheless, the author indicated that this panel may not be ready for clinical applications in PC screening since the relative expression profiles of both case or control groups are not adequately separated[34]. It is foreseeable to combine multiple efficient exosomal miRNAs, or to combine promising proteomics markers with miRNAs. Madhavan et al[33] evaluated the diagnostic performance of an exosomal miRNA panel (exo-miR1246, exo-miR-4644, exo-miR-3976, exo-miR-4306), a protein panel (CD44v6, Tspan8, EPCAM, MET, CD104), and the combination of two panels. It is surprising that the AUC, sensitivity, and specificity in the protein/miRNA combination were significantly improved compared with each of the single panel. In the light of widely studied biomarker panels for cancer diagnosis, such as tumor-associated antigens (TAAs) with acceptable diagnosis performance[45,49-51], combining TAAs with exo-miRNAs may have broad prospects for early diagnosis of pancreatic cancer.

Some of the markers in this review can be used to identify pancreatic cancer patients either from healthy participants or from benign patients. Exo-miR-17-5p, exo-miR-21 can identify PC from non-PC participants (including ampullary carcinoma, benign pancreatic tumor, chronic pancreatitis, and healthy participants). Nevertheless, after stratification by histology, it is not ideal for applying exo-miR-21 to distinguish ampullary cancer from PC (p-value: 0.21; Table II). Compared with the conventional biomarkers CEA and CA19-9, the diagnostic performance of exo-miR-19, exo-miR-21, exo-miR-415a, was superior in the early stage. However, CA19-9 was the better choice in advanced stage (Table V).

Table IV. The difference between exosomal miRNAs and CEA, CA19-9 in PC detection.

				Stage (I, II-a)				Stage (IIb, III, IV)			
Biomarkers	First Author[ref]	Cases	Controls	Se (%)	Sp (%)	AUC	*p*-value	Se (%)	Sp (%)	AUC	*p*-value
miR-191	Goto et al[36]	32	29	66.7	84.2	0.754	0.032	78.6	76.0	0.801	0.001
miR-21	Goto et al[36]	32	29	66.7	81.0	0.742	0.004	86.4	81.0	0.862	<0.001
miR-451a	Goto et al[36]	32	29	66.7	85.7	0.735	0.044	69.6	81.0	0.768	0.002
CA19-9	Goto et al[36]	32	29	77.8	64.3	0.729	0.404	82.6	100	0.720	0.019
CEA	Goto et al[36]	32	29	55.6	64.7	0.601	0.059	60.9	76.5	0.893	<0.001

Abbreviations: *p*-value: (PC *vs.* non-PC); Se=sensitivity; Sp=specificity; AUC: receiver operator characteristic curve (AUC); Case: pancreatic cancer (PC); Controls: non-PC (included healthy participants, chronic pancreatitis).

Table V. Exosomal miRNAs that were associated with the prognosis of PC patients and compare them with CEA, CA19-9.

				OS			DFS		
Biomarkers	First Author[ref]	Cases	Regression Controls	HR coefficient	(95% CI)	Regression *p*-value	HR coefficient	(95% CI)	*p*-value
miR-451a	Takahasi et al[37]	50	20	1.61	5.03 (1.83, 7.60)	0.001	1.05	2.86 (1.33, 6.52)	0.007
miR-451a	Kawamura et al[40]	55	20	1.90	6.66 (1.87, 12.59)	0.002	1.37	3.92 (1.75, 9.98)	0.001
miR-21	Kawamura et al[40]	55	20	1.53	4.61 (1.50, 10.19)	0.006	1.27	3.57 (1.62, 9.00)	0.001
miR-4525	Kawamura et al[40]	55	20	1.67	5.29 (1.74, 12.89)	0.002	1.33	3.79 (1.78, 8.77)	0.001
CA19-9	Takahasi et al[37]	50	20	0.05	1.05 (0.43, 2.68)	0.921	0.38	2.05 (0.15, 11.56)	0.530
CEA	Takahasi et al[37]	50	20	0.42	1.52 (0.53, 3.87)	0.409	0.74	2.09 (0.87, 4.72)	0.095

Abbreviations: OS: overall survival rate; DFS: disease-free survival rate; case: pancreatic ductal adenocarcinoma (PDAC); CI: confidence interval; HR: hazard ratio; controls: healthy participates; *p*-value: between case and controls. Univariate analyses of the prognostic factors for OS and DFS (data from original studies).

Conclusions

This review has a high reference value for early PC detection and diagnosis. The combination of exosomal miRNAs and protein panels may be ideal tools for early diagnosis of PC. For prognosis, exo-miR-451a is currently the best candidate biomarker. Screening candidate exosomal miRNAs from previous studies and testing their diagnostic value for pancreatic cancer holds significant promise. Due to the high mortality rate of pancreatic cancer, it is urgent to explore early diagnostic markers to improve the survival rate from pancreatic cancer.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Author Contributions

H. Ye conducted the initial literature search and wrote this manuscript. H. Wang, P. Wang, C.-H. Song verified the results of the literature search. K.-J. Wang, L.-P. Dai, J.-X. Shi assisted with data extraction and evaluation. X.-X. Liu, C.-Q. Sun conceived this study. X. Wang, Y. Peng contributed to the statistical analysis. X.-B. Chen, J.-Y. Zhang revised the manuscript and all authors approved the submitted version.

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