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REVIEW ARTICLE

Cell-free microRNAs as Non-invasive Diagnostic and Prognostic Biomarkers in Pancreatic Cancer

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Abstract: Pancreatic cancer (PaC) is one of the most lethal cancers, with an increasing global incidence rate. Unfavorable prognosis largely results from associated difficulties in early diagnosis and the absence of prognostic and predictive biomarkers that would enable an individualized therapeutic approach. In fact, PaC prognosis has not improved for years, even though much efforts and resources have been devoted to PaC research, and the multimodal management of PaC patients has been used in clinical practice. It is thus imperative to develop optimal biomarkers, which would increase diagnostic precision and improve the post-diagnostic management of PaC patients. Current trends in biomarker research envisage the unique opportunity of cell-free microRNAs (miRNAs) present in circulation to become a convenient, non-invasive tool for accurate diagnosis, prognosis and prediction of response to treatment. This review analyzes studies focused on cell-free miRNAs in PaC. The studies provide solid evidence that miRNAs are detectable in serum, blood plasma, saliva, urine, and stool, and that they present easy-to-acquire biomarkers with strong diagnostic, prognostic and predictive potential.

Keywords: Cell-free microRNA, pancreatic cancer, diagnosis, prognosis, prediction, non-invasive biomarker.

1. INTRODUCTION

Pancreatic cancer (PaC) is one of the most lethal human malignancies worldwide, but its incidence is relatively low, accounting for 2.4% of all cancer types. Due to its aggressive growth and lack of early symptoms, PaC mortality rates closely follow incidence rates, emphasizing the very poor prognosis for this disease. Owing to its fatality, PaC is ranked as the fourth most common cause of cancer-related deaths in both sexes combined [1, 2]. Moreover, PaC is likely to become one of the major causes of cancer-related death in the near future due to its increasing global trend of incidence and a lack of progress in the management and treatment of PaC patients [1, 3].

The most common histological type of pancreatic malignancies is pancreatic ductal adenocarcinoma (PDAC). It presents the lowest survival, with only 5-7% of patients living longer than five years after diagnosis [4]. The reasons behind its fatal prognosis are rapid disease progression and early dissemination, both resulting in late diagnosis at advanced unresectable stages [5]. The current standard of PDAC care is surgery followed by adjuvant chemotherapy, which has demonstrated improved prognosis, increasing the five-year survival rate to 16-21%. However, only 15-20% of PDAC patients are diagnosed early enough to be considered candidates for surgical resection, currently the only chance for long-term survival [6-10]. Besides, a vast majority of PDAC patients undergo diagnosis at either a regional or a distant metastasized level, rendering them ineligible for tumor radical resection. Unfortunately, no satisfactory treatment is available for the advanced stages of the disease, and the median survival rate of these patients drops rapidly [11]. Improving the survival rate, thus, requires an early diagnosis at a molecular level.

Conventional diagnostic methods, including imaging methods and serum biomarkers, suffer from unsatisfactory sensitivity and specificity. Common imaging modalities-computed tomography (CT), magnetic resonance imaging (MRI), and endoscopic ultrasound EUS)-are usually introduced after the appearance of local and systemic symptoms. It is usually too late because, in most PDAC patients, the disease is already advanced.

Even though carbohydrate antigen 19-9 (CA 19-9) often fails to detect precancerous or early-stage lesions because of its inadequate sensitivity and specificity, it is routinely used to assess disease prognosis or monitor the disease. For the past few years, significant efforts have been dedicated to seek novel biomarkers to support early diagnosis of PDAC, mainly in the fields of liquid biopsy (KRAS mutations), proteomics (*e.g.*, CEMIP, C4BPA, IGFBP2, and IGF), metabolomics (*e.g.*, palmitic acid, glucitol, xylitol and inositol), and

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non-coding RNAs, including microRNAs (miRNAs) [12, 13].

MiRNAs are RNA molecules of about 18-25 nucleotides in length. They act as regulators of gene expression at a post-transcriptional level, and-unlike other classes of emerging biomarkers-they are characterized by a wide range of favorable analytical and biological properties. They are negative regulators through interactions with protein-coding mRNAs. Partial complementarity between miRNAs and 3′UTR of the target transcripts inhibits translation, while perfect complementarity results in the cleavage of the targeted mRNAs. MiRNAs have been linked to the regulation of the expression of over half of all protein-coding genes in mammals [12].

Several studies have recognized that miRNAs are involved in the regulation of cell homeostasis by controlling important cellular processes. MiRNA concentration can rapidly change in response to various stimuli [13]. Therefore, their signature can reflect early molecular changes in various diseases, including cancer. Specific miRNAs have also been found in body fluids, and it is already known that these molecules have the potential to become promising biomarkers for cancer detection in liquid biopsies. The concept of non-invasive biomarkers seems to be particularly important in the context of PaC, in which there are many unmet medical needs in terms of diagnostic and prognostic approaches.

2. CIRCULATING microRNAs: ORIGIN AND METHODS OF DETECTION

The first body fluids in which miRNAs were discovered were cell-free blood plasma and serum [14, 15]. MiRNAs were later found in cerebrospinal fluid, breast milk, colostrum, saliva, seminal fluid, tears, urine, *etc*. [16]. These molecules exhibit remarkable stability in severe conditions, such as boiling, low and high pH levels, extended storage, and repeatedly freeze-thaw cycles. Nevertheless, the mechanisms of miRNAs entry into circulation have not yet been fully understood. Due to their stability and the consistency of their signatures with the expression of primary-tumor miRNAs [17], miRNAs are believed to be rather selectively and specifically released into the extracellular milieu, conjugated with the structures protecting them against RNase activity.

The most frequent form of cell-free miRNAs are miRNAs encapsulated into exosomes or microparticles, two distinct classes of membrane-derived extracellular vesicles (EVs) that differ in their vesicular structures and secretory mechanisms. About 30-100 nm in diameter, exosomes are formed by the inward budding of the plasma membrane into multivesicular bodies within endosomes. MiRNAs encapsulated in exosomes are released into the extracellular environment upon the fusion of endosomes with the plasma membrane. Microparticles are generally larger vesicles (100-4000 nm) that result from the outward budding and blebbing of the plasma membrane. Exosomes do not occur in circulation randomly but are released in an adenosine triphosphate-dependent coordinated process, and they play a crucial function in intercellular communication [18, 19]. Furthermore, miRNAs have been found in circulation in a vesicle-free form loaded into high-density lipoproteins (HDL) and low-density lipoproteins (LDL). However, some studies showed that the HDL-bound miRNA fraction is rather low (<10%) compared to the total circulating miRNA pool.

MiRNAs can be secreted into circulation by binding with a protein belonging to the Argonaut protein family, particularly Argonaut 2 (Ago2), one of the major components of the RNA-induced silencing complex (RISC), which is involved in the regulation of mRNA translation. Some studies have revealed that miRNAs associated with the Ago2 protein represent the largest population of all the circulating miRNAs [20-22]. The role of the Ago2 protein complex in miRNA transport into the extracellular environment remains unclear since several investigations have provided opposite results. Turchinovich *et al.* suggested that the presence of Ago2-miRNAs in circulation resulted from cell death [23]. This being the case, these molecules would not help determine disease status. Moreover, miRNAs may be packaged into apoptotic bodies and released into the extracellular space as a result of cellular death [24, 25].

A number of papers have demonstrated that circulating miRNAs might be a valuable biomarker for cancer. Since a standardized protocol for miRNA quantification has not been established, many of these studies, however, lack consistency and reproducibility. A complex process, miRNA quantification requires many aspects of both preanalytical and analytical phases to be considered as well as advanced statistics to be applied for both data analysis and interpretation.

Since all these factors may add variation to the data, it is desirable to determine general guidelines for such a multi-step analysis. Cellular-derived contamination may lead to the undesirable enrichment of a particular miRNA pool in body fluids; for example, erythrocyte-specific miRNAs may increase the expression level of serum miRNAs up to 50-fold. [26, 27]. Therefore, standardizing sampling and processing procedures are a prerequisite to provide consistent and reliable results reflecting the miRNA status in circulation.

The currently available protocols for miRNA extraction from body fluids vary in performance. Thus, choosing among the available methods, one should evaluate which one of them provides RNA that is free of contaminants and has the highest yield. Commercially available kits mainly based on guanidine/phenol/chloroform protocols or column extraction method, miRNeasy Mini Kit (Qiagen) and mirVana (Life technologies), are two of the widely used kits [28, 29]. Some studies have demonstrated that the addition of RNA carriers to an isolation protocol improves the extraction efficiency. Exogenous RNA, however, can interfere-*via* nonspecific hybridization or amplification-with the results of circulating miRNA quantification [30]. To increase miRNA extraction yield, pure glycogen can be used as an RNA carrier; it does not affect the quantification results [31, 32].

The low abundance of miRNAs in circulation often leads to detectability problems, making their concentration difficult to estimate, using both spectrophotometric and fluorimetric standard methods (NanoDrop, Qubit) [26, 33]. High accuracy of assessing circulating miRNA concentration may be achieved by automated capillary electrophoresis. Bio-Pico Chip allows for RNA detection even at low concentrations of 50 pg μL^{-1} to 2 ng μL^{-1} [33]. Nonetheless, some studies indicate that using an equal volume input instead of the same

amount of RNA is more reasonable for quantifying circulating miRNAs. Since nucleic acids are released into circulation under various pathological conditions (including cancer), circulating RNA is more abundant in cancer patients than in healthy people [26, 34].

Several methods have been applied to analyze miRNAs in body fluids. These methods, however, differ in performance, sensitivity, specificity, the number of miRNAs that can be detected in an assay, input of starting material, and cost. Thus, a platform for miRNA analysis should be chosen on the basis of the nature of research [35]. Quantitative polymerase chain reaction (qPCR) relies on miRNA-specific reverse transcription (RT) and the use of RT stem-loop primer followed by a real-time PCR protocol [29]. Providing high sensitivity and specificity, this method is widely used for the verification of miRNA profiling results obtained using other platforms [36]. The definite advantage of this method is its high accuracy in miRNA detection, although the analysis is limited to miRNAs with known sequences.

High-throughput platforms based on amplification or hybridization principles have been introduced in the market to allow for the analysis of a wide spectrum of miRNAs in an assay. Even though these platforms have shown good performance in the analysis of miRNAs in tissues [37], their protocols require a large amount of input RNA-thus they might be inadequate for the detection of miRNAs in body fluids. Advances in circulating miRNA quantification have revealed that adapting the step of RT product preamplification could overcome the problem of low miRNA abundance, thereby increasing the sensitivity of analysis [38]. Most recently, RNA-seq has become a preferred method for analyzing circulating miRNAs, because it requires significantly lower input of the starting material and allows for the detection of novel miRNAs (in addition to quantifying known miRNAs) and the discrimination of their isoforms [39]. These advantages do not come without cost; the method is expensive, it requires bioinformatics support, and its multi-step library preparation procedure increases the risk of producing bias in sequencing results.

3. MicroRNAs IN PANCREATIC CANCER PATHOGENESIS

Over the last few years, miRNAs have been shown to correlate with the initiation, progression and promotion of PDAC [40]. They can act as either tumor promoters or tumor suppressors, by silencing or promoting cellular pathways, including proliferation, apoptosis, angiogenesis and cell-cycle modulation.

The most frequently mutated gene in PDAC is the KRAS gene. Nearly 95% of cases harbor mutationally activated KRAS [41, 42]. KRAS is an oncogene that encodes a small GTPase transductor protein and its mutant forms that frequently occur in PDAC. Activated KRAS induces the MAPK pathway, involved in cell proliferation and migration [43, 44]. The mRNA of KRAS has been reported to be directly targeted by miR-217, which acts as a tumor suppressor-by decreasing the KRAS level and reducing the constitutive phosphorylation of the downstream signal transducer AKT-and inhibits cell proliferation [45]. MiR-21 has been reported to be overexpressed in PDAC and to correlate with a poorer prognosis for PDAC patients and chemoresistance [46, 47]. MiR-21 inhibits PTEN (phosphatase and tensin homolog) mRNA translation, thereby decreasing its tumor-suppressive action on cell proliferation of pancreatic tumor cells. Moreover, the overexpression of miR-21 in PDAC cell lines decreases the level of PDCD4 (Programmed cell death 4), which plays an important role as a tumor suppressor involved in apoptosis, cell transformation, invasion and tumor progression. FOXO1 was deduced to be targeted by miR-21. The downregulation of both the tumor suppressors by miR-21 significantly increases 5-FU resistance in pancreatic cancer cell lines [48]. MiR-155, miR-17-5p and miR-146 have been found to be involved in gemcitabine resistance in PDAC [49]. Yan Tang *et al.* revealed that miR-34a was involved in the regulation of the Notch signaling pathway and epithelial-mesenchymal transition in pancreatic cancer. The overexpression of miR-34a decreases the levels of Notch1 and Snail1, thereby inhibiting the migration and invasion of pancreatic cancer cells [50]. In another study, miR-34 restoration inhibited the expression of target genes-namely, Bcl-2, Notch1, and Notch2-in p53-deficient pancreatic cancer cells. This led to the significant inhibition of clonogenic cell growth and invasion, induction of apoptosis, and G1 and G2/M arrest, and sensitized the cells to chemotherapy and radiation [51]. MiR-17-5p belongs to the miR-17-92 cluster and is often up-regulated in PDAC. In a study by Gu *et al.*, a high GFRα2 expression level inactivated PTEN by enhancing a miR-17-5p level [52].

4. MicroRNAs AS NON-INVASIVE DIAGNOSTIC BIOMARKERS IN PANCREATIC CANCER

One of the reasons behind poor prognosis for PDAC patients is the lack of effective biomarkers. Such biomarkers would improve the early diagnosis rate and the clinical management of PDAC. Currently, methods recommended for clinical use in PDAC include imaging techniques and carbohydrate antigen 19-9 (CA-19-9), the only available blood-based biomarker so far [53]. CA-19-9 performance as a diagnostic biomarker, however, is poor; its sensitivity is insufficient, with the median sensitivity below 80%. Levels of serum CA-19-9 are often elevated in many various non-pancreatic conditions, leading to false positivity in PDAC detection based on this criterion. Besides, about 10% of the Caucasian population represents a Lewis negative blood phenotype, so they do not produce CA-19-9; these patients can also be recognized as falsely negative PDAC [54]. Thus, CA-19-9 should not be considered a PDAC-specific tumor biomarker.

Some non-cancerous lesions within the pancreas are known high-risk factors that may contribute to developing pancreatic cancer. Early diagnosis is a prerequisite for radical surgery, currently the only chance for long-term survival. However, small pancreatic neoplasms in asymptomatic patients are difficult to identify with current diagnostic possibilities. Some studies have achieved promising results, indicating cell-free miRNAs as non-invasive, cheap, and accurate diagnostic markers for PDAC (Table **1**).

4.1. MicroRNAs in Blood Plasma and Serum

Human blood was frequently considered a preferred source for emerging microRNA-based tests to be used in

Table 1. Cell-free microRNAs with diagnostic potential in pancreatic cancer.

Pancreatic Cancer *vs*. Healthy Controls				
miRNA	**Sensitivity (%)**	**Specificity (%)**	**Area Under the Curve (AUC)**	**References**
miR-21	- - 80.7 71.4 81.5 -	- - 81.0 100 95.5 -	0.889 0.790 0.826 - 0.897 0.99	[59, 62, 65, 73, 92]
miR-18a	-	-	0.9369	[57]
miR-17-5p	92.6	72.7	0.887	[92]
miR-34a	-	-	0.865	[59]
miR-25	*75.58*	*93.03*	0.915	[60]
miR-1290	-	-	0.96	[67]
miR-3679-5p	82.5	45	0.673	[71]
miR-940	90.0	40	0.680	[71]
miR-181b	84.6	84.6	0.745	[74]
miR-210	51.7	65.5	0.772	[74]
miR-21, miR-210, miR-155, miR-196a	64.0	89.0	0.82	[55]
miR-10b, miR-155, miR-106b, miR-30c, miR-212	95.0	100.0	>0.90	[58]
miR-642b-3p, miR-885-5p, miR-22-3p	91.0	91.0	0.97	[61]
miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, miR-191	89.0	100.0	0.992	[68]
miR-21, miR-155, miR-216	83.33	83.33	0.8667	[75]
miR-143, miR-30e	83.3	96.2	0.923	[79]
Pancreatic Cancer *vs*. IPMN				
miR-483-3p	43.8	-	0.703	[62]
IPMN *vs*. Healthy Controls				
miR-145-5p	-	-	0.79	[63]
miR-191 miR-21 miR-451a	64.3 75.9 62.1	79.0 81.0 85.7	0.741 0.741 0.742	[64]
Pancreatic Cancer *vs*. Chronic Pancreatitis				
miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, miR-191	-	-	0.993	[68]
miR-486-5p, miR-126-3p, miR-106b-3p	0.827	0.844	0.891	[70]

Abbreviation: IPMN, Intraductal Papillary Mucinous Neoplasm.

clinical routine. Several investigations have emphasized the diagnostic potential of blood plasma/serum miRNA levels, showing that they differ between PDAC patients and the matched healthy population. Wang *et al.* provided the first evidence that a combined analysis of miR-21, miR-210, miR-155, and miR-196a levels in blood plasma can distinguish-with high sensitivity and specificity-PDAC patients from healthy controls (HC) [55]. Ho *et al.* found that the miR-210 level was elevated in human PDAC plasma samples in two independent patient cohorts [56]. Studies by Morimura *et al.* showed miR-18a to be highly expressed in the plasma of preoperative PDAC patients than that of healthy subjects, with high discriminative power (AUC of 0.9369). In addition, the concentration of miR-18a in PDAC post-operative plasma was decreased, suggesting that this miRNA could be used for monitoring disease dynamics [57]. In the study by Cote *et al.*, a panel of three miRNAs- miR-10b, miR-106b, and miR-155-exhibited excellent accuracy for distinguishing PDAC from healthy controls and CHP [58]. The serum of miR-21 and miR-34a was differentially expressed between groups of 24 PDAC and 10 HC ($P < 0.001$ and $P = 0.001$, respectively). Both the miRNAs accurately discriminated between the two groups, with an AUC of 0.889 for miR-21 and of 0.865 for miR-34a [59].

A large-sample investigation showed that miR-25 had higher levels in the serum of PaC patients than in that of healthy controls, and even in that of patients with other cancers, as evaluated by Deng *et al.* ROC curve analysis revealed that miR-25 had a significant diagnostic value for the differential diagnosis of PaC and normal controls, with an AUC of 0.915, higher than an AUC of 0.725 for CEA and an AUC of 0.844 for CA19-9 [60].

Ganepola *et al.* compared plasma miRNA expression between HC and early-stage PaC patients. This led them to establish a panel of three miRNAs: miR-642b-3p, miR-885-5p, and miR-22-3p, which provided high diagnostic accuracy with AUC of 0.97, SEN of 91%, and SPF of 91%. It had higher sensitivity than the CA19-9 marker (which had 73%), but the latter had higher specificity (100%) [61].

A study by Abue *et al.* showed that plasma miR-483-5p was higher in a group of 32 PDAC patients than in a group of 12 patients diagnosed with intraductal papillary mucinous neoplasm (IPMN) ($P < 0.05$). ROC AUC analysis showed that miR-483-5p differentiated PDAC patients from IPMNs, with an AUC of 0.703 [62]. Permuth *et al.* profiled levels of 800 miRNAs in 42 IPMN preoperative plasma samples (including benign IPMN and malignant IPMN) and 24 healthy controls. They found a panel of 30 miRNAs that distinguished IPMN cases from the controls (AUC = 74.4). They also found that miRNAs might have distinct signatures in benign and malignant IPMN cases-a panel of 5-miRNAs discriminated between 21 malignant (high-grade dysplasia and invasive carcinoma) and 21 benign (low- and moderate-grade dysplasia) IPMNs (AUC=73.2) [63].

Interestingly, Goto *et al.* demonstrated that the expressions of miR-191, miR-21 and miR-451a derived from serum exosomes better distinguished PaC and IPMN patients from healthy controls, but the corresponding serum circulating miRNAs did not differ markedly among the three groups. These findings suggest that the information based on an exosome-related miRNA signature accurately reflects a disease status [64].

A nested case-control study showed that miRNA-10b, miRNA-21-5p and miRNA-30c were overexpressed in plasma samples collected years before PDAC was diagnosed, compared to samples from healthy subjects. In a five-year follow-up study, the AUCs between the blood collection and PDAC diagnosis were 0.76 (miRNA-10b), 0.79 (miRNA-21-5p) and 0.77 (miRNA-30c), suggesting their pre-diagnostic potential [65].

Another group found a panel of 15 miRNAs associated with PC at the time of diagnosis. Further investigation in pre-diagnostic samples, however, showed that none of these miRNAs were altered before diagnosis-and, therefore, they could not be considered pre-diagnostic biomarkers [66]. MiR-1290 has been identified to accurately discriminate between PDAC and healthy controls (AUC=0.96), PDAC and chronic pancreatitis (AUC=0.81), and PDAC and pancreatic neuroendocrine tumors (PNETs) (AUC=0.80). The miR-1290 level was higher in PC than in IPMNs patients and discriminated between intermediate/high-grade dysplasia and low-grade dysplasia. Interestingly, serum miR-1290 levels distinguished patients with low-stage pancreatic cancer from controls better than did CA19-9 [67].

Using Illumina sequencing by synthesis technology, Liu *et al.* discovered a panel of seven miRNAs (miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, and miR-191) that had high sensitivity and specificity for distinguishing various stages of PaC from cancer-free controls and CHP [68]. In a multicenter multistep validation study, Xu *et al.* demonstrated that plasma miR-486-5p effectively distinguished PaC patients from healthy controls (AUC of 0.861) and chronic pancreatitis patients (0.707) [69]. In another study, the authors constructed two diagnostic panels, one comprising three plasma microRNAs (panel I: miR-486-5p, miR-126-3p, and miR-106b-3p) and another comprising six microRNAs (panel II: miR-486-5p, miR-126-3p, miR-106b-3p, miR-938, miR-26b-3p, and miR-1285). Both the panels distinguished PaC patients from CHP ones with high accuracy (AUC of 0.891 and 0.889, respectively) [70]. Unfortunately, the ability of plasma miRNAs to distinguish between between PaC (n=156) and PNETs (n=27) was not satisfactory (miR-938 – AUC=0.66; miR-126-3p – AUC=0.64; miR-19b-3p – AUC=0.64; miR-26b-3p – AUC=0.64) [70].

4.2. MicroRNAs in Saliva

Biomarker research has made considerable progress over the past few years. Effective PaC management calls for novel, easy-to-use, and non-invasive tools for patient specimen collection-but, despite many attempts, such tools are still missing, increasing the urgency for their development.

Salivary miR-3679-5p and miR-940 efficiently distinguished between resectable PaC and other patients in the following comparisons: PaC *versus* healthy control (sensitivity of 72.5% and specificity of 70.0%), PaC *versus* benign pancreatic tumors (BPT) (62.5% and 80.0%), and PaC *versus* non-cancer (healthy control + BPT) (70.0% and 70.0%) [71].

Humeau *et al.* reported that miR-21, miR-23a, miR-23b and miR-29c were highly abundant in the saliva of PDAC

patients but were not detected in the samples of healthy controls. The miRNAs reached sensitivities of 71.4%, 85.7%, 85.7%, and 57%, respectively, and all had excellent specificity (100%). Additionally, miR-210 and the Let-7c miRNA precursor were identified to be increased in the saliva of patients with pancreatitis as compared to the control group, with the sensitivity of 100% and 75%, and specificity of 100% and 80%, respectively [72].

4.3. MicroRNAs in Stool

Pancreatic juice is released into the ductal lumen, thus representing a potentially rich source of cancer-specific molecules, such as miRNAs [73]. Due to the anatomical localization of the pancreas, however, the collection procedure of pancreatic juice is invasive and often impossible. Nevertheless, since this juice is released into the gastrointestinal tract, it seems reasonable to test the use of fecal miRNAs in PaC diagnostics.

Expression analysis of seven miRNAs (miR-21, miR-143, miR-155, miR-196a, miR-210, miR-216a, and miR-375) in feces revealed that four of them-miR-216a, miR-196a, miR-143, and miR-155-were downregulated compared to healthy specimens; the other three showed no changes in expression levels. The expression of the four miRNAs was the highest in controls and the lowest in PaC patients, while that of chronic pancreatitis patients was in between. Nevertheless, very few patients were enrolled in the study to detect the statistical significance of the differences, thus the results did not provide sufficient evidence of fecal miRNAs' usefulness in the clinical diagnosis of PaC patients. Ren *et al.* evaluated fecal miRNAs' potential for screening PaC patients. By doing so, they analyzed three populations of patients: 29 with PaC, 22 with chronic pancreatitis and 13 with HC. They pointed out miR-181b and miR-210 as possible biomarkers for discriminating PaC and HC patients, with AUC of 0.745 for miR-181b and 0.772 for miR-210 [74].

Yang *et al.* reported that the expression levels of miR-21 and miR-155 in the stool of PDAC patients were higher and the expression level of miR-216 was lower than that of HC patients. Stool miRNAs signatures were consistent with those found in PDAC tissue and pancreatic juice. The combination of these miRNAs would be most effective for detecting and screening PDAC with AUC of 0.8667, sensitivity of 83.33%, and specificity of 83.33% [75]. These results were not in agreement with those by Link *et al.* who found downregulated miR-155 in PaC stool specimen [76].

4.4. MicroRNAs in Urine

Finally, attention has also been paid to reveal the possible implementation of urinary samples in PDAC diagnostics. The topic of urine-based miRNA biomarkers is well developed, and they have been evaluated in diagnosing other types of cancer, such as bladder cancer [77, 78]. Debernardi *et al.* studied whether urinary miRNAs could serve as non-invasive biomarkers in PDAC. To this aim, they performed miRNAs global profiling, using microarray technology, in 13 PDAC, 6 CHP, and 7 HC patients. Based on this analysis, the authors chose a panel of the most differentially expressed miRNAs for further validation in an independent group of 101 urine samples (from 46 PDAC, stages I-IV; 29 CHP; and 26 HC patients). A combination of urinary miR-143 and miR-30e accurately discriminated between healthy controls and PDAC stage I (AUC of 0.923, SEN of 83.3%, and SPF of 96.2%) [79].

5. CELL-FREE MicroRNAs AS PROGNOSTIC AND PREDICTIVE BIOMARKERS IN PDAC

The individualization of PDAC patient care remains an unsolved medical problem since currently available methods are insufficient to both predict the course of the disease and estimate survival probability. Most PDAC patients do not benefit from the treatment they are offered. In several studies, the miRNA signature in circulation correlated with PDAC survival and could predict the response of a PDAC patient to treatment. In combination with currently available approaches, circulating miRNAs as prognostic/predictive biomarkers can find important applications in the personalization of PDAC therapy. They might be particularly helpful in the context of radical resection, currently the only effective treatment-but a significant percentage of PDAC patients who underwent curative-intent surgery did not experience survival benefit, compared to advanced PDAC patients who received systemic chemotherapy only. This is mainly because even patients with localized tumors may have metastases that are not detectable with currently available methods.

For a long time, prognostic and predictive biomarkers have been extensively studied, already leading to some promising results. Among the prognostic biomarkers studied are CA19-9, DNA methylation panels, methylation of MUC genes and BRCA mutations, whereas among the predictive biomarkers are microsatellite instability, secreted protein acidic and rich in cysteine (SPARC), human equilibrative nucleoside transporter 1 (hENT1), and human concentrative nucleoside transporter 3 (hCNT3) [13]. Although promising, these biomarkers suffer from several analytical and standardization bottlenecks, preventing their clinical application. As a new class of non-invasive biomarkers, circulating miRNAs could allow for overcoming these limitations. Table **2** summarizes the studies on cell-free miRNAs in this context.

Using quantitative real-time polymerase chain reaction (qPCR), Kong *et al.* analyzed several miRNAs in sera of 35 patients diagnosed with PDAC, 15 with CHP, and 15 with HC. MiR-21, miR-155, and miR-196a were significantly overexpressed in PDAC. Additionally, miR-196a levels in the serum of unresectable PDAC (stages III and IV) patients were significantly higher than those of resectable PDAC patients (stages I and II). The expression levels of miRNA were found to have the potential to predict the median survival for PDAC patients; those with higher miR-196a expression had a median survival time of 6.1 months, whereas those with lower miR-196a expression had a median survival time of 12 months [80]. Likewise, Yu *et al.* reported the median survival of 6.3 months in PDAC patients with high plasma miR-196a levels, which was shorter than that of 12.5 months in the low miR-196a group. Additionally, PDAC patients with high miR-210 expression had a longer median survival (11.7 months) than those with low miR-210 expression who had a median survival of 6.6 months [81].

Table 2. The summary of cell-free microRNAs with prognostic/predictive potential in pancreatic cancer.

miRNA	Specimen	Observations and Correlations with Clinical Outcome	References
miR-196a	Serum	A high level of miR-196a correlated with shorter survival. Moreover, its expression was higher in patients with unresectable (stage III-IV) than those with resectable PDAC (stage I-II).	[80, 81]
miR-210	Plasma	Levels of miR-210 correlated with the overall survival of PDAC patients.	[81]
miR-221	Plasma	A high level of miR-221 correlated with the presence of distant metastases and the non-resectable status of PDAC.	[82]
miR-124	Serum	Low serum levels of miR-124 correlated with lymph node metastasis, TNM stage, and shorter survival time after surgery.	[83]
miR-182	Plasma	Elevated levels of miR-182 correlated with positive lymph node metastasis and advanced clinical stages. MiR-182 was identified as an independent predictor for disease-free and overall survival.	[84]
miR-744	Plasma	High levels in post-operative plasma were correlated with lymph node metastasis and a more frequent recurrence. MiR-744 contributed to poorer progression-free survival of non-resectable PaC patients who underwent gemcitabine-based chemotherapy.	[85]
miR-107	Plasma	Lower levels in post-operative plasma correlated with the occurrence and recurrence of liver metastases. Low levels were an independent predictor for worse disease-free and overall survival of patients who underwent curative pancreatectomy.	[86]
miR-373	Serum	Lower levels correlated with the advanced stage, lymph node metastasis, and distant metastasis. Patients with lower levels had shorter overall survival.	[87]
miR-451a	Plasma	Higher levels correlated with early recurrence after surgery, tumor size and stage, and shorter disease-free and overall survival.	[88]
miR-21	-	Correlated with liver metastasis, positive lymph node status, resistance to gemcitabine therapy. MiR-21 was indicated as an independent factor of poor disease-free and overall survival.	[89-91]
miR-155	Plasma	Higher levels were linked to gemcitabine resistance and were indicated as a predictor of disease-free survival.	[49]

Kawaguchi *et al.* indicated that the expression levels of miR-221 were lower in postoperative PaC plasma than in preoperative samples. Besides, correlation analysis between plasma miR-221 levels and clinicopathological features revealed that higher expression of miR-221 was significantly correlated with both distal metastasis (P=0.041) and non-resectable status (P=0.021) [82]. In another study, the expression of serum miR-124 was evaluated in 53 resectable PDAC patients and 73 PDAC patients with a non-resectable status. MiR-124 levels were significantly higher in resectable PDAC patients than in non-resectable patients. Further analysis indicated that the survival of patients who underwent resection and exhibited high serum miR-124 expression was significantly better than those with low serum miR-124 levels [83]. Chen *et al.* demonstrated that both overall and disease-free survival was shorter in patients with higher expression of miR-182 in plasma. They also demonstrated that miR-182 expression could provide information about disease staging, and the elevated expression of miR-182 correlated with a PaC stage. Multivariate Cox regression analysis confirmed that circulating miR-182 was an independent prognostic factor for DFS ($P = 0.001$) and OS ($P < 0.001$) [84].

Miyamae *et al.* used microarray analysis and, afterward, the qPCR method. They selected miR-744 to further investigate its expression in both preoperative and postoperative plasma samples of PDAC patients. MiR-744 levels were reduced in patients after pancreatectomy; its higher expression was correlated with worse OS after surgery. Multivariate analysis, using the Cox proportional hazard regression model, revealed that a high level of miR-744 was an independent factor that predicted poor prognosis for PaC patients (P=0.0007, the hazard ratio of 21.2 and 95% CI of 3.17-436). They determined that a high level of plasma miR-744 contributed to poorer progression-free survival of non-operable PaC patients who underwent gemcitabine-based chemotherapy (P=0.0533). *In vivo* analysis revealed that the overexpression of miR-744 in PaC cell lines induced chemoresistance to gemcitabine [85]. In the study by Imamura *et al.*, miR-107 was suppressed in the plasma of PaC patients. The miR-107 level correlated with advanced N stages ($P = 0.0376$), T stages ($P = 0.0755$) and liver metastases ($P = 0.0027$). The low miR-107 plasma level was significantly associated with the worse overall survival rate in all PaC patients ($P < 0.0001$), worse overall survival rate in PaC patients with curative pancreatectomy ($P = 0.0038$), and worse disease-free survival rate in PaC patients with curative pancreatectomy. Multivariate analysis indicated that low miR-107 expression was an independent prognostic factor for worse OS in PaC patients [86]. The prognostic potential of miR-373 was evaluated in a PDAC patient cohort, which included 103 serum samples. Patients with lower expression had a shorter five-year overall survival. Moreover, reduced miR-373 levels correlated with the TNM stage, lymph node,

and distal metastasis [87]. Takahasi used microarrays to analyze exosomal miR-451a levels in the plasma of 50 PDAC patients who underwent tumor resection. The Kaplan-Meier analysis showed that patients with higher miR-451 expression had worse OS and DFS, suggesting that miR-451a is associated with recurrence and poor prognosis of PDAC patients. Moreover, both univariate and multivariate Cox analyses showed the significance of miR-451a for OS and DFS in PDAC patients [88].

The oncogenic role of miR-21 in PDAC disease has been established, and numerous studies have been dedicated to evaluate the possible application of circulating miR-21 to clinical practice with prognostic/predictive intent. Most recently, Negoi *et al.* summarized the current state of knowledge regarding the prognostic potential of miR-21 in pancreatic cancer. It was found associated with worse survival, liver metastasis, lymph node status, and increased resistance to gemcitabine [89]. Subsequently, it was also shown that miR-21 could predict a patient's response to treatment. Wang *et al.* demonstrated that the time to progression (TTP) of advanced PDAC after gemcitabine treatment was longer in patients with lower serum miR-21 levels (161 days) than those with higher expression (80 days). The patients who received gemcitabine-based palliative therapy had a median OS of 348 days in case of lower miR-21 levels and 186 days in case of a higher expression. Therefore, miR-21 could be considered a factor in predicting the response of PDAC to gemcitabine treatment [90]. Most recently, Karasek *et al.* demonstrated that preoperative plasma levels of miR-21 could predict a PDAC patient's survival time after curative surgery [91]. Mikamori *et al.* found that miR-155 was involved in a chemoresistance loop by influencing exosome synthesis and promoting chemoresistance in PDAC cells after exposure to gemcitabine. Further, they repeated this investigation on clinical specimens of 45 tissue and 23 preoperative plasma samples of PDAC patients who received gemcitabine-based adjuvant therapy. The results revealed that high expressions of exosomal miR-155 were associated with worse DFS and OS in cancer cell samples, and plasma with high levels of exosomal miR-155 correlated with DFS but not with OS. This is probably due to the limited number of samples [49, 92].

CONCLUSION

Until now, miRNAs have been found to be mechanistically involved in major pathogenic pathways that are deregulated in pancreatic cancer. Cell-free miRNAs correlate with their cell-free counterparts in blood plasma, blood serum, and other human biofluids. In pancreatic cancer, cell-free miRNAs were detected in blood plasma/serum saliva and stool, indicating strong diagnostic potential to discriminate between pancreatic cancer, chronic pancreatitis, IPMN, and healthy controls, and signifying their potential for early detection or population screening of pancreatic cancer.

In terms of their prognostic potential, two cell-free miRNAs-miR-196a and miR-21-have been repeatedly observed to correlate with disease-free and overall survival of PDAC patients. Circulating miRNAs are also linked to primary resistance to gemcitabine and 5-fluorouracil therapy in PDAC. According to recent research, cell-free miRNAs represent a promising non-invasive diagnostic tool in pancreatic cancer. Before they can be introduced into clinical practice, however, the most promising miRNA candidates have to be validated in multicenter prospective studies, with independent cohorts of patients and laboratory facilities.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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