REVIEW

miRNA and Gene Expression in Pancreatic Ductal Adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) remains a challenging disease that is mostly diagnosed late in the course of the illness. Unlike other cancers in which measurable successes have been achieved with traditional chemotherapy, targeted therapy, and, recently, immunotherapy, PDAC has proved to be poorly responsive to these treatments, with only marginal to modest incremental benefits using conventional cytotoxic therapy. There is, therefore, a great unmet need to develop better therapies based on improved understanding of biology and identification of predictive and prognostic biomarkers that would guide therapy. miRNAs are small noncoding RNAs that regulate the expression of some key genes by targeting their 3'-untranslated mRNA region. Aberrant expression of miRNAs has been linked to the development of various malignancies, including PDAC. A series of miRNAs have been identified as potential tools for early diagnosis, prediction of treatment response, and prognosis of patients with PDAC. In this review, we present a summary of the miRNAs that have been studied in PDAC in the context of disease biology. (*Am J Pathol* 2019, 189: 58–70; <https://doi.org/10.1016/j.ajpath.2018.10.005>)

Pancreatic ductal adenocarcinoma (PDAC) is the main histologic type (>90%) of cancer arising from the pancreas. A total of 55,440 new cases of PDAC are estimated to be diagnosed in 2018, whereas 44,330 deaths are estimated to be caused by PDAC in the United States.¹ In the United States, PDAC is currently the third leading cause of cancer-related mortality; however, it is projected to be the second leading cause of cancer-related death by 2020.^{1,2} Progress in PDAC treatment has been slow. The 5-year overall survival rate has only improved to 9% during the period 2006 to 2012 in the United States, from 3% for the period 1975 to 1977.³ Modern cancer chemotherapy showed modest incremental improvement in patient outcomes, especially in patients who have advanced disease. The median overall survival of patients with metastatic PDAC treated with newer regimens has improved to approximately 11 months compared with the historic benchmark of 5 to 6 months.^{4,5} Because of rapid clinical decline, most patients do not receive further chemotherapy after first-line therapy has failed.⁶ Patients who receive second-line treatment have

been shown to live for >1 year.^{7,8} Despite poor specificity and sensitivity, CA19-9 remains the only biomarker in routine clinical use as a surrogate for prognosis and response to therapy.^{9–12} Clinical trials in PDAC that were completed in the past few years have shown no therapeutic benefit from newer agents that include drugs aimed at intracellular molecular targets and the immune system.¹³

miRNAs and Pancreatic Ductal Adenocarcinoma

miRNAs are noncoding, single-strand RNAs of approximately 22 nucleotides that interact with untranslated regions of a target mRNA, resulting in their reduced translation or a rapid degradation. More than 700 miRNAs have been described so far. miRNAs represent approximately 3% of

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the human genome¹⁴ and control the expression of >60% of the protein-coding genes in humans.¹⁵ They serve as regulators of gene expression, thereby playing a key role in the regulation of a wide range of cellular processes, including growth, differentiation, proliferation, and apoptosis. They may also play oncogenic or tumor-suppressive functions and have been profiled in various cancers, including breast, lung, colorectal, pancreas, and liver cancers.¹⁶

The differential expression of miRNA in malignancies has led to their evaluation as biomarkers of diagnostic and therapeutic importance. In addition, description and targeting of oncogenic miRNAs and those with tumor suppressor functions has been a major research focus. Multiple genetic aberrations involved in the development of PDAC are shown to be influenced by miRNAs.¹⁷ Table 1 summarizes a few examples of aberrant expression of some of the miRNAs described in malignancies.^{18–33}

The interaction between miRNA and mRNA molecules was investigated in pancreatic cancer using bioinformatics techniques. There were 17,401 relevant miRNA-mRNA interactions estimated to occur in pancreatic cancer, highlighting the complexity of miRNA-mRNA interactions in PDAC and the challenge to identify the most relevant target for therapeutic development.³⁴

miRNAs and Molecular Changes in Pancreas Ductal Adenocarcinoma

Oncogene Activation and miRNAs

Activating *KRAS* mutations are the earliest genetic changes in the malignant transformation of the pancreatic ductal epithelial cells to adenocarcinoma and are seen in nearly all cases of PDAC at diagnosis. Activation of the *RAS* family

oncogenes leads to cellular proliferation, survival, and invasion through the stimulation of several effector pathways.³⁵ miRNAs targeting *KRAS* and its downstream genes have been identified. Down-regulation of miR-96, miR-126, and miR-217 was shown in *KRAS* mutant PDAC, unlike in normal pancreatic tissues.^{36–38} *KRAS* activity was suppressed by the reexpression of miR-96 and miR-217, with resultant reduced cell migration and invasion, highlighting a tumor suppressor–like function.^{37,39} Overexpression of *miR-217* also influences AKT, downstream to RAS proteins, with effects on cell survival and proliferation.³⁹ The loss of *miR-143/145* cluster expression is seen in *KRAS* mutated PDAC, and its reexpression has been shown to mitigate carcinogenesis and inhibit RAS-responsive element–binding protein, mitigating the effects of *KRAS* activation in preclinical models.²⁹ The expression of the miRNA *let-7* has been shown to suppress *KRAS* expression and mitogen-activated protein kinase activation, whereas its loss plays a role in *KRAS*-activating mutations.⁴⁰

miRNAs and Tumor Suppressor Gene Inactivation

Tumor suppressor genes play a key role in triggering cell cycle arrest or apoptosis when there is irreparable DNA damage. Their inactivation leads to the loss of their regulatory function, thereby favoring the growth and sustenance of malignant cells. The loss of tumor suppressor genes *Ink4A* (*p16/CDKN2A*), *TP53*, *SMAD4*, and *BRCA2* has been well described in PDAC.⁴¹ *Ink4A* acts as a checkpoint that controls the continued cellular proliferation from *KRAS* activation and leads to cellular senescence. The functional loss of *Ink4A*, seen in up to 95% of PDAC, plays a key role in the perpetual growth of malignant PDAC cells by overriding the cellular senescence induced by activated *KRAS*.^{42,43}

Table 1 Examples of Dysregulated miRNAs with Their Potential Targets and Suggested Biological Functions

miRNAs	Expression	Potential targets	Biological function	References
<i>miR-34a</i>	Down-regulation	Bcl-2/Notch1/Notch2	Inhibits proliferation and invasion and induces apoptosis	18
<i>miR-221/222</i>	Up-regulation	MMP-2 and MMP-9	Cancer cell invasion	19
<i>miR-320a</i>	Up-regulation	PDCD4	Reduces apoptosis and promotes EMT	20
<i>miR-365</i>	Up-regulation	SHC1/BAX	Reduces apoptosis	21
<i>miR-21</i>	Up-regulation	PI3K/AKT/PTEN, PDCD4, and Bcl-2/FasL	Reduces apoptosis and promotes proliferation	22–24
<i>miR-29a</i>	Up-regulation	Wnt/β-catenin	Regulates transcription factors	25
<i>miR-210</i>	Up-regulation	Pancreatic stellate cells	Promotes invasion and EMT	26
<i>miR-100</i>	Down-regulation	FGFR3	Inhibits proliferation	27
<i>miR-146a</i>	Down-regulation	MAPK/Kras/EGFR/IRAK/MTA-2	Inhibits invasion	28
<i>miR-145/143</i>	Down-regulation	KRAS	Inhibits tumor growth	29
<i>miR-33a</i>	Down-regulation	AKT/β-catenin and Pim-kinase	Inhibits proliferation	30
<i>miR-101-3p</i>	Down-regulation	RRM1	Interferes with DNA synthesis	31
<i>Let-7</i>	Down-regulation	N-cadherin/ZEB1	Reverses EMT and inhibits invasion	32
<i>miR-211</i>	Down-regulation	RRM2	Reduces drug targets	33

EGFR, epidermal growth factor receptor; EMT, epithelial-to-mesenchymal transformation; FGFR3, fibroblast growth factor receptor 3; IRAK, interleukin-1 receptor-associated kinase 1; KRAS, kirsten rat sarcoma virus; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; MTA-1, metastasis-associated protein 1; PDCD4, programmed cell death 4; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; RRM1, ribonucleoside-diphosphate reductase large subunit; SHC1, SHC transformic protein 1; ZEB1, zinc finger E-box-binding homeobox 1.

The inactivation of *TP53* is seen in >50% of PDAC, mostly in the later stage of carcinogenesis.³⁵ The rate of genomic aberrations increases dramatically after loss of the *p53* function, leading to tumor heterogeneity and resistance to multiple chemotherapeutic agents.⁴⁴ Mutant *p53* affects the transcription of *miR-15a*, which is suppressed in PDAC. The overexpression of *miR-15a* down-regulates *WNT3A* and *FGF7*, resulting in reduced cellular proliferation and survival.⁴⁵ A functional *P53* induces the overexpression of *miR-200*, which leads to the down-regulation of *Jag1*, a target of *Zeb1* and a ligand of the Notch pathway. The miRNAs *Zeb1* and *Zeb2* activate epithelial-to-mesenchymal transformation (EMT) and facilitate metastases of cancer cells.⁴⁶ The expression of *Zeb1*, on the other hand, has a negative feedback inhibition on the levels of *miR-200*.⁴⁷ Hence, loss of *p53* function indirectly facilitates EMT through a complex interaction of miRNAs. On the other hand, *miR-491-5p* inhibited the expression of *TP53*, *Bcl-XL* genes, in addition to other effects.⁴⁸ In human PDAC cell lines with *p53* mutations, the reexpression of *miR-34* restores tumor suppressor function of *p53*.¹⁸

MDM2 is a proto-oncogene that results in the functional inactivation of tumor suppressor proteins, like *p53*. *MDM2* overexpression induces PDAC cellular proliferation, migration, and invasion. At the post-transcriptional level, *miR-509-5p* negatively regulates *MDM2*, reversing the effects of *MDM2* overexpression: cell proliferation, migration, and invasion. *miR-509-5p* is down-regulated in pancreatic cancer tissues.⁴⁹

The inactivation of *SMAD4* [deleted in pancreatic cancer (DPC) 4] is seen in approximately 30% to 50% of PDAC, mostly in a later stage of carcinogenesis. The loss of *SMAD4* leads to loss of transforming growth factor (TGF)- β -mediated growth inhibition and subsequent tumor progression by influencing tumor-stroma interactions.³⁵ *miR-137* targets *SMD4A* mRNA during Ras-induced senescence, resulting in the activation of tumor suppressors *p53* and retinoblastoma. The expression of *miR-137* is significantly reduced in PDAC, resulting in *SMAD4*-mediated loss of tumor suppressor functions. Restoring its expression restores the tumor suppressor functions lost in these pancreatic cancer cells.⁵⁰ In another study, *miR-421* and *miR-483-3p* promoted PDAC progression through directly regulating the tumor suppressor *SMAD4*.^{51,52}

Overexpression of *miR-192* leads to a reduction in the expression of *SMAD*-interacting protein 1.³⁹ *miR-323-3p* inhibited TGF- β signaling, via direct suppression of *SMAD2* and *SMAD3*, leading to decreased metastasis.⁵³ In irradiated pancreatic cancer tumor models, a high expression of *miR-193a* was seen, which promotes pancreatic cancer cell repopulation and subsequent metastases in preclinical models. *miR-193a* inhibited *TGF β /SMAD/c-Myc* signaling and disrupted normal intercellular junctions with metastases promotion. Pancreatic cancer repopulation and metastasis after radiation were blocked by the restoration of TGF- β /*SMAD* signaling or suppression of *miR-193a*.⁵⁴

P16, otherwise known as cyclin-dependent kinase inhibitor 2A, functions as a tumor suppressor gene. Aberrations of *p16* expression have been well described in PDAC.⁵⁵ Studies have shown the inhibitory role of *miR-10b* and *miR-24* on the expression of *p16* in malignancies other than pancreatic cancer.^{56,57} Both *miR-10* and *miR-24* were overexpressed in pancreatic cancer.^{58,59}

Down-regulation of *miR-7-5p* in PDAC correlates with poor survival outcomes in patients. *In vitro* cell function assays demonstrated that *miR-7-5p* inhibited proliferation, migration, and invasion of PANC-1 cells by inversely regulating *SOX18*. Silencing of *SOX18* leads to the suppression of the *gp130/JAK2/STAT3* signaling pathway in PANC-1 cells. Hence, *miR-7-5p* likely has a tumor suppressor role, and its down-regulation may lead to enhanced *gp130/JAK2/STAT3* signaling.⁶⁰

miRNAs in PDAC Stemness

Accumulating evidence shows that most solid tumors have a heterogeneous cell mix, some of which are a cancer stem cell (CSC) population that is generally resistant to conventional cytotoxic chemotherapy. Several research groups have identified the overexpressed molecular markers that are relatively specific to the PDAC CSCs. These include CD24, CD44, CD133, epithelial cell adhesion molecule (EPCAM), epithelial-specific antigen (ESA), tyrosine-protein kinase met (c-met), aldehyde dehydrogenase (ALDH1), leucine-rich repeat-containing receptor (Igr5), and serine/threonine-protein kinase (Dcl1). Because these cancer stem cells have self-renewal capabilities, they can repopulate tumor cells that possibly carry newer genetic mutations, if the bulk of cancer cells have been eliminated by treatment.^{61,62} This characteristic of cancer stem cells may be influenced by specific miRNAs.⁶³ A differential expression of miRNAs in cancer stem cells has been described, including *miR-99a*, *miR-100*, *miR-125b*, *miR-192*, and *miR-429*.⁶⁴

Various miRNAs have been implicated in promoting stemness. In PDACs, the loss of *miR-34* was seen in CSCs, and its restoration could suppress the spheroid-forming ability through suppression of notch and *bcl-2* and restoration of *p53*.¹⁸ The EMT-activator *ZEB1* (a zinc finger E-box binding homeobox 1) represses the expression of stemness-inhibiting *miR-203*. The candidate targets of *miR-200* family members are also stem cell factors, such as sex determining region Y-box 2 (Sox2) and Kruppel-like factor 4 (Klf4).⁶⁵ These studies show that the *ZEB1-miR-200* axis may become a novel therapeutic target against PDAC CSCs. Larger-scale differential expression studies performed using microarray analysis, in sphere culture methods enriching the CSC, identified 210 miRNAs and 258 stem cell-associated mRNAs that were differentially expressed in the PDAC CSCs.⁶⁴ Several other studies have identified a series of miRNAs with prominent roles in promoting EMT and cancer stemness in PDAC (Table 2).^{66–75}

Table 2 Prominent miRNAs with Confirmed Roles in PDAC Stemness

miRNAs	Status	Stemness markers regulated	References
<i>miR-1246</i>	Up-regulation	CCNG2	66
<i>miR-200a</i>	Down-regulation	N-cadherin, ZEB1, and vimentin	67
<i>miR-1181</i>	Down-regulation	SOX2 and STAT3	68
<i>Mir-29c</i>	Down-regulation	Wnt	69
<i>miR-21</i> and <i>miR-221</i>	Up-regulation	CDK6, C5ORF41, EFNA1, IRAK3, KLF12, MAPK10, NRP1, SMAD7, SOCS6, and ZBTB41	70
<i>miR-17-92</i>	Up-regulation	NODAL/ACTIVIN/TGF- β 1	71
<i>miR-200c</i>	Down-regulation	ZEB1	72
<i>miR-744</i>	Up-regulation	SFRP1, GSK3 β , and TLE3 (Wnt)	73
<i>miR-30a</i> , <i>miR-30b</i> , and <i>miR-30c</i>	Up-regulation	EMT and CD133	74
<i>miR-106a</i>	Up-regulation	EMT	75

C5ORF41, chromosome 5 open reading frame 41; CCNG2, cyclin G-2 protein; CDK6, cyclin-dependent kinase 6; EFNA1, ephrin A1; EMT, epithelial-to-mesenchymal transformation; GSK3 β , glycogen synthase kinase-3 beta; IRAK3, IL-1 receptor-associated kinase 3; MAPK, mitogen-activated protein kinase; NODAL, nodal growth differentiation factor; NRP1, neuropilin-1; PDAC, pancreatic ductal adenocarcinoma; SFRP1, secreted frizzled related protein 1; SOCS6, suppressor of cytokine signaling 6; SOX2, sex determining region Y-box 2 (alias SOY); TGF- β 1, transforming growth factor- β 1; TLE, transducin-like enhancer protein; ZBTB41, zinc finger and BTB domain containing 41; ZEB1, zinc finger E-box binding homeobox 1.

miRNA Epigenetic Regulation in PDAC

Several different tumor suppressor genes in human cancers are silenced by chromatin alterations, including promoter methylation and histone deacetylation. Likewise, miRNAs have also been shown to be regulated epigenetically.⁷⁶ Such epigenetic alterations may be used in the diagnosis and treatment of PDAC. However, the research on the impact of epigenetic modulation of miRNAs in PDAC is scarce.

Overexpression of several miRNAs that are generally suppressed, *miR-29a*, *miR-29b*, *miR-103*, *miR-107*, and *miR-320*, was shown in PDAC cell lines as a result of treatment with the histone deacetylase inhibitor trichostatin A or the hypomethylating agent decitabine. The enforced expression of *miR-107* in MiaPACA-2 and PANC-1 cells inhibited their *in vitro* growth and suppressed the putative *miR-107* target, cyclin-dependent kinase 6, thereby providing a functional basis for the epigenetic inactivation of this miRNA in PDAC.⁷⁷

A study of epigenetic regulation of different miRNAs in a broader panel of tumors (including mammary tumors, colorectal tumors, pancreatic tumors, and soft tissue sarcomas) showed consistent CpG methylation of *miR-34a* and *miR-34-b/c* in the tumor tissues.⁷⁸ Inducing overexpression of *miR34a* by treatment with decitabine or the histone deacetylase inhibitor vorinostat resulted in cell cycle arrest, reversal of stemness, suppression of EMT and invasion, and PDAC growth inhibition.⁷⁹ CpG island methylation was found to lead to the underexpression of the tumor suppressor *miR-1247*.⁸⁰ Contrary to noncancerous tissues, PDAC contains highly methylated *miR-124-1*, *miR-124-2*, and *miR-124-3*. Hypermethylation of these genes was linked to worse survival of PDAC patients.⁸¹ Functional studies showed that *miR-124* inhibited cell proliferation, invasiveness, and metastasis through *KRAS* downstream RhoGTPase effector Rac1, highlighting its tumor suppressor property, often silenced epigenetically in PDAC.

Mortality factor 4 (MORF4)-related gene-binding protein (MRGBP) binds directly to the histone acetyltransferase complex, components of tat-interacting protein 60, and histone deacetylase complexes. The loss of MRGBP suppresses the replication and growth of pancreatic cancer cells, promotes apoptosis, and inhibits migration and invasiveness. The overexpression of MRGBP has been documented in malignancies, including pancreas cancer. MRGBP expression is down-regulated by *miR-137*, its loss leading to overexpression of MRGBP. Adverse tumor characteristics were seen in patients with PDAC in whom there was loss of *miR-137*. The reexpression of *miR-137* significantly blocked migration and invasion of pancreatic cancer cells. Hence, the loss of *miR-137* may function as a novel tumor promoter by facilitating the overexpression of MRGBP in PDAC.⁸²

Higher serum levels of *miR-21* as a result of promoter histone acetylation are seen in patients with gemcitabine-resistant PDAC compared with subjects with gemcitabine-sensitive tumors. In PDAC cells, after treatment with gemcitabine, the *miR-21* levels increased significantly, causing a marked increase in invasion and metastatic potential through activation of Akt and reduction in phosphatase and tensin homolog (PTEN). These changes were reversible with anti-miR-21 transfection.⁸³

Given that several epigenetically silenced miRNAs have a role in promoting EMT, the link to miRNA epigenetic alterations and stemness has also been postulated. A genome-wide study of the DNA methylation profile in PDAC CSC investigated the impact of DNA methyltransferases in PDAC CSC maintenance and tumorigenicity. CSCs had higher levels of DNA methylation because of higher DNA (cytosine-5)-methyltransferase 1 (DNMT1) levels that lead to the suppression of the *miR-17-92* cluster. In functional studies, pharmacologic or genetic targeting of DNMT1 blocked the self-renewal and *in vivo* tumorigenic potential of CSCs through the reexpression of *miR-17-92*.⁸⁴

The restoration of *miR-377* levels resulted in a reduction of the expression of DNMT1 and reactivation of target genes *BNIP3* and *SPARC* via promoter demethylation, leading to inhibition of cell proliferation and induction of apoptosis.⁸⁵ Epigenetic reprogramming of *miR-663a* and *miR-4787-5p* by synthetic histone methylation reversal agents attenuated TGF- β 1-induced EMT features in human PDAC.⁸⁶ The levels of *miR-663b* are lower in PDAC than in normal tissue. Expression of *miR-663b* is suppressed epigenetically (on H3K4me3 and H3K27me3 promoters) by the long noncoding RNA, homeobox (HOX) transcript antisense RNA (*HOTAIR*). Overexpression of *miR-663b* resulted in inhibition of cell proliferation, invasion, and migration and induced apoptosis. Reexpression of *miR-663b* or silencing of *HOTAIR* blocked tumor growth by targeting insulin-like growth factor 2 in PDAC.⁸⁷ These proof-of-concept studies validate the concept of epigenetic modifications targeting miRNAs and may be explored further in the development of therapeutics for PDAC.

miRNA Deregulation Supporting the PDAC Microenvironment

Desmoplastic reaction, which is characterized by a dense concentration of extracellular matrix proteins, activated pancreatic stellate cells, and immune cells surrounding tumor, has been a well-described feature of pancreatic adenocarcinoma. This has been considered a barrier to the delivery of anticancer drugs, as one explanation for poor treatment outcome.⁸⁸ In experiments in which human pancreatic stellate cells were co-cultured with pancreatic adenocarcinoma cells, there was increased expression of fibrosis-related genes along with reduction in the expression of miRNA let-7d. The inhibition of the miRNA let-7d resulted in enhanced expression of fibrosis-related genes [α -smooth muscle actin, platelet-derived growth factor receptor- β , and collagen type XXI alpha 1 chain (COL1A1)]. Data from The Cancer Genome Atlas showed the expression of *let-7d* was consistently reduced in pancreatic cancer compared with normal tissue. In addition, patients with pancreatic ductal adenocarcinoma were shown to have lower serum levels of *let-7d* compared with healthy controls. Reduced *let-7d* expression correlated with poor overall survival in patients who were treated with gemcitabine-based chemotherapy.⁸⁹

miRNA Deregulation, Exosomes, and Other Cellular Transport Systems

Exosomes that are alias microvesicles are small vesicular structures that are approximately 40 μ m in size.⁹⁰ These vesicular structures harbor several different types of biological molecules, ranging from RNA to proteins and even drug breakdown products.⁹¹ There is compelling evidence suggesting the role of exosomes in cancer-related signaling, due in part to the varied cargoes that they carry.⁹² It is not surprising to note that miRNAs are also transported through exosomes and have been implicated in disease progression and resistance to therapy.⁹³ PDAC is shown to have high exosomal levels of *miR-10b*, *miR-21*, *miR-30c*, and *miR-181a* and low *miR-let7a* compared with normal pancreatic tissue.⁹⁴

In patients with PDAC, serum levels of *miR-155* and *miR-196a* were lower, whereas *miR-17-5p* and *miR-21* were higher, when compared with healthy controls. The overexpressed *miR-17-5p* also correlated with metastasis and poor overall survival.⁹⁵ These miRNAs can be found free or exosome bound in the serum, which may be easily accessed for diagnostics, prognostics, and therapy response in patients with pancreatic cancer.⁹⁶

The connective tissue growth factor and *miR-21*, expression of which is highly up-regulated in activated pancreatic cancer stellate cells, are seen in pancreatic cancer stellate cell-derived exosomes. Such exosomal secretory *miR-21* was shown to generate a positive feedback loop stimulating connective tissue growth factor expression. These exosomes facilitate the delivery of these agents to other pancreatic stellate cells.⁹⁷ Pancreatic cancer-derived exosomes were shown to down-regulate toll-like receptor 4 and downstream cytokines in dendritic cells via *miR-203*.⁹⁸ In addition, the regulatory factor X-associated protein, an important transcription factor for major histocompatibility complex II, was inhibited by *miR-212-3p* transferred from PC-secreted exosomes. Such *miR-212-3p*-mediated suppression of regulatory factor X-associated protein resulted in major histocompatibility complex II suppression and resulting immune tolerance in dendritic cells.⁹⁹

A successful transfection of Panc-1 cells with *miR-155* and *miR-125b2* caused alterations in their derivative exosome content, resulting in differential communication and

Table 3 List of Up-Regulated Exosome-Transported miRNAs Identified in Serum or Tumor Microenvironment of Pancreatic Cancer

Exosome miRNAs	Disease stage and outcome	References
<i>miR-196a</i>	Pancreatic ductal adenocarcinoma	101
<i>miR-1246</i>	Intraductal papillary mucinous neoplasms	101
<i>miR-1246</i> , <i>miR-3976</i> , <i>miR-4306</i> , and <i>miR-4644</i>	Pancreatobiliary cancer diagnosis	102
<i>ExmiR-191</i> , <i>ExmiR-21</i> , and <i>ExmiR-451a</i>	Pancreatic cancer diagnosis	103
<i>miR-451a</i>	Pancreatic cancer recurrence	104
<i>miR-155</i>	Gemcitabine resistance	105
<i>miR-23b-3p</i>	Correlated to carbohydrate antigen 19-9 (CA19-9) levels	106

reprogramming of the macrophage cells (J774.A1) to an M1 phenotype. This technical success has led to the hypothesis that genetic therapies targeted toward selective manipulation of tumor cell–derived exosome content may become a promising strategy in pancreatic cancer.¹⁰⁰ Table 3 summarizes exosomes described in miRNA aberrant expression in PDAC.^{101–106}

The human ether-a-go-go-related potassium channel 1 has been identified to play a carcinogenic role in multiple malignancies, including PDAC. High expression of human ether-a-go-go-related potassium channel 1 is associated with proliferation and invasiveness of pancreatic cancer cells. The expression of human ether-a-go-go-related potassium channel 1 has been shown to be inhibited by *miR-493* and *mi-96*. The loss of *miR-493* and *mi-96* leads to increased expression of human ether-a-go-go-related potassium channel 1 in pancreatic cancer cells, leading to proliferation and invasion.^{107,108}

miRNA Deregulation and Immunosuppression in Cancers

Tumor-derived exosomes deliver miRNAs to immune system cells that facilitate immune evasion by the tumor cells, by affecting various parts of the immune system. There has been much work published specific to pancreatic cancer. We have summarized the impact of miRNAs on immune responses to various malignancies reported in the literature.

Pancreatic cancer cell–derived exosomes containing *miR-212-3p* down-regulate the transcription factor regulatory factor X–associated protein when pulsed onto immature dendritic cells, resulting in reduced expression of major histocompatibility complex class II molecules and possibly ineffective antigen presentation abilities of the dendritic cells. Exposing immature dendritic cells to *miR-203* resulted in reduced toll-like receptor 4 expression, which may affect subsequent T- and B-cell activation by the dendritic cells.⁹⁹ Aberrant expression of *miR-146a* in dendritic cells from PDAC patients was observed, along with repression of *SMAD4*, which resulted in impaired differentiation and antigen presentation by dendritic cells.¹⁰⁹

In patient-derived esophageal adenocarcinoma specimens and cell lines, overexpression of *miR125a-5p* and *miR148a-3p* resulted in reduced levels of transporter associated with antigen processing (TAP)2 and major histocompatibility complex-I, which affect tumor cell recognition by specific cytotoxic T lymphocytes.¹¹⁰ Natural killer cells have been shown to be down-regulated by *miR-23a* carried in exosomes derived from hypoxic tumor cells,¹¹¹ whereas *miR-362-5p* has been shown to promote natural killer cell activation, which may lead to their overstimulation and subsequent exhaustion.¹¹² Tumor-derived exosomes from nasopharyngeal carcinoma were shown to contain overexpressed *miR-24-3p*, *miR-106a-5p*, *miR-891a*, *miR-20a-5p*, and *miR-1908*, which all have

been linked to down-regulation of mitogen-activated protein kinase 1 and subsequent transformation of T cells from type 1 and 17 helper T-cell to type 2 helper T-cell and regulatory T-cell phenotypes.¹¹³ Tumor-derived exosomes from non–small-cell lung carcinoma were shown to deliver *miR-21* and *miR-29a* to macrophages entering the tumor microenvironment. These miRNAs activated toll-like receptor 8 (murine toll-like receptor 7) and subsequently nuclear factor κ –light chain enhancer of activated B cell pathway, which culminated in IL-6 and tumor necrosis factor- α secretion, generating a protumor inflammatory environment.¹¹⁴

miRNAs as Diagnostic and Prognostic Tools in Pancreatic Adenocarcinoma

Diagnostic Utility of miRNAs

Differential expression of miRNA profiles has been well described in PDAC, with miRNAs isolated from various patient-derived specimens, including the peripheral blood, pancreatic tissue, and digestive juices. Circulating miRNAs are attractive targets for scientific exploration because of their abundance, stability, and technical ease of their isolation and amplification with inexpensive and noninvasive techniques.¹¹⁵

Several miRNA profiles had differential expression in PDAC tissue, unlike in healthy pancreatic tissue and benign pancreatic pathology. The overexpression of *miR-483-3p* in the blood differentiates PDAC from intraductal papillary mucinous neoplasms and healthy pancreas tissue with sensitivity of >40%, which is comparable to that of CA19-9 (45%).¹¹⁶ Plasma levels of *miR-196a* and *miR-1246* are significantly higher in patients with early-stage PDAC when compared with healthy volunteers. Higher levels of *miR-196a* have better specificity to PDAC, whereas *miR-1246* is seen with intraductal papillary mucinous neoplasms. In pancreatic neuroendocrine tumors, these miRNAs appear to be unchanged.¹⁰¹

The serum levels of *miR-200a* identify patients with PDAC from healthy controls with a sensitivity and specificity of >80%. The serum levels of *miR-200b* identify patients with PDAC from healthy controls with a sensitivity and specificity of 71.1% and 96.9%, respectively.¹¹⁷ Serum *miR-1290* identified patients with early-stage pancreatic cancer from controls better than CA19-9, while additionally hinting at the pancreatic cancer cell invasion capability.¹¹⁸ When a panel of *miR-16* and *miR-196a* was used in conjunction with CA19-9, PDAC was identified with a sensitivity and specificity of >90% from healthy controls and chronic pancreatitis.¹¹⁹ In metastatic PDAC, more than twofold down-regulation of *miRNA-205* was seen contrary to nonmetastatic disease.⁶³

Diagnostic kits were developed that detect aberrant expression of miRNAs to discriminate malignant from benign

pancreatic lesions. One such kit is the miRInform Pancreas (Asuragen, Inc. Austin, TX), which uses miR-196a and miR-217 to differentiate PDAC from other benign conditions with sensitivity and specificity of 95%.¹²⁰ Another kit developed uses miR-21-5p, miR-375, miR-485-3p, and miR-708-5p to differentiate PDAC from intraductal papillary mucinous neoplasms with sensitivity of 95% and specificity of 85%.¹²¹ One of the main challenges in fully exploiting the miRNAs for diagnostic purposes is that, despite their differential expression in cancerous tissues, their mechanistic role in the molecular carcinogenesis is not completely well understood. In addition, because miRNAs control the expression of >60% of the normal protein-coding genes in humans, they may lack the specificity to the oncogenesis in question.¹⁵

miRNAs as Prognostic Biomarkers

Poor survival outcomes have been reported in PDAC patients with lower expression of *miR-183* and *miR-34a* as well as high expression of *miR-1290*, *miR-155*, *miR-203*, *miR-222*, and *miR-10b*.^{118,122} In a retrospective study of patients who underwent pancreatic resection for PDAC and received adjuvant gemcitabine treatment, the overexpression of *miRNA-142-5p* and *miR-21* were shown to be an independent prognostic marker for longer overall survival.^{116,123,124}

From analysis of The Cancer Genome Atlas, 494 miRNAs were evaluated for their impact on overall survival of patients. The top five miRNAs whose overexpression was associated with better overall survival were *miR-1301*, *miR-125a*, *miR-376c*, *mi-328*, and *miR-376b*. However, the underlying molecular mechanisms have not been fully investigated.¹²⁵ A differential expression of *hsa-miR-as93*, *hsa-miR-203*, *hsa-miR-424*, *hsa-miR-1266*, and *hsa-miR-4772* was shown to correlate with overall survival in PDAC using The Cancer Genome Atlas data. This was also validated in newly diagnosed patients with PDAC. The target genes for these miRNAs are suggested to involve various pathways, including TGF- β , phosphatidylinositol 3-kinase–Akt, and stem cell signaling pathways.¹²⁶

Tumor analysis of >100 Korean patients with pancreatic ductal adenocarcinoma using microarray analysis yielded 19 miRNAs that can be used as a classifier to predict prognosis. Some of these included the following: miRNAs, *miR-106b-star*, *miR-324-3p*, and *miR-615*, which were related to a p53 pathway, whereas *miR-324*, *miR-145-5p*, *miR-26b-5p*, and *miR-574-3p* were related to a cyclooxygenase (Cox)-2 centered pathway.¹²⁷

The pattern of miRNA expression can also be used as a surrogate for estimation of overall tumor burden and the emergence of resistant clones of cancer cells.¹²⁸ A postoperative decrease in the plasma levels of *miR-18a* and *miR-221* was shown after surgical resection of pancreatic cancer. In a patient with disease recurrence postoperatively, an elevation of *miR-18a* was shown without a parallel increase in CA19-9.^{129,130}

Therapeutic Implications of miRNAs

miRNAs and Chemotherapy Resistance in PDAC

Differential expression of miRNA is well described in pancreatic cell lines that are resistant to chemotherapy. Different possible mechanisms of miRNA-related chemotherapy resistance have been postulated. Increased expression of drug efflux pumps by the cancer cell has been described as a mechanism of resistance. The expression of P-glycoprotein, a multidrug efflux pump that confers cancer cell resistance to a broad spectrum of drugs, is modulated by *miR-27a* and *miR-451*.¹³¹

Another drug resistance mechanism described with aberrant miRNA expression is evasion of apoptotic signals and cell cycle deregulation. *miR-506* enhances chemosensitivity in PDAC by inhibiting cell proliferation and inducing cell cycle arrest at the G₁/S transition. *miR-506* down-regulates *SPHK1*, which participates in Akt/NF- κ B-dependent apoptosis, inducing chemoresistance. Reduced expression of *miR-506* and overexpression of *SPHK1* correlate with poor survival outcomes in PDAC.¹³²

Inhibiting *miR-221/222* induces G₁/S arrest and suppresses the proliferation of pancreatic cancer cells, followed by induction of resistance to gemcitabine.¹⁹ The overexpression of antiapoptotic proteins, such as Bcl-2 proteins, induced by *miR-21*, can confer resistance to chemotherapy, as shown in pancreatic cancer cells.²⁰ *miR-301a*, which directly controls the expression of the Bim gene (Bcl-2–like protein 11), is overexpressed in PDAC, thereby fostering pancreatic cell proliferation. The reexpression of the Bim gene through depletion of *miR-301a* leads to reduced pancreatic cancer cell proliferation.¹³³ In *in vitro* experiments, *miR-181b* sensitizes pancreatic cancer cells to gemcitabine by down-regulating Bcl-2.¹³⁴ Restoration of *miR-34* in pancreatic cancer MiaPaCa2 and BxPC3 cells inhibits the expression of Bcl-2/Notch1/Notch2, sensitizing them to chemotherapy and inducing apoptosis.¹⁸ In pancreatic cell lines, *miR-320a* increased resistance to fluorouracil through its effects on programmed cell death 4.¹³⁵

Down-regulation of *miRNA-200* family expression was seen in gemcitabine-resistant pancreatic cancer cells.²⁴ It was reported that *miR-365* induced gemcitabine resistance in pancreatic cancer cells by directly targeting Src homology 2 domain containing 1 (*SHC1*) and apoptosis promoting protein BAX. The knockdown of both SHC1 and BAX was also shown to increase resistance to gemcitabine.²¹ An *in vitro* gemcitabine sensitivity was induced by the knockdown of *miR-1246* expression in gemcitabine-resistant pancreatic cancer cell lines. This was believed to be due to restoration of cyclin G-2 protein expression, a tumor suppressor gene. A high expression of *miR-1246* was associated with low expression of cyclin G-2 protein and poor overall survival in patients.⁶⁶

The transcription factor–activating protein 2 γ is negatively regulated by *miR-10a-5p*. The overexpression of *miR-10a-5p*

(low levels of transcription factor—activating protein 2 γ) is seen in gemcitabine-resistant PDAC cells and enhances metastatic behavior and gemcitabine resistance *in vitro* and *in vivo*. The overexpression of transcription factor—activating protein 2 γ resensitizes PDAC cells to gemcitabine.¹³⁶

The expression of 13 miRNAs that target genes enriched for EMT predicts response to epidermal growth factor receptor inhibition in cancer cell lines and tumors, with distinction between the primary and metastatic tumors. The ectopic expression of *miR-200c* alters expression of EMT proteins and possibly improves sensitivity to erlotinib.^{137,138}

Potential of miRNAs as PDAC Therapeutics

miRNA-based interventions have been viewed as possible therapeutic targets because miRNAs affect multiple gene expression and cellular pathways. It has been hypothesized that they may have an advantage over single gene—directed therapeutic interventions in the design of better cancer treatment. Different approaches have been taken to alter the expression of oncogenic and tumor suppressor miRNAs. Transfecting pancreatic CSCs with an *miR-200c* mimic and *miR-200a* reduced their colony formation, invasiveness, and resistance to chemotherapy by regulating epithelial mesenchymal transformation.^{67,139} The expression of tubulin beta 3 class III (TUBB3) and p21 activated kinase 1 (Pak-1) was reduced by transfection of pancreatic cells resistant to gemcitabine with *miRNA-205* and *miR-7*, respectively. This decreased the CSC population.⁶³

The administration of micelles of gemcitabine and the tumor suppressor *miRNA-205* lead to significant inhibition of tumor growth and increased apoptosis in preclinical models.¹⁴⁰ Targeting *miR-21* using lentivirus vector was shown to inhibit cell proliferation.¹⁴¹ The suppression of *miR-199a* and *miR-214* in pancreatic stellate cells dampened their protumor effect and decreased tumor growth.¹⁴² The use of synthetic curcumin analog has been shown to down-regulate *miR-21* and restore *miR-200* and tumor suppressor PTEN, resulting in depletion of the cancer stem cell population and tumor growth suppression.¹⁴³ Reexpression of the *miRNA-200* family, along with reversal of EMT and resensitization of pancreatic cancer cells to gemcitabine, was seen as a result of treatment with isoflavone or 3,31-diindolylmethane.³²

Nanodelivery of Anti-miRNAs

Given the significant roles miRNAs play in the biology of PDAC, attempts have been made to use these tiny RNAs as therapeutics in this deadly disease. Nevertheless, these studies remain mostly restricted to preclinical evaluations in cellular and animal models. A poly (D, L-lactide-coglycolide)—based nanoformulation of *miR-150* (*miR-150-NF*), developed by Arora and colleagues,¹⁴⁴ showed PDAC cellular uptake, engagement with its target gene *MUC4*, and consequent reduction in cellular proliferation. The i.v.

delivery with *miR-34a* and *miR-143/145* nanovectors inhibited the growth of MiaPsCa-2 s.c. xenografts in animal models.¹⁴⁵

Similarly, miR-145—based magnetic nanoparticle formulation showed antitumor potential in cellular models of PDAC.¹⁴⁶ Combination approaches in which anti-miRNA oligo miRNAs are loaded in nanoparticles with chemotherapeutics have also been attempted. For example, combined therapy of miR-21 antisense oligonucleotides and gemcitabine using a targeted codelivery nanoparticle carrier was shown to have synergistic antitumor activity in PDAC cells and animal tumor models.¹⁴⁷ In another study, miRNA-mimic to increase *miR-34a* together with siRNA to silence *PLK1* oncogene were loaded in biodegradable amphiphilic polyglutamate amine polymeric nanocarrier. Systemic administration of amphiphilic polyglutamate amine polymeric nanocarrier—miRNA—siRNA polyplexes to orthotopically inoculated PDAC-bearing mice showed no toxicity and accumulated at the tumor, resulting in an enhanced antitumor effect due to inhibition of MYC oncogene, a common target of both *miR-34a* and *PLK1*.¹⁴⁸ Despite this promising preclinical work, it is unclear how these different formulations will work in humans. Therefore, more work is needed to justify the use of nanoparticles as delivery vehicles for noncoding RNAs in the treatment of PDAC.

Clinical Updates

Treatments aimed at RNA interference have been of interest for new drug development recently. RNA interference using siRNAs and miRNA inhibitor has been studied for different indications, including pancreatic ductal adenocarcinoma, hepatitis C virus infection, ophthalmic disease, and amyloidosis in clinical trials (phases 1 through 3). Despite the interest, there are some unanswered questions in the development of RNA interference as a therapeutic strategy, including the ideal target, best route of administration, delivery method, and potential toxicities.¹⁴⁹

Currently, there is no ongoing clinical study targeting miRNAs in the treatment of pancreatic ductal adenocarcinoma reported. There are ongoing studies exploring the diagnostic utilities of different combinations of miRNAs. A few clinical trials aimed at RNA interference in patients with pancreatic ductal adenocarcinoma are summarized below to give an overview of such therapeutic interventions.

There is an open-label phase 1/2a study of siRNA against *KRAS* (G12D) delivered through an intratumoral slow-releasing device in combination with gemcitabine for locally advanced pancreatic adenocarcinoma. The recommended phase 2 dose was then combined with modified 5-fluorouracil, leucovorin, irinotecan and oxaliplatin (FOLFIRINOX). Of the total 15 patients treated, a third experienced serious adverse events, whereas 90% experienced grade 1 to 2 adverse events. Radiographic stable

disease was the best response in 83% of the patients, whereas 17% had partial response. The median overall survival was 15.12 months.¹⁵⁰

There is *Atu027*, a novel liposomal RNA interference agent including an siRNA, which silences expression of protein kinase N3 in the vascular endothelium and has been shown to inhibit local tumor invasion and lymphatic and visceral metastases in animal models. In a first-in-human study, *Atu027* was shown to be safe in patients with advanced solid tumors. Stable disease was seen in 41% of patients for at least 8 weeks.¹⁵¹

Conclusions

Accumulating evidence supports that miRNAs may exert a key role in the pathogenesis of PDAC, by affecting important genetic alterations like *KRAS*, *Tp53*, and *TGFβ/SMAD*, cancer stemness, and support for an unfavorable tumor microenvironment. miRNAs represent an appealing therapeutic target and important biomarkers that may be used in the diagnosis, prediction of treatment response, and prognosis of PDAC. Their ubiquitous presence and the incomplete understanding of their effect on the normal cellular mechanisms and intercellular interactions limit their current clinical applicability and need to be explored further. Despite extensive preclinical work, a practical clinical application is still lacking.

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