

MIR-744 AND MIR-30D ACT AS TUMOUR SUPPRESSORS IN PANCREATIC
DUCTAL ADENOCARCINOMA

BY

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ABSTRACT Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer and has one of the highest mortality rates and poorest prognosis among cancers. Unfortunately, the diagnosis of this cancer is often a lengthy process and requires invasive techniques like tissue biopsies and endoscopies, and the use of various imaging modalities that requires specialized training. The development of liquid biopsy, a non-invasive diagnostic technique that requires a simple blood test, would be beneficial to help diagnose PDAC. My research project aims to identify the biological significance of previously identified up- and downregulated miRNAs found in extracellular vesicles extracted from PDAC patient plasma compared to controls. First, a number of miRNA targets ($n = 17$) identified by small RNA sequencing in patient samples were validated by droplet digital PCR. Some of these miRNAs ($n = 4$) were then used to transfect one healthy (H6C7) and one cancerous cell line (PANC 10.05) with a mimic miRNA (increases abundance) and a siRNA (decreases abundance) for each target. The number of live cells was measured 72h post-transfection by flow cytometry. It was found that miR-744-5p mimic decreases the number of live cells ($n = 3$, $P < 0.05$), while miR-30d siRNA ($n = 3$, $P < 0.05$) increases the number of live cells. Interestingly, miR-744-5p mimic and miR-30d siRNA had no significant effect on the number of H6C7 cells. These results suggest that miR-744-5p and miR-30d may both be tumour suppressor miRNAs in pancreatic cancer.

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Completing my undergraduate degree at Mount Allison University has been one of the most rewarding experiences in my life so far. The people I met, the skills I learned and the memories I made will stay with me forever.

I hope I am making my grandma LaPlante proud of all the hard work I put into my research as she lost her battle to pancreatic cancer in 2014.

Glossary of terms and abbreviations

PDAC – Pancreatic ductal adenocarcinoma

miRNA – microRNA

NET – neuroendocrine tumour

PanIN – pancreatic intraepithelial neoplasias

KRAS – Kirsten Rat Sarcoma gene

CDKN2A – Cyclin Dependent Kinase Inhibitor 2A gene

TP53 – Tumor protein 53 gene

SMAD4/DPC4 – Deleted in Pancreatic Cancer 4 gene

EUS – Endoscopic ultrasonography

CAF – Cancer-associated fibroblasts

PSCs – Pancreatic stellate cells

CTC – Circulating tumour cells

ctDNA – circulating tumour DNA

ctRNA – circulating tumour RNA

EV – extracellular vesicle

Pri-miRNA – primary miRNA

Pre-miRNA – precursor miRNA

NGS – Next generation sequencing

FBS – fetal bovine serum

ddPCR – Droplet digital PCR

ATG5 – Autophagy related gene 5

SFRP1 – secreted frizzled-related protein 1

GSK3 β - Glycogen synthase kinase 3 β

TLE3 – transducin-like enhancer 3

HNRPN C - heterogenous nuclear ribonucleoprotein C

NFIX – nuclear factor X

Shh – Sonic Hedgehog pathway

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INTRODUCTION

Cancer is a disease that affects everyone in one way or another at some point in our lifetime. Generally, cancer cells develop due to mutations in oncogenes and tumour suppressor genes that alter their function¹. These mutations cause disruptions in cell cycle control which leads to an increased proliferation and a decreased apoptosis rate^{2,3}. Cancer cells acquire certain characteristics as they develop, allowing them to metastasize to other areas of the body⁴. These characteristics can include, but are not limited to, the development of self-sufficient growth signals while silencing antigrowth signals, metabolic reprogramming, and the ability to bypass immune system destruction⁴. Breakthroughs in cancer research have allowed the development of a variety of therapies for certain cancers that target some of these acquired characteristics. Unfortunately, advances in diagnosing and curing pancreatic cancer have not been as successful. Pancreatic cancer cells exhibit unique characteristics that delay diagnosis and have a complex tumour microenvironment that may be associated with their resistance to the current available treatments⁵. This study will aim to determine the functionality of liquid biopsy in diagnosing pancreatic ductal adenocarcinoma (PDAC) by identifying potential PDAC specific biomarkers in blood plasma, like microRNAs (miRNA), and identifying which genes they regulate. This study could also be a starting point in the development of targeted therapies for PDAC.

Pancreatic Ductal Adenocarcinoma

PDAC is the most common form of pancreatic cancer and has one of the highest mortality rates and poorest prognosis rates among cancers⁶. Approximately 90% of pancreatic cancer cases are PDAC, and it has a 5-year survival rate of less than 10%, mostly due to it almost always being diagnosed in the late stage⁵. In 2020 alone, approximately 6000 Canadians will be diagnosed with pancreatic cancer, and another 5300 will die from it⁷. The pancreas is a dual function organ playing important roles in the digestive and the endocrine systems⁸. The exocrine portion of the pancreas is responsible for the secretion of digestive enzymes, while the endocrine portion secretes a variety of hormones, including insulin and glucagon⁸. The proper functioning of the endocrine portion of this organ is crucial in maintaining glucose homeostasis in the body⁹. Tumours arising from endocrine cells are classified as neuroendocrine tumours

(NET), whereas those arising from exocrine cells are classified as adenocarcinomas or PDAC⁸. Adenocarcinomas originate from pancreatic intraepithelial neoplasias (PanIN) that develop in acinar cells of the pancreas due to inactivation of the KRAS (Kirsten Rat Sarcoma) and CDKN2A (Cyclin Dependent Kinase Inhibitor 2A) genes¹⁰. Mutations in TP53 (Tumor Protein P53) and SMAD4/DPC4 (Deleted in Pancreatic Cancer-4) arise later in the development of PanIN lesions and these allow the cancer to progress and invade the body¹⁰. Tumours that originate in the head of the pancreas are usually diagnosed earlier and have a better prognosis rate than the ones originating in the body or the tail of the pancreas (Figure 1)⁵. Physical symptoms of this cancer include jaundice, abdominal and back pain, weight loss, nausea, vomiting, and in some cases, the onset of type 2 diabetes^{5,11}. Currently, the median survival rate for this cancer is approximately four months, and is even lower if the cancer has already metastasized when diagnosed¹². Along with late diagnosis, the low survival rate of pancreatic cancer can be attributed to PDAC's resistance to chemotherapy. Although certain chemotherapies can be administered, they do not have a long-lasting effect as patients typically develop resistance to these treatments, making resection the only treatment available at this time¹³.

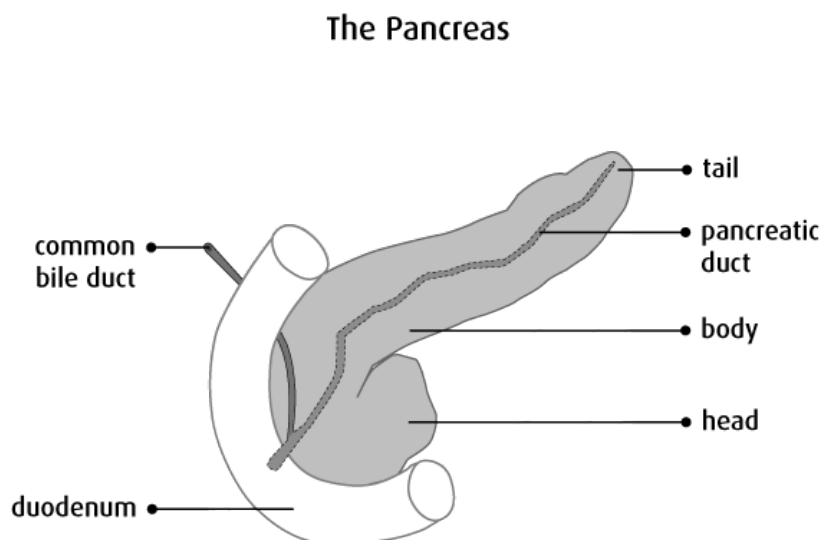


Figure 1. Anatomy of the human pancreas⁶

Currently, there are no available screening tools to diagnose PDAC when it is in its early stage. Moreover, the late onset of symptoms associated with PDAC delays the diagnosis of this disease¹⁴. Current diagnostic methods include a variety of imaging modalities like CT scans or MRI scans combined with an endoscopy to confirm the presence of cancer¹⁵. While MRI scans can detect smaller tumours at the early stage, CT scans are currently preferred because of their high accuracy and availability¹⁵. CT scans can also help determine if the cancer has metastasized, which is a common occurrence in PDAC due to its late diagnosis¹⁵. Endoscopic ultrasonography (EUS) is also a preferred diagnostic tool as it is more sensitive than CT scans in detecting small tumours and it can be used to collect a sample of the cancer to categorize the stage of cancer progression¹⁶. A combination of EUS and CT scans is most often used to diagnose PDAC. A variety of blood biomarkers, including miRNAs, have already been identified for early detection in lung and colorectal cancer, among others¹⁷. However, no specific biomarkers for PDAC have been identified, demonstrating the need for new, non-invasive diagnostic tools that could be used for prevention and early detection¹⁵. As discussed below, the use of liquid biopsy, an inexpensive and minimally invasive technique, could help alleviate this need. Liquid biopsy can not only detect dysregulated biomarker expression, but also genetic mutations that can be linked to diagnosis, prognosis and likelihood of responding to treatment¹⁸.

As mentioned previously, PDAC is somewhat unique because it does not respond to chemotherapy¹⁹. The only way to treat the cancer is to resect it, which is not always possible depending on the patient¹⁹. The inadequate response of pancreatic cancer cells to chemotherapy may be due to the accumulation of numerous genetic mutations. Pancreatic cancer cells, like many other cancer cells, display genomic instability, meaning multiple mutations are responsible for the onset of this cancer and each patient could express different genes and mutations in different amounts²⁰. Although PDAC cells have a wide variety of mutations, mutations in four key loci have been identified as having high prevalence in PDAC patients. These include KRAS, TP53, CDKN2A and SMAD4/DPC4²¹.

KRAS is a common mutation found in most cancer cells. KRAS mutations are found in over 90% of PDAC patients and influence the development and the progression

of the cancer¹⁹. For example, a study found that pancreatic cancer cells become dependent on KRAS mutations; once KRAS was inactivated, the cancerous cells would die²². The same study found that KRAS is also responsible for the dysregulation of the Hedgehog pathway since pancreatic cancer cells and PanIN lesions secrete Hedgehog ligands, which, in turn, promotes the development of a stroma (a structure consisting of mostly cancer associated fibroblasts and extracellular matrix that plays a role in maintaining the tumour microenvironment²³) and its tumorigenic characteristics²². The development of the stroma in PDAC has been linked to the progression and invasion of the cancer, suggesting that mutations in KRAS may be one of the first mutations to trigger the onset of PDAC²⁴.

CDKN2A mutations are also found in over 90% of pancreatic cancer patients. More specifically, mutations in this gene have been linked to familial pancreatic cancer, which accounts for 5-10% of PDAC cases^{25,26}. The CDKN2A gene encodes two different proteins: the p16 tumour suppressor protein and p14ARF. P16 regulates the cell cycle by inhibiting the transition from the G1 phase to the S phase^{25,27}. Furthermore, abnormally low p16 expression in PDAC patients has been linked to poorer prognosis since patients who expressed p16 had a longer survival rate than those who did not express p16 or expressed mutated versions of the protein²⁷. Interestingly, CDKN2A also encodes p14ARF²⁵. Alternative splicing allows one gene to code for multiple proteins and in cancer, this can become dysregulated and drive oncogenesis²⁸. The p14ARF protein negatively regulates cell proliferation by activating p53 which allows it to regulate the progression of the cell cycle at the G1 and G2 checkpoints²⁵. Another study adds to this claim by showing how the loss of p14ARF promotes tumour development even if wild type p53 is present, supporting its role in regulating proliferation²⁹.

Mutations in the tumour suppressor p53 gene are also associated with increased tumour growth and metastasis³⁰. In normal cells, p53 can sense cellular stress such as DNA damage and hyperproliferative signals which ultimately sends the cell into cell cycle arrest³¹. Gain of function mutations in the p53 protein disrupts its ability to sense cellular stress which in turn increases tumour growth³¹. Interestingly, a deficiency in p53 as well as gain of function mutations in p53 promote tumorigenesis³¹. Complete deficiency of the p53 protein has been linked to more malignant tumours than tumours

having the mutant p53 protein³¹. Since KRAS and p53 mutations are common in PDAC, there have been efforts to find a link between both mutations. It was found that the development of PDAC cells is dependent on the presence of KRAS mutations, and the mutation or complete loss of the p53 protein simultaneously³⁰. However, if wildtype p53 is present, KRAS mutations alone cannot drive the progression of PDAC³⁰. KRAS mutations are more commonly found in primary tumours while p53 mutations are found in metastasized tumours which suggests that KRAS is one of the first mutations present in PDAC, and p53 maintains the progression of the cancer³².

The fourth common mutation in PDAC is in the SMAD4/DPC4 gene. SMAD4 is a tumor suppressor protein that works with TGF- β to regulate cell proliferation³³. In normal conditions, TGF- β downregulates growth and promotes apoptosis but mutation or the loss of SMAD4 reverses these functions and TGF- β becomes a tumor promoter³³. The presence of SMAD4 decreases invasion and metastasis, which may explain why PDAC patients who express SMAD4 have a better prognosis and were more likely to respond to chemotherapy^{34,35}. *In vitro*, it has also been found that KRAS mutations alone cannot promote cell proliferation and that downregulation or complete loss of SMAD4 is needed to transform healthy H6C7 cells into malignant tumours, suggesting yet another interaction between KRAS and a separate mutation found in PDAC³⁵.

Finally, the tumour microenvironment of PDAC is also linked to its resistance to chemotherapy. The cancer-associated fibroblasts (CAFs), pancreatic stellate cells (PSCs) and the stroma are important components of the tumour microenvironment and play a role in PDAC's resistance to treatment by drastically reducing vascularization of the tumour making drug delivery a challenge⁵. As mentioned above, KRAS mutations lead to the generation of the stroma in PDAC by dysregulating the Hedgehog pathway. It has been shown that targeting the Hedgehog pathway to decrease the production of the stroma increases drug delivery to the tumour³⁶. The various interactions between these key genes and cells in the tumour microenvironment show the complexity of the onset and progression of PDAC. The presence of more than one of these prevalent mutations makes for a worse prognosis and may explain why this type of cancer is difficult to treat²¹.

The Potential of Liquid Biopsy as a Diagnostic Tool for PDAC

Overview of Liquid Biopsy

The lack of early diagnostic techniques and effective treatments for PDAC patients highlights the need for the development of practical diagnostic tools as well as functional treatments. Liquid biopsy refers to the sampling of a bodily fluid, often blood, to identify biomarkers linked to cancer³⁷. Liquid biopsy biomarker panels for other cancers are already being used in clinical settings¹⁷. These biomarkers may be cancer cell derived materials like circulating tumour cells (CTCs), circulating tumour (ct) nucleic acids (ctDNA, ctRNA), extracellular vesicles (EVs) and proteins³⁷. The identification of a certain biomarker(s) in a patient sample could signal the presence of an underlying disease.

The use of liquid biopsy as an early detection tool has potential as it has many advantages. A typical biopsy is invasive, and cannot always be performed depending on the patient's current state of health¹⁸. Typical biopsies only provide a snapshot of what is happening in the body at that specific time point¹⁸. Although a liquid biopsy also provides a snapshot of what is currently happening in the body, this type of biopsy can be repeated at multiple points in time, providing information that can help with diagnosis, the formation of a prognosis, chance of recurrence, treatment efficiency and treatment resistance¹⁸. Being able to get information at multiple points in time using a minimally invasive method would be beneficial for both patients and physicians. This technique could be used during routine blood tests for patients with family history of disease, increasing the possibility of an early diagnosis if any underlying diseases are present. In the case of familial pancreatic cancer, liquid biopsies may provide a simple method to screen for the development of disease.

Challenges and Limitations of Liquid Biopsy

The challenges and limitations associated with liquid biopsy are not related to the technique itself, but rather to the identification of biomarkers associated with specific diseases. The amount of information one can acquire from a liquid biopsy is great however it is crucial that specific biomarkers linked to disease are identified for it to be functional. Not knowing what information to look for renders liquid biopsy less useful to diagnose disease. One challenge is that ctDNA and CTCs are present in low quantities in

the blood and the amount differs greatly among individuals³⁸. Also, the current extraction methods are not yet standardized, leading to inconsistencies in results and detection methods are not sensitive enough to detect low numbers of CTCs or ctDNA in the blood compared to tissue biopsies³⁸. Consequently, there is still significant validation to occur before clinical adoption.

One of the biggest challenges associated with liquid biopsy is identifying a biomarker that is unique to a certain disease. KRAS is a common mutation found in PDAC and the presence and type of KRAS mutation can be used to assess prognosis in patients³⁹. Detecting KRAS mutations (ctDNA) linked to PDAC in blood plasma has been successful, however many other cancers express KRAS mutations³⁹. For example, KRAS mutations in colorectal cancer⁴⁰ and non-small cell lung cancer⁴¹ have been linked to patient response to chemotherapy treatments administered for these types of cancer, suggesting that KRAS mutations are not an ideal biomarker for PDAC diagnosis. It is important to note that a variety of PDAC-specific biomarkers are currently being studied, but none have been efficient or reliable for diagnosis as of yet¹⁵. This is why the identification of a novel, PDAC specific biomarker is important for diagnosis. Although various components can act as biomarkers in plasma, this research project focused on miRNA found in EVs extracted from PDAC patient plasma.

Extracellular vesicles

The development of liquid biopsy has greatly improved our knowledge of human diseases. More importantly, the discovery of genetic material in blood plasma allowed researchers to gain a better understanding on how diseases develop and progress in the human body. Many studies have focused on analyzing CTCs and ctDNA to try and understand cancer. However, recent studies have discovered the importance of EVs in cell-cell communication, explaining how they are very likely to be involved in cancer⁴².

Liquid biopsy also enables the analysis of EV released from cells. Similar to ctDNA and CTCs, extracting EVs from samples is a challenge since extraction methods are not standardized, a variety of methods exist depending on the sample type, and the method utilized can affect the yield of cargo from the EVs⁴³. The contents of EVs can include proteins, lipids, mRNA and other noncoding RNAs like miRNAs^{44,45}. Methods to isolate EVs include those that take advantage of their physical properties such as

sedimentation coefficient (i.e. ultracentrifugation), those that reduce their solubility in solution (i.e. chemical precipitation), and by affinity to EV markers (i.e. peptide-affinity capture)⁴⁶. The most commonly used method for EV isolation is ultracentrifugation, however this method requires specialized equipment, is time consuming and is not clinically amenable. Recently, a study found that different commercially available EV extraction kits yield different amounts of EVs, and that the miRNA content extracted from these EVs differs depending on what kit is used⁴⁷. This suggests that more research is required to identify the best EV isolation method for clinical adoption. However, a method developed at the Atlantic Cancer Research Institute utilizes peptide-affinity capture technology with the help of the Vn96 peptide. Vn96 captures small to medium EVs which contain numerous canonical EV markers⁴⁸⁻⁵².

EVs are nanosized membrane-bound particles that are released by all cells. Recent research has demonstrated that they play a role in intercellular communication, although they have many potential functions. The nomenclature for EVs has been conflicting since some researchers use similar terminology when referring to two different types of EVs. To alleviate this problem, the International Society for Extracellular Vesicles published guidelines on EV nomenclature⁵³. The term “EV” is the generic term given to particles naturally released by cells and can be divided in two categories: ectosomes (microparticles and microvesicles) and exosomes⁵⁴. Ectosomes are released from the plasma membrane through budding, while exosomes are released from the cell by exocytosis in multivesicular bodies⁵⁵. Ectosomes tend to be larger than exosomes but both types share many similarities which poses a challenge when isolating them from samples⁵⁶. Currently, there are no standardized isolation methods or defined characteristics to distinguish between both types of EVs⁵⁶. Also, when referring to published literature, the term (EVs, exosome, etc.) used by the original authors will be used.

The function or purpose of EVs is not completely understood, however recent research has begun to unravel this important question. Under normal physiological states, evidence suggests that exosomes aid in sorting out unwanted material to maintain internal homeostasis⁵⁴. The EVs then use this material to communicate with other cells in the body⁵⁴. This selective sorting process is well described in multiple cancers. In colon

cancer exosomes, KRAS mutations influence exosome miRNA composition⁵⁷. Cells harboring wild-type KRAS and cells harboring mutated KRAS secrete exosomes with different miRNA composition, suggesting KRAS plays a role in cancer progression beyond the effects it has within a cell⁵⁷. Furthermore, gastric cancer cells selectively sort let-7a, a tumour suppressing miRNA, into EVs and out of the cancer cells to maintain their cancerous characteristics⁵⁸. In PDAC, EVs secreted from the stroma contain miR-145, a tumour suppressor, which is then subsequently delivered to PDAC cells⁵⁹. This miRNA is not only selectively sorted in these cells to have its tumor suppressing function, but it is also inhibited by KRAS, suggesting yet again that KRAS has a strong influence on miRNA sorting in EVs⁵⁹. However, it is suspected that the filtering of material is not random, meaning cells participate in selective sorting. Selective sorting in cancer cells could include packaging tumour suppressing miRNAs in EVs so they cannot exert their functions on cells, or packaging oncogenic miRNAs in EVs to be delivered to other areas of the body to promote metastasis. Both outcomes could ultimately lead to faster disease progression. If selective sorting is occurring, extracting genetic material from EVs could lead to a better understanding of how PDAC progresses by studying differences in miRNA concentration in diseased *versus* healthy people.

EVs are important in maintaining the tumor microenvironment and promoting metastasis⁶⁰. It has been shown that exosomes secreted by CAFs in PDAC promote chemoresistance⁶¹. When treated with gemcitabine, these CAFs release more exosomes which promotes cancer by increasing proliferation in the neighboring cells⁶¹. Exosomes can also promote metastasis by packaging miRNAs and delivering them to various areas in the body. For example, colorectal cancers harboring a p53 mutation can reprogram macrophages⁶². The exosomes secreted by these modified macrophages carry miR-1246 which promotes tumor progression by communicating with other macrophages in the body to reduce the immunological response to the tumor cells⁶². The suppression of the immune system allows rapid metastasis, and miRNA-carrying exosomes secreted by PDAC tumor cells could mitigate this process. Therefore, miRNA in plasma EVs may be the result of (1) selective sorting (getting rid of tumour suppressing miRNA) and thus aiding in tumour growth or (2) cell-to-cell communication with the immune system to

evade immunosurveillance. These are but two possibilities and future research may uncover others.

Function of miRNAs in gene regulation and cell biology

miRNAs are small, non-protein coding RNAs that are approximately 20-25 nucleotides in length⁶³. Although miRNAs were once thought to have no known function, research has shown that they are crucial in gene regulation, and their dysregulation has been implicated in the pathogenesis of multiple disease⁶⁴. This makes them an important component to consider for the development of new diagnostic methods and treatments since some miRNAs target oncogenes and tumour suppressor genes^{18,65}. miRNAs are synthesized in a series of steps that begins with the transcription of an miRNA gene, creating a primary miRNA (pri-miRNA)⁶³. In the nucleus, a class 2 ribonuclease III enzyme called Drosha then cleaves the pri-miRNA into a precursor miRNA (pre-miRNA) which is then exported to the cytoplasm by exportin-5⁶³. Dicer, an endonuclease, then cleaves the pre-miRNA and creates the final, mature miRNA⁶³. These non-coding RNAs regulate gene expression post-transcriptionally by binding to mRNAs and directing the mRNA for degradation., leading to lower amounts of mRNA in the cell for translation and thus an eventual decrease in protein abundance⁶⁶. While this mechanism downregulates gene expression, it is suspected that miRNA-mediated gene upregulation is caused by miRNAs binding to mRNA and making them more stable⁶⁷. A single miRNA can have multiple target mRNA and can therefore regulate multiple genes and as a result a multitude of separate and/or connected cellular pathways.

A variety of miRNAs are known to influence cancer development, progression, and severity and these are called oncomirs. Oncomirs can be both oncogenes (promote cancerous phenotype) and tumor suppressors (inhibit cancerous phenotype). The same oncomir can be both an oncogene and a tumour suppressor depending on the cell type. One of the most commonly found oncomirs is miR-21. miR-21 has been defined as an antiapoptotic factor in human glioblastoma since it was found to be upregulated in glioblastoma patients and the inhibition of miR-21 caused cell death in multiple cell lines⁶⁸. In gastric cancer, miR-21 simultaneously downregulated apoptosis and upregulated proliferation⁶⁹. It was also found that miR-21 directly targets RECK, a tumour suppressor gene in gastric cancer and that complete knockdown of miR-21 reduced cell

invasion and migration ⁶⁹. In PDAC, high levels of miR-21 cause downregulation of the FasL pathway, a pathway involved in programmed cell death, which causes an increase in chemoresistance ⁷⁰. Another miRNA involved in cancer progression is miR-744. In PDAC, this upregulation of this miRNA increases the activity of the Wnt/ β -catenin pathway which eventually leads to an increase in proliferation of the cancer cells ⁷¹. miR-374 also plays a role in PDAC progression. The downregulation of this miRNA increases the expression of anti-apoptotic proteins which leads to increased chemoresistance in these cells ⁷².

Interestingly, miRNAs are also found in exosomes. Exosomes released from cancer cells do not contain high levels of mature miRNA, but they do contain the dicer enzyme required for cleaving pre-miRNA into mature miRNAs ⁷³. The inhibition of the dicer enzyme inside exosomes showed reduce tumour growth, suggesting that the mature miRNAs are not processed inside the cancer cell but rather inside the exosome prior to coming in contact with another cell ⁷³. Since PDAC is considered to be inherently metastatic, the miRNAs found inside exosomes released from PDAC tumour cells may be partially responsible for metastasis of this cancer.

Rational for Current Study

A variety of components could be involved in the development and the progression of PDAC. Unpublished small RNA Sequencing data from the Atlantic Cancer Research Institute (Moncton, NB) showed that a variety of miRNA found in EVs were up- or downregulated in PDAC patient plasma, suggesting that these miRNAs may be involved in regulating the progression of PDAC (Supplemental Figure 1). Since next generation sequencing (NGS) can lead to false positives or negatives, validating these targets through other means is crucial to further research ^{74,75}. This research will aim to understand the differences in miRNA expression in PDAC patients compared to controls.

The validation of the NGS data was done using droplet digital PCR (ddPCR). Validated targets will be short-listed according to three criteria: 1) highest fold change in patients versus controls, 2) present in patients but absent in controls, or 3) found in literature or novel target. In order to understand the potential functional role of the identified miRNA, non-cancerous and cancerous pancreatic cell lines will be transfected with the short-listed targets using mimic miRNAs (increase abundance of miRNA) and

siRNAs (decrease abundance of miRNA). The effect on cell viability and number was then measured by flow cytometry.

As these miRNAs were previously identified in plasma samples from pancreatic cancer patients, knowing if they are up or downregulated in cells and EVs from pancreatic cells lines could help identify their specificity for PDAC. Targeting these miRNAs and attempting to identify their functions could also allow for development of new and precise patient-dependent therapies.

METHODS

Cell culture

General

Three human pancreatic cell lines (H6C7, Panc 10.05, Capan-2) were used for experiments. All cells were maintained in RPMI-1640 medium (Wisent Bioproducts, 350-000-CL) supplemented with 10% fetal bovine serum (FBS, Wisent Bioproducts, 080-150) and final concentrations of 1 mM sodium pyruvate (Thermo Fisher, 11360-070), 2 mM L-glutamine (Sigma Aldrich, 59202C-100ml) and 1.63-1.98 μ M of insulin (Sigma Aldrich, I9278-5ml). Cells were grown at 37 °C and 5 % CO₂ and passaged at 70-80 % confluency. H6C7 cells were subcultivated at a 1:2 ratio and Panc10.05 and Capan-2 cells at a 1:3 ratio.

Transfection

Approximately 1×10^5 cells were plated in multi-well plates, allowed to attach and grow for 24 hours reaching ~60-70 % confluency prior to transfection. Cells were transfected in 12-well plates for flow cytometry to assure enough cells for detection, and 24-well plates for RNA extraction. Transfections were done using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher, 13778075) and miRVana miRNA mimics (increases miRNA abundance) (Thermo Fisher, 4464066), siRNA (inhibitors, decreases miRNA abundance) (Thermo Fisher, 4464084), hsa-miR-1 positive control (Thermo Fisher, 4464062) and negative control (Thermo Fisher, 4464058) according to the manufacturer's protocol. All miRVana controls, mimics and inhibitors were diluted to a final concentration of 20 μ M, rather than 10 μ M as suggested by the

manufacturer. (Table 1). Cells were collected 72 hours post-transfection for flow cytometry.

Table 1. Targets used for transfection and corresponding Assay ID. All targets were tested in each cell line.

Target	Assay ID
Positive control (hsa-miR-1-5p)	n/a – refer to catalog number
Negative control	n/a – refer to catalog number
hsa-miR-744-5p mimic	MC13027
hsa-miR-744-5p inhibitor	MH13027
hsa-miR-30d-5p mimic	MC10756
hsa-miR-30d-5p inhibitor	MH10756
hsa-miR-196b-5p mimic	MC12946
hsa-miR-196b-5p inhibitor	MH12946
hsa-miR-374a-5p mimic	MC10112
hsa-miR-374a-5p inhibitor	MH10112
hsa-miR-374b-5p mimic	MC11339
hsa-miR-374b-5p inhibitor	MH11339

Droplet Digital PCR

Droplet Digital PCR (ddPCR) was used to detect miRNA in patient samples and cells, and mRNA in cells. For patient samples, plasma in which miRNA was already extracted and converted to cDNA by another research assistant was used to validate NGS targets identified in a previous experiment.

For miRNA detection using ddPCR, 1X QX200 ddPCR EvaGreen supermix (Bio-Rad, 1864034), 1X miRNA forward primer (Table 2), 0.5X miScript Universal Primer (Qiagen, 218073), 2.2 μ l of molecular grade water and 5.5 μ l of cDNA were mixed, vortexed, and centrifuged prior to droplet generation using the QX200 Bio-Rad droplet generator and the QX200 Droplet Generation Oil for EvaGreen (Bio-Rad, 1864005). The droplets were carefully pipetted into a 96-well semi-skirted plate (Bio-Rad) and placed in the Bio-Rad C1000 Touch Thermal Cycler for 5 minutes at 95 °C, then 44 cycles of 30 seconds at 95 °C, 1 minute at 56 °C and 2 minutes at 72 °C, then 5 minutes at 4 °C and 5 minutes at 90 °C. The lid was at a temperature of 105 °C and ramp speed was 2 °C/sec. Once amplification was done, the plate was placed into the QX200 Bio-Rad droplet reader. All cDNA samples were at a final concentration of 1 in 8.

Reactions were done in duplicates and the values obtained were averaged for results. A minimum of 10 000 droplets needed to be detected for the sample to be valid for analysis.

Targets and primer sequences can be found in Table 2.

Table 2. The sequences, sample tested and associated catalog number for all targets used for ddPCR assays done for this study. All miScript miRNA primers were obtained from Qiagen.

Target	Sequence	Sample	Catalog #
hsa-let-7a-5p	5'UGAGGUAGUAGGUUGUAUAGUU	Patient	MS00031220
hsa-let-7b-5p	5'UGAGGUAGUAGGUUGUGUGGUU	Patient	MS00003122
hsa-miR-10b-5p	5'UACCCUGUAGAACCGAAUUUGUG	Patient	MS00031269
hsa-miR-146b-5p	5'UGAGAACUGAAUCCAUAGGCU	Patient	MS00003542
hsa-miR-155-5p	5'UUA AUGCUAAUCGUGAUAGGGGU	Patient	MS00031486
hsa-miR-196b-5p	5'UAGGUAGUUUCCUGUUGUUGGG	Patient, H6C7, Panc10.05	MS00031570
hsa-miR-200b-3p	5'UAAUACUGCCUGGUAUGAUGA	Patient	MS00009016
hsa-mir-21-5p	5'UAGCUUAUCAGACUGAUGUUGA	Patient	MS00009079
hsa-miR-25-3p	5'CAUUGCACUUGUCUCGGUCUGA	Patient	MS00003227
hsa-miR-3174	5'UAGUGAGUUAGAGAUGCAGAGCC	Patient	MS00020846
hsa-miR-374b-5p	5'AUAUAAUACAACCUGCUAAGUG	Patient, Capan-2	MS00009618
hsa-miR-432-5p	5'UCUUGGAGUAGGUCAUUGGGUGG	Patient	MS00031850
hsa-miR-493-5p	5'UUGUACAUGGUAGGCUUUCAUU	Patient (only cohort 2)	MS00009800
hsa-miR-6852-5p	5'CCCUGGGGUUCUGAGGACAUG	Patient	MS00047733
hsa-miR-744-5p	5'UGC GGGGCUAGGGCUAACAGCA	Patient	MS00010549
hsa-miR-98-5p	5'UGAGGUAGUAAGUUGUAUUGUU	Patient	MS00003367
hsa-miR-30d-5p	5'UGUAAACAUCCCCGACUGGAAG	Patient, H6C7, Panc10.05	MS00009387

Flow Cytometry

Proliferation of all cell lines was quantified by flow cytometry post-transfection using the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor 488 and Propidium Iodide (PI) (Thermo Fisher, V13245) and Beckman-Coulter CytoFLEX flow cytometer. The staining process was done according to the manufacturer's protocol, with the exception of adding 300 µl of Annexin V 1X binding buffer instead of 400 µl prior to analyzing. The flow cytometer was calibrated and the gains (voltages) were set equal for every assay. A total of 10 000 events were recorded at a speed of 30 µl/min for every sample. The number of healthy cells (Annexin V - / PI-), early apoptotic (Annexin V+/PI-

) and late apoptotic (Annexin V+/PI+) were calculated to measure the effects of miRNA transfection on cell proliferation and viability.

RESULTS

Validation of patient biomarker miRNA

Previous studies at the Atlantic Cancer Research Institute identified up- and downregulated EV-miRNAs in PDAC patients compared to controls using EV isolation followed by small RNA sequencing (*personal communication*). The first aim of our study was to validate the EV-miRNAs identified in the small RNA sequencing experiments using ddPCR. ddPCR allows for accurate and reproducible measures of absolute values of cDNA content and does not require normalization unlike qPCR. It is therefore better suited for clinical assays ⁷⁶. The small RNA sequencing targets to validate were chosen based on three criteria: 1) highest fold change in patients versus controls and/or 2) present in patients but absent in controls, and/or 3) supported by literature as a diagnostic biomarker for PDAC or novel biomarker that has never been studied in PDAC. Prior experiments at the Atlantic Cancer Research Institute determined if the targets were detectable in the cell lines used for transfection which was a general criterion applied to all targets. Each miRNA assay (n=17) was first tested for compatibility with the ddPCR system. In order for a miRNA assay to be considered compatible, the PCR amplification must: 1) not amplify genomic DNA, 2) produce individual droplets that contain detectable fluorescence greater than background fluorescence, and 3) have an efficiency close to 100%. Four out of 17 did not pass one or more of these criteria and were therefore not validated. A summary of the validation data can be found in Table 3.

Compared to controls, four miRNA that were found to be up- or downregulated in PDAC patient plasma by small RNA sequencing, and one that was found to not be different, were validated by ddPCR. The average concentration of duplicates was significantly higher in PDAC patients for miR-21 ($P=0.0296$), miR-374b ($P=0.0094$), miR-744 ($P=0.0180$), and significantly lower in PDAC patients for miR-150 ($P=0.0076$) (Figure 2). The small RNA sequencing data showed that miR-25 expression does not differ between groups, and this was confirmed by ddPCR ($P=0.1009$) (Figure 2). The average concentration of duplicates of let-7a ($P=0.5494$), let-7b ($P=0.9769$), miR-146a

($P=0.1735$), miR-432 ($P=0.2731$), miR-98 ($P=0.2772$) and miR-30d ($P=0.7534$) was higher in PDAC patients compared to controls, but not statistically significant (Figure 3).

The average concentration of duplicates of miR-200b was significantly upregulated in PDAC patients in cohort 2 ($P=0.0004$), but failed quality control for cohort 1 and was not further analyzed (Figure 3).

Table 3. The target name, NGS result, ddPCR result and call for next experiment for all tested miRNA targets suspected to be differentially expressed in PDAC patients compared to controls.

Target	NGS result	ddPCR result	Call
hsa-let-7a-5p	Upregulated in PDAC	Upregulated, not statistically significant (Cohort 1 only)	Did not move forward with target
hsa-let-7b-5p	Upregulated in PDAC	Upregulated, not statistically significant (Cohort 1 only)	Did not move forward with target
hsa-miR-10b-5p	Downregulated in PDAC	Failed quality control (Cohort 1 only)	Did not move forward with target
hsa-miR-146a-5p	Upregulated in PDAC	Upregulated, statistically significant (Cohort 1 only)	Did not move forward with target
hsa-miR-150-5p	Downregulated in PDAC	Downregulated, statistically significant	Did not move forward with target
hsa-miR-196b-5p	Upregulated in PDAC	Failed quality control	Target used for cell culture transfection
hsa-miR-200b-3p	Upregulated in PDAC	Failed quality control in cohort 1, upregulated cohort 2	Did not move forward with target
hsa-mir-21-5p	Upregulated in PDAC	Upregulated, statistically significant	Did not move forward with target
hsa-miR-25-3p	No change (Normalizer)	No change	Did not move forward with target
hsa-miR-3174	Upregulated in PDAC	Failed quality control	Did not move forward with target
hsa-miR-374b-5p	Upregulated in PDAC	Upregulated, statistically significant	Target used for cell culture transfection
hsa-miR-432-5p	Upregulated in PDAC	Upregulated, not statistically significant (Cohort 1 only)	Did not move forward with target
hsa-miR-493-5p	Upregulated in PDAC	Failed quality control (Cohort 2 only)	Did not move forward with target
hsa-miR-6852-5p	Upregulated in PDAC	Failed quality control	Did not move forward with target
hsa-miR-744-5p	Upregulated in PDAC	Upregulated, statistically significant	Target used for cell culture transfection
hsa-miR-98-5p	Upregulated in PDAC	Upregulated, not statistically significant (Cohort 1 only)	Did not move forward with target
hsa-miR-30d-5p	Upregulated in PDAC	Upregulated, not statistically significant (Cohort 1 only)	Target used for cell culture transfection

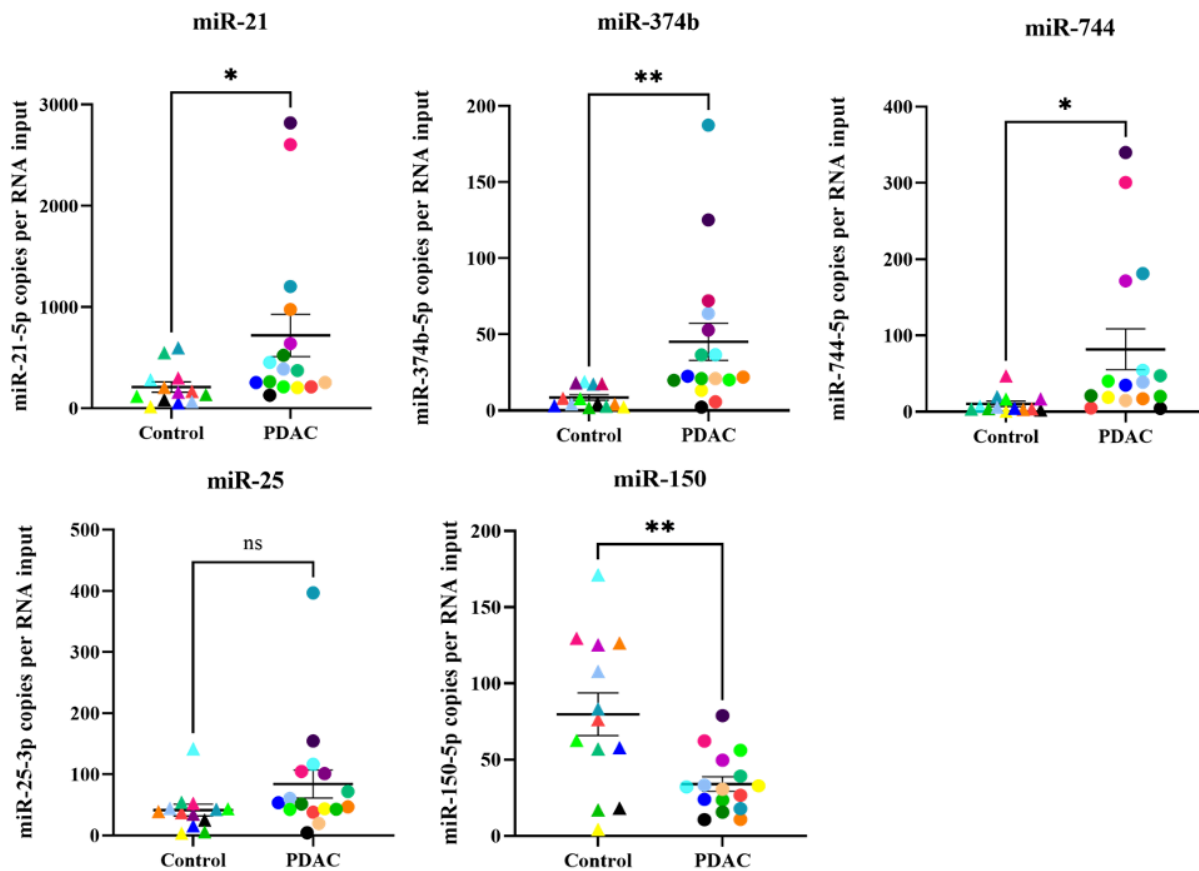


Figure 2. Five EV-miRNAs were validated using ddPCR. miRNA concentration in controls (n=13) and PDAC patients (n=16) was measured using ddPCR. Each coloured symbol is the average of duplicates and represents the same patient in each scatter plot. Statistical significance (* $p < 0.05$, ** $p < 0.01$, ns = not significant) was calculated using a two-way unpaired *t*-test on technical duplicates. Note differences in y-axis scale values.

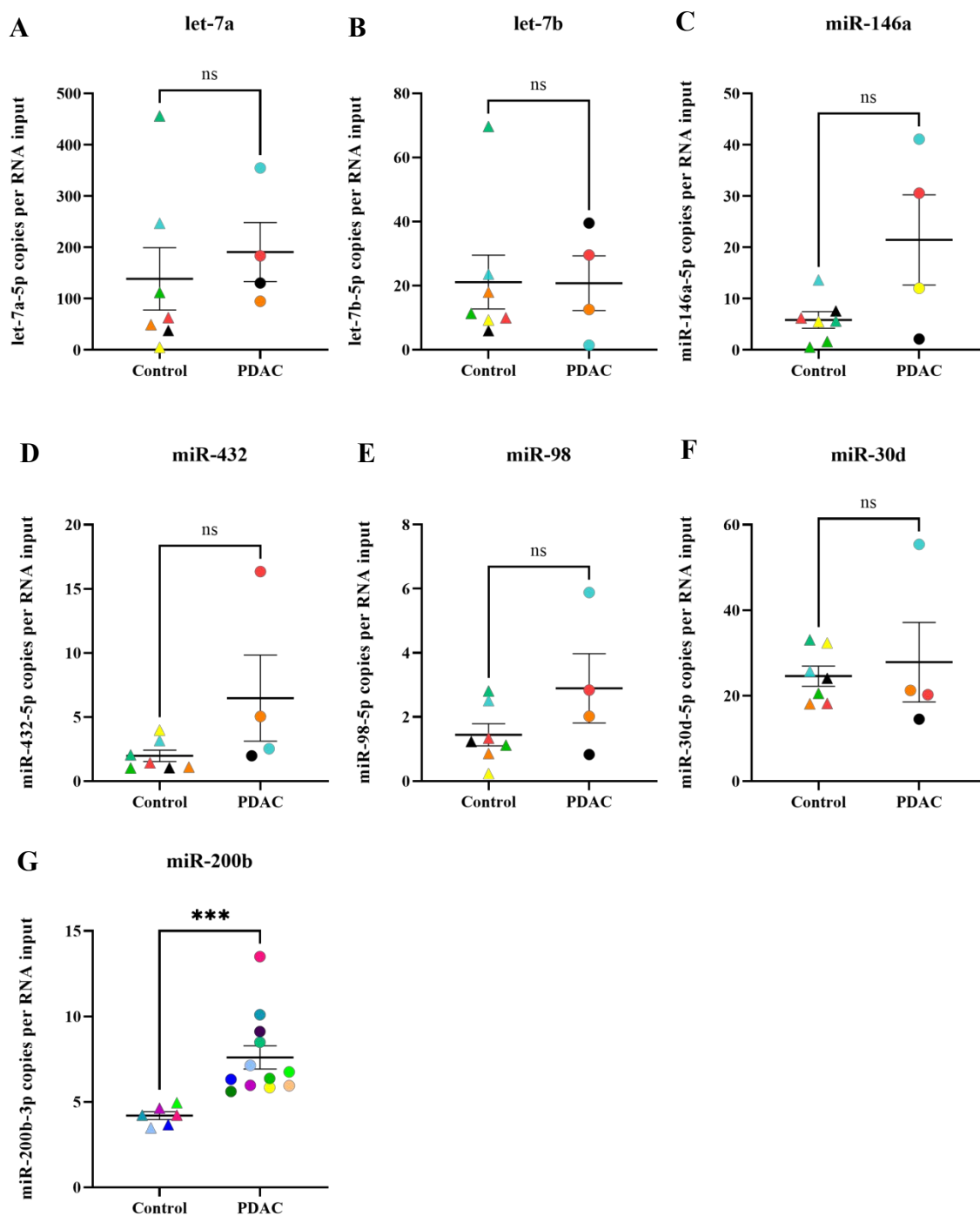


Figure 3. Multiple miRNAs were not validated by ddPCR as useful biomarkers for PDAC. miRNA concentration in controls (n=7, cohort 1 only) and PDAC patients (n=4, cohort 1 only) was measured using ddPCR. miR-200b (G) concentration shown is only for cohort 2 (Controls n=6, PDAC n=12). Each coloured symbol is the average of duplicates and represents the same patient in each scatter plot. Statistical significance (***) $p < 0.001$, ns = not significant) was calculated using a two-way unpaired *t*-test on technical duplicates. Note differences in y-axis scale values.

Functional role of miR-196b, miR-744, miR-374b and miR-30d – effect on cell viability

The second aim of our study was to investigate the biological effects of certain miRNAs on cell viability and/or proliferation. Targets for transfection were determined from target validation ddPCR data on the patient samples. After cellular RNA was converted to cDNA, ddPCR was done to determine if changes in miRNA expression could be detected in cells post-transfection, confirming if the transfections were successful. Cells were transfected with a mimic or an inhibitor of miR-196b, miR-744, miR-374b or miR-30d, as well as positive and negative controls using a lipid vector. 72h post-transfection, the cells were collected and stained with Annexin-V and PI for quantification of cell viability using flow cytometry. Technical and user errors prevented the analysis of Capan-2 cells and cells transfected with miR-374b.

The results of the negative control transfections show that the transfection itself does not kill the cells in both cell lines. This indicates that the effects we see on cell viability and/or proliferation are due to the changes in miRNA expression and not due to the lipid vector used for transfection. In the H6C7 cells, cell viability and/or cell proliferation was not affected by changes in miRNA expression for all targets (Figure 4A). Transfection with miR-196b mimic or inhibitor did not have a significant effect on cell viability in either cell line, while transfection with miR-30d inhibitor significantly increased cell viability from 83.53% live cells in the negative control to 88.94% live cells in PANC10.05 ($P=0.01298$) (Figure 4B). Interestingly, PANC10.05 cell viability was significantly decreased from 85.53% live cells in negative control to 77.07% live cells when transfected with a miR-744 mimic ($P=0.01769$) (Figure 4B). Transfections with the miR-30d mimic and miR-744 siRNA did not have a significant effect on cell viability. Percent live cells in H6C7 ranged from 85.91% to 90.72%, and 75.96% to 90.01% in PANC10.05 cells. Percent apoptotic and necrotic cell populations were not different across conditions.

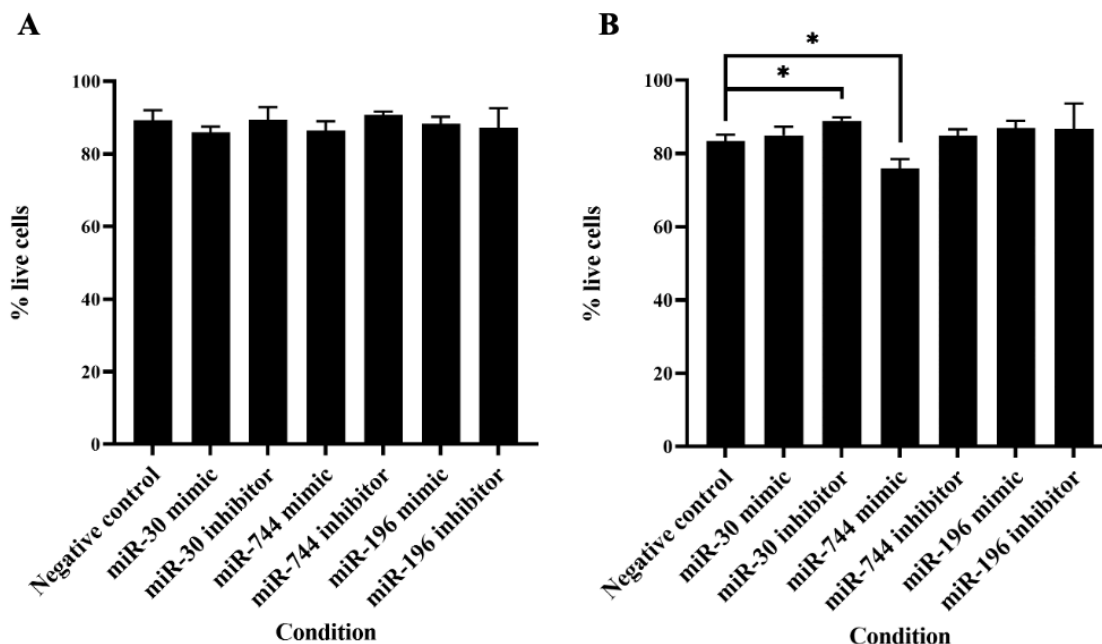


Figure 4. miR-744 and miR-30d act as tumour suppressor miRNAs in PANC10.05 cells. Percent live cells 72h post-transfection was measured using flow cytometry using Annexin-V and PI for staining in H6C7 (A) and PANC10.05 (B) cells lines. Statistical significance (* $P < 0.05$) was evaluated using a *t*-test on the data combined from three biological replicates.

DISCUSSION

Given the dearth of biomarkers for PDAC and the need for a new diagnostic technique for this cancer, this study aimed to determine if EV-miRNA profiles can be used as a companion diagnostic tool for PDAC, and if these miRNAs will have effects on pancreatic cell line models. This was achieved by validating multiple EV-miRNAs identified by NGS as being significantly different in PDAC patients compared to controls using ddPCR. The validated miRNAs were then used to transfect cancerous and healthy pancreatic cells. The ddPCR experiments allowed the validation of five miRNAs. Following the validation, miR-744 was used to transfect PANC10.05 and H6C7 cells. miR-196b and miR-30d were also used to transfect the cells as miR-196b had been previously identified in PDAC as an important biomarker, while miR-30d had never been studied in PDAC⁷⁷. Transfecting with the miR-744 mimic decreased the percent of live cells, while transfecting with the siRNA for miR-30d increased the percent of live cells in PANC10.05.

Small RNA sequencing revealed differences in EV-miRNA expression in PDAC patients compared to controls. A total of 264 miRNAs were found to be significantly upregulated in PDAC patients, while 7 miRNAs were found to be significantly downregulated in PDAC patients (*personal communication*). Unsupervised hierarchical clustering showed that PDAC patients cluster together, while controls cluster together. This indicates that miRNA expression is different between these two groups (Supplemental figure 1). These findings prompted further investigation of these miRNAs for their potential use in a clinical setting.

ddPCR is the preferred method to validate miRNAs identified by small RNA sequencing. Of the 17 miRNA targets chosen for validation, a total of 5 were successfully validated by ddPCR. miR-21, miR-744 and miR-374b were confirmed to be upregulated in PDAC samples while miR-150 and miR-25 were confirmed to be downregulated in PDAC samples and not differentially expressed in PDAC compared to controls, respectively. Although let-7a, let-7b, miR-10b, miR-146b, miR-155, miR-196b, miR-200b, miR-3174, miR-432, miR-493, miR-6852, miR-98 and miR-30d were not successfully validated by ddPCR, this does not indicate that they do not have diagnostic or therapeutic potential. An increased sample size of both patient and control samples, or alternative oligos, could show their usefulness for PDAC diagnosis and treatment. Overall, these results show that ddPCR is an effective tool to measure miRNA expression in liquid biopsy samples for PDAC.

The three miRNA targets confirmed to be upregulated in PDAC samples have previously been described in PDAC and in cancer in general. miR-21 is overexpressed in multiple cancers and is involved in the development of metastatic characteristics ⁷⁸. In PDAC, the overexpression of miR-21 causes increased resistance to gemcitabine, a chemotherapy administered to PDAC patients, by decreasing FasL signaling, which in turn decreases apoptosis ⁷⁰. Since miR-21 is associated with many cancers, it was not further investigated. However, miR-744 and miR-374 have been identified as promising biomarkers for PDAC. miR-744 has already been identified as a diagnostic and prognostic biomarker in PDAC ⁷⁹ as well as an activator of the Wnt/ β -catenin pathway ⁷¹. Meanwhile, miR-374b increases chemoresistance to gemcitabine in PDAC by targeting anti-apoptotic proteins like BCL2, BIRC3 and XIAP ⁷². Therefore, these results support

that the validated upregulated miRNAs in PDAC patient plasma are cancer related and warrant further investigation.

The downregulated miRNA identified in PDAC patients also plays a role in this cancer. Decreased expression of miR-150 in PDAC tissues caused increase proliferation and metastasis by binding to c-Myb, a transcription factor, and MUC4, a gene encoding mucin protein⁸⁰. Furthermore, miR-150 also regulates IGF-1R and an increase in miR-150 expression negatively regulates the expression of c-Myb and IGF-1R, leading to an increase in apoptosis in those cells⁸¹. This suggests that miR-150 may be selectively sorted out of PDAC cells in order for them to maintain their metastatic characteristics. Since this study focused on understanding upregulated miRNAs in PDAC because this is what is typically measured in diagnosis, miR-150 was not further investigated. However, future studies should aim to target miR-150 and the genes it regulates for potential therapeutic intervention.

Preliminary transfection experiments showed no gross changes in both cancerous and non-cancerous cell lines 72h post transfection. Thus, a more sensitive technique, flow cytometry, was used to quantify changes in percent live cells, apoptotic cells and necrotic cells after transfection. Our data did not show any significant changes of the percent of apoptotic and necrotic cells. Therefore, when we did detect effects on the percent of live cells, we, at this time, cannot determine if this is due to an increase/decrease in cell proliferation (cell cycle progression) or an increase/decrease in cell viability (apoptosis). Since miR-744 was found to be significantly upregulated in PDAC patient plasma and previous research has identified it is a possible prognostic marker in this cancer, it was of interest to further investigate its role *in vitro*⁷⁹. Transfection with a miR-744 mimic showed a decrease in the percent of live PANC10.05 cells, while transfecting with an siRNA for miR-744 showed no difference in the percent of live PANC10.05 cells. Future studies should determine which genes and signaling pathways are regulated by miR-744 in order to better understand its role in cancer progression. Although preliminary, this finding suggests that miR-744 acts as a tumour suppressor miRNA in PANC10.05 cells. It may do this by regulating multiple mRNAs that regulate cell proliferation.

Further analysis of the small RNA sequencing data showed that miR-30d is significantly upregulated in PDAC patients compared to controls. Although this miRNA

was not validated by ddPCR, determining if this miRNA affects PDAC cell proliferation *in vitro* was of great importance since it has not been described in PDAC yet, making it a novel diagnostic and therapeutic target for this cancer. Interestingly, inhibiting miR-30d with an siRNA against this target showed an increase in percent live PDAC10.05 cells, suggesting that this miRNA also acts as a tumour suppressor miRNA in PANC10.05 cells. In renal cell carcinoma, miR-30d negatively regulates proliferation and autophagy by targeting Autophagy-Related Gene 5 (ATG5) *in vitro*, again suggesting that this miRNA is a tumour suppressor⁸². Future studies will need to determine the therapeutic potential of miR-30d and miR-744 for PDAC.

Interestingly, transfections with miR-196b mimics and siRNA against this target did not show statistically significant changes in the percent of live cells. Although it failed to be validated by ddPCR, this target was chosen for transfections because it has already been identified as a biomarker for PDAC⁷⁷ and is known to downregulate CADM1 which leads to reduced apoptosis of PANC-1 and ASPC-1 cells⁸³. Failure to detect any significant changes in cell phenotype and viability could be influenced by multiple factors. One possible explanation for this is that miR-196b has different effects in different cell types. Since miRNAs regulate gene expression post-transcriptionally by binding to the 3'-UTR of mRNA, if the mRNAs of the genes of interest are not present in the cell type, then the miRNA will not have any effects on the cell⁸⁴. The downregulation of CADM1 mediated by miR-196b was observed in different cell lines than the ones used in this study. Another possible explanation is that the effects of miR-196b *in vitro* were measured too late and a shorter transfection time is needed to determine if miR-196b has any effects on the percent of live PDAC10.05 cells. Unfortunately, due to Covid-19-related challenges, the effects of miR-374b *in vitro* were not determined in this study.

Remarkably, transfecting H6C7 cells with any target (miR-744, miR-30d, miR-196b) did not result in any significant changes in percent live cells. This indicates that the miRNAs do not target the same mRNA, or mRNA that serve similar functions, in each cell line, or that H6C7 cells simply do not express the mRNA targets of the miRNA used for transfection. Otherwise, the transfections would have had similar effects in both cell lines. Although untested here, it is possible that the cancerous cells acquired mutations through carcinogenesis making them dependent on the up- or downregulation of certain

genes for proliferation, which is why increasing the abundance of miR-744 or inhibiting miR-30d changed the percent live cells. Since no effects were observed in the non-cancerous cells, this suggests that miR-30d and miR-744 have therapeutic potential since targeted therapies would only affect cancerous cells and not the non-cancerous cells. Nonetheless, the effect of the transfections on the percent live cells in the cancerous cells was modest. Even with the treatments, the cancerous cells were able to proliferate indicating that they are not dependent on the mechanisms that would be affected by the change in miRNA expression. This suggests that optimization of the targets is needed to obtain better results. In preliminary experiments (data not shown) we did confirm successful transfection efficacy in all cell types, therefore we are confident that the differences we observe between cell types is due to differences inherent to their specific phenotypic characteristics.

The use of bodily fluids to look at tumour-derived materials to diagnose different types of cancer and even determine prognosis has been shown to be useful many times. Our finding that multiple plasma EV-miRNAs in PDAC, including up- and downregulated miRNAs, can be measured by ddPCR supports other previously reported data that miRNAs can be used as biomarkers for liquid biopsy for PDAC. Our finding that miR-744 is a good biomarker for PDAC is already well supported by the literature⁷⁹. However, previous studies describe this miRNA as being oncogenic rather than tumour suppressing, which disagrees with our *in vitro* results. If this miRNA is indeed oncogenic, the upregulation of this miR-744 in plasma EVs could explain how EVs also promote metastasis by allowing oncogenic miRNAs to be delivered to other areas of the body. The validation of miR-21 as a useful biomarker has also been described in previous research⁸⁵. However, since this miRNA is described as a general oncogenic miRNA (i.e. it is associated with many cancers), measuring miR-21 levels may not be useful in diagnosing PDAC itself but elevated levels of miR-21 may be able to signal the presence of cancer and determine prognosis of the disease⁷⁸. Finally, miR-374b has also been described as a diagnostic and prognostic marker in PDAC⁷². Since low expression of this miRNA in tissues indicates poorer prognosis and increased therapeutic resistance, it is likely that the increase in miR-374b expression in plasma EVs of PDAC patient is due to the selective sorting of materials out of the cancer cells, a process previously described by others⁵⁸.

This could indicate that miR-374b has tumour suppressing effects on cancerous cells. Overall, these results show multiple miRNAs that can be used to build a functional liquid biopsy biomarker panel for PDAC. These results add to the existing literature by showing that miRNAs found within EVs in plasma are useful in diagnosis. These results also emphasize the importance of building a large panel of biomarkers since the expression level of each miRNA varied for each patient. Future studies should aim to understand the function of these miRNAs in cancer and how these change over time which will eventually allow the tracking of disease as it progresses or as treatment is administered.

In this study, it was also determined that dysregulated miRNAs associated with PDAC do indeed have an effect on the percent of live cells in cancerous cell line models. When increasing miR-744 abundance in cells, we measured a significant decrease in the percent live cells which indicates that miR-744 has tumor suppressing effects. Contrary to our result, previous research described miR-744 as an oncogenic miRNA in PDAC. One study explained that increasing miR-744 abundance activates the Wnt/ β -catenin pathway by downregulating secreted frizzled-related protein 1 (SFRP1), glycogen synthase kinase 3 β (GSK3 β) and transducin-like enhancer of split 3 (TLE3) which are all inhibitors of this pathway⁷¹. However, miR-744 does have tumour suppressing effects in ovarian cancer cells by decreasing the expression of heterogenous nuclear ribonucleoprotein C (HNRNPC) and nuclear factor X (NFIX) which lead to the activation of pro-apoptotic pathways⁸⁶. More specifically, decreased HNRNPC expression also decreased miR-21 expression and downregulated the AKT pathway, while decreased NFIX decreased the expression of Bcl2 levels, a protein that triggers apoptosis⁸⁶. Therefore, it is possible that miR-744 has similar effects in PDAC by regulating this mechanism. The evidence on the role of miR-744 in cancer is conflicting. Thorough investigation of this miRNA and its function in PDAC cell line models is needed to better understand its significance and whether it promotes or inhibits oncogenesis in this cancer.

Interestingly, we were able to identify a miRNA that has not been previously studied in PDAC. Decreasing miR-30d expression in PANC10.05 cells increased cell viability, suggesting that this miRNA has tumor suppressing properties. The small RNA sequencing data showed that this miRNA is significantly upregulated in PDAC compared to controls. Taking these things into consideration, it is possible that this miRNA is being

selectively sorted out of cells by EVs to prevent it from having its tumour suppressing effects on the cancer cells. In other cancers, miR-30d regulates autophagy-related genes. This miRNA is downregulated in colon cancer cells and transfecting these cells with a miR-30d mimic reduces cancer cell viability while transfecting with an siRNA for miR-30d promotes cancer cell viability, similarly to our results⁸⁷. Furthermore, miR-30d mimics can induce cell autophagy by increasing cleaved caspase-3 and decreasing LC3-II expression, two proteins involved in autophagy⁸⁷. As mentioned previously, a similar mechanism has been outlined in renal cell carcinoma. In this cancer, however, miR-30d downregulates the ATG5 gene⁸². Again, increasing miR-30d abundance in renal cell carcinoma *in vitro* models reduces ATG5 expression but increasing ATG5 expression reverses the tumour suppressing effects of miR-30d⁸². It is likely that miR-30d regulates a similar process in PDAC.

Autophagy is a process that is often dysregulated in cancer⁸⁸. The PI3K/AKT/mTOR pathway is an important regulator of autophagy and this pathway is also often dysregulated in cancer, causing increased cell proliferation and tumour growth⁸⁹. In PDAC, the PI3K/AKT/mTOR pathway is overactivated and inhibits apoptosis but these actions can be reversed by administering a PI3K/AKT/mTOR agonist⁹⁰. Interestingly, the same drug also inhibits the sonic hedgehog pathway (shh) in transgenic mice expressing KRAS and p53 mutations, suggesting that the shh and PI3K/AKT/mTOR pathways interact to promote PDAC progression⁹⁰. In esophageal cancer, the downregulation of miR-30d correlates with the increased signaling of the PI3K/AKT/mTOR pathway⁹¹. Furthermore, administering Ricolinostat, an antitumour drug, increased miR-30d expression which led to the downregulation of PI3K, while reducing miR-30d expression increased AKT signaling in this cancer which clearly elucidates the role of this miRNA in this pathway⁹¹. Combining this knowledge, it is likely that miR-30d regulates the PI3K/AKT/mTOR and possibly the shh pathway to suppress autophagy and apoptosis in PDAC. It is also possible that the downregulation of miR-30d triggers KRAS and p53 mutations early in the development of this cancer. Future studies should aim to understand this mechanism in PDAC and if Ricolinostat serves similar functions in pancreatic cancer cell line models as it does in esophageal cancer cells.

In conclusion, the screening of short-listed EV-miRNAs from NGS showed that 5 were validated by ddPCR, with 4 of these being potential biomarkers for PDAC. Of these miRNAs, only miR-744 was chosen, along with other non-validated targets like miR-30d and miR-196b, to measure the effects of these miRNAs on pancreatic cancer cell line models. This revealed that miR-744 and miR-30d act as tumour suppressors in PANC10.05 but do not affect non-cancerous cells. Our results are partially supported by previous research since miR-744 has been described as a PDAC biomarker but also as an oncogenic miRNA, which conflicts with the results of this study. Other studies have described the role of miR-30d in cancer, but this has yet to be explored in PDAC. The focus of future studies should aim to continue to build to PDAC liquid biopsy biomarker panel and understand which genes and pathways these miRNAs regulate in order to develop targeted therapies. Overall, the results of this study show that EV-miRNA profiles can be used as a companion diagnostic for PDAC and that these EV-miRNAs are involved in cellular processes affecting the percentage of live cells, which suggests them as potential therapeutic targets. This study has important implications for the development of a liquid biopsy biomarker panel for PDAC to improve diagnostic times and reduce the reliance on invasive techniques for diagnosis. Furthermore, this study also emphasizes the importance of precision medicine by showing variability in miRNA expression among patients meaning each could respond to treatments differently based on their molecular profile.

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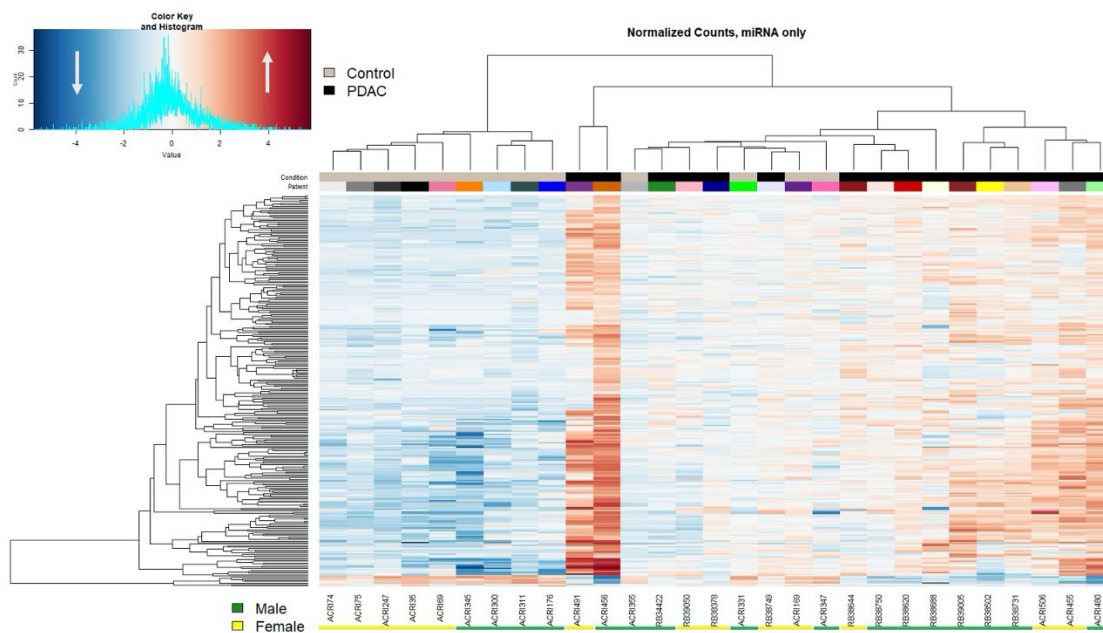
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APPENDIX



Supplemental Figure 1. Unsupervised hierarchical clustering of differentially expressed miRNA measured by small RNA sequencing in PDAC patients (black) and controls (grey) (n=29). Red represents upregulated miRNA and blue represents downregulated miRNA.