MICRORNA125 IS A NOVEL BIOMARKER IN PANCREATIC CANCER PATIENTS

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University of Pittsburgh, 2014

Approximately 46,420 Americans are diagnosed with pancreatic cancer and 39,590 individuals die from the disease annually. Pancreatic cancer is associated with a less than 5% five-year survival rate.[1] Early diagnosis is rare and surgical treatment is most beneficial before the cancer becomes locally invasive or metastatic. Previously, we identified *in vitro* DAMPmiRs (Damage Associated Molecular Pattern molecule induced microRNAs, miR-34c and miR-214) that are differentially expressed in peripheral blood mononuclear cells (PBMCs) upon DAMP stimulation and play an important role in regulating the inflammatory response via targeting inflammatory pathways. DAMPs are passively released into the local micro-environment, and progressively, into the systemic circulation to initiate early innate and adaptive immune responses.[2,3] Whether the microRNA (miRNA) expression in pancreatic cancer patients' PBMC is different from those of normal healthy individuals is unknown. Here, we examined the miRNA expression profile of age and sex matched samples to identify potential miRNA markers. One of the most promising markers (miR-125a-5p) was selected for further analysis in patients enrolled in our recently completed phase I/II pre-operative treatment with hydroxychloroquine and gemcitabine. We also evaluated how individual immunological stimuli affect miR-125a-5p expression in normal PBMC and validated several miR-125a-5p predicted down-stream targets.

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PREFACE

This dissertation is dedicated to the three people who made it possible – Drs. Michael T. Lotze, Sebnem Unlu, and Xiaoyan Liang. A scientific career is an endless expedition; thus, this is not my destination but the beginning of my quest. These three people made my PhD a magnificent quest.

I would also like to dedicate this dissertation to my parents Wilson & Annie, my sister and brother-in-law Amy & Bill, and my best friends Yong and Steven. They always stand by my side when I felt frustrated and every time I face obstacles. They are always good listeners and comforters in my life.

First, I would like to thank my mentor Dr. Michael T. Lotze, who not only mentored me in science but also mentored me about life. He also gave me an opportunity to explore clinical research and well as the business and intellectual property sides of scientific research. Without these opportunities, I would not have found my ultimate career goal.

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1.0 INTRODUCTION

This chapter will cover important information, including key terms and a brief summary of the current literature related to how microRNAs (miRNAs), damage-associated molecular patterns (DAMPs), and immune cells play important roles in pancreatic cancer. This section will also give an overview of the rationale and goals of the dissertation regarding how to identify and validate miRNA biomarkers from pancreatic cancer patients' peripheral blood mononuclear cells (PBMCs). Part of this chapter is from our previous publication.[4,5]

1.1 ORGANIZATION OF THE INTRODUCTION

This dissertation covers concepts in identifying miRNA biomarkers in pancreatic cancer patients' PBMCs and changes in miRNA expression during treatment. It also covers concepts in identifying stimulations that alter miRNA expression and validate the downstream targets of microRNA markers. In this introduction, I will first review basic information on pancreatic cancer, the importance of miRNAs, and the role of DAMPs and pathogen-associated molecular patterns (PAMPs) in tumors. Finally, I will introduce the rationale and goals of the dissertation and summarize the dissertation in a schematic picture.

1.2 PANCREATIC CANCER

Each year, approximately 43,140 Americans are diagnosed with pancreatic cancer and 36,800 die from the disease.[6] Pancreatic cancer is associated with a less than 5% five-year survival rate .[1] Early diagnosis is rare and surgical treatment is most beneficial before the cancer becomes locally invasive or metastatic. Physical exam, chest X-ray, CT Scan, MRI, PET scan, endoscopic ultrasound, laparoscopy, endoscopic retrograde cholangiopancreatography, percutaneous transhepatic cholangiography, and biopsy are used to diagnose the disease; however, few signs or symptoms are noticeable in the early stages. The fact that the signs are similar those of other illnesses and the location of pancreas make the disease difficult to detect and diagnose early. Therefore, there is a substantial unmet clinical need to develop early diagnostic reagents for identifying pancreatic cancer. Although CA19.9 (a biomarker) is widely used to monitor therapy, it has proven to be detectable only late in disease and to be increased with pancreatitis.[7] Recently, miRNAs present within the tumor and in the blood are potential quantitative measures of tumor that may be identified earlier in disease.[5]

1.2.1 Pancreatic Cancer

Pancreatic cancer is a malignant neoplasm originating from mutant cells (both endocrine and exocrine) that form the pancreas. Ninety-five percent of pancreatic cancers are derived from exocrine cells, which produce pancreatic enzymes for digestion. The majority of exocrine-derived tumors are pancreatic adenocarcinomas. **In this dissertation, the pancreatic cancer patients' clinical samples to which we refer are pancreatic adenocarcinomas.**

1.2.1.1 Pancreatic Cancer Progression Model

The progression of pancreatic duct lesions into invasive cancer is the widely-accepted pancreatic cancer progression.[8] (Figure 1.1) First, a genetically-altered clonal cell population usually proliferates and forms a stage 1A pancreatic intraepithelial neoplasia (PanIN), which is usually associated with Her-2/new and K-RAS mutation. Then, inactivation of p16 causes the PanIN to progress into a higher grade ductal lesion (PanIN 2). Accumulation of additional (e.g. p53, DPCA, and BRCA2) occurs during PanIN 3 development and the PanIN eventually progress into pancreatic cancer.

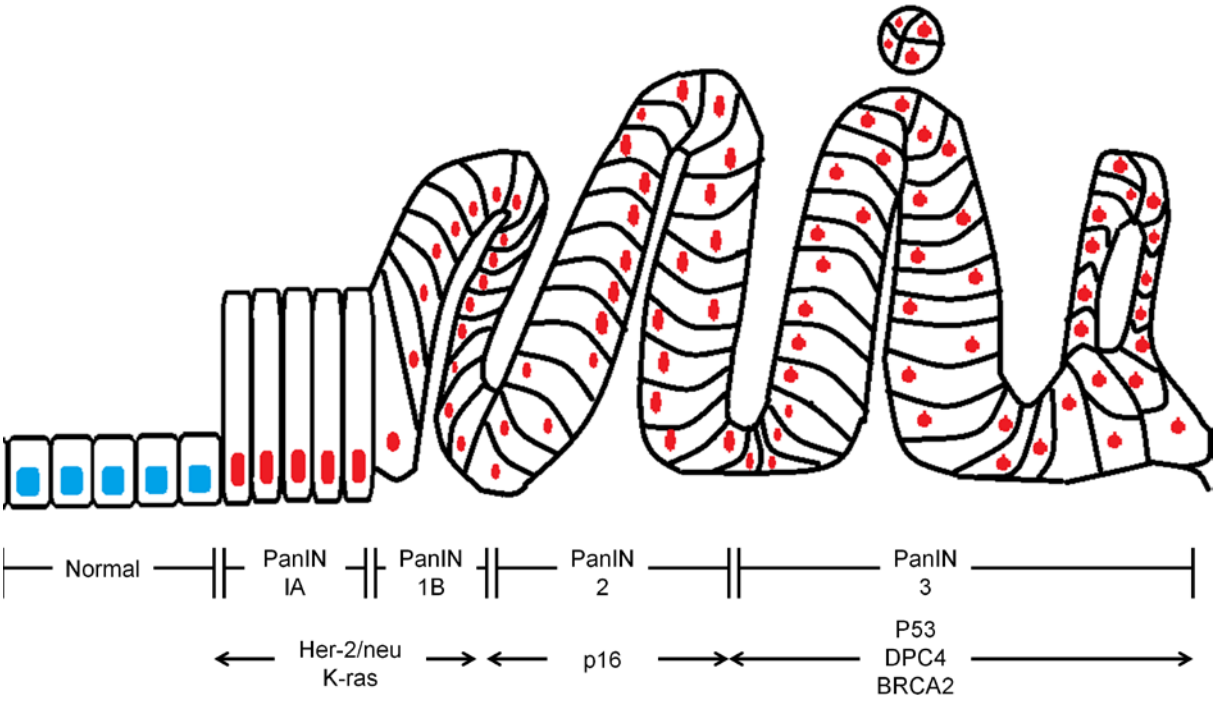


Figure 1-1:Pancreatic Cancer genetic mutations model

Genetic mutations are accumulated as pancreatic cancer progress. K-RAS and Her-2/neu mutations happened during PanIN 1A and as the lesions progress into pancreatic cancer, additional mutations e.g. p16, p53, DPC4, BRCA2 are also accumulated.

1.2.1.2 Mutations Associated with Pancreatic Cancer

Pancreatic cancer progression is associated with several defined mutations or losses of genes, including TGF/SMAD4, KRAS, BCRA, p53, and p16.[9] Those mutations may also be associated with miRNA expression in pancreatic cancer patients' tissue or blood samples (Figure 1.2).[5] We will discuss the role of miRNA in pancreatic cancer later in this chapter.

Pancreatic cancer cells do not respond to transforming growth factor beta (TGFβ) signaling even in the presence of high-level TGFβ receptor expression, which limits the ability of TGFβ to inhibit cell growth and.[10] The loss/mutation of SMAD4 in the TGFβ pathway in pancreatic cancer cells attenuates the inhibitory function of TGFβ. Furthermore, TGFβ is also associated with cancer invasiveness (and metastasis) and regulating extracellular matrix (ECM) expression, angiogenesis, and immunosuppression.[11]

KRAS is the most frequently mutated gene (>95%) in pancreatic ductal adenocarcinoma (PDAC).[12] Mutation in KRAS disables GTPase to hydrolyse GTP, resulting in a constitutively-activated protein. As PDAC progresses, KRAS-mutated tumor cells may accumulate mutations in other genes such as p53 and SMAD4. The KRAS mutation occurs in the early stage of pancreatic cancer development and is associated with the loss of tumor suppressor genes in late stages.[13-19] Ras regulates cellular proliferation, differentiation, migration, and apoptosis via activation of the MAP kinase cascade (AKT and the P13K pathway). Ras is deregulated in many cancer types, leading to decreased apoptosis and increased cell invasion and metastasis.[20] Active mutations of Ras are found in 90-95% of all pancreatic tumors (and a quarter of all other tumors). Thus, KRAS is one of the most frequent mutations in pancreatic cancer.

Breast cancer type 2 susceptibility proteins (BRCA2) are essential for cell proliferation, differentiation, and DNA repair.[21-23] In murine models, BRCA2 mutation, in concert with other

mutations (e.g. KRAS, p53), defines a role for BRCA in PDACs.[24] When p53 is intact, BRCA2 mutation alone is not sufficient to drive PDAC, while double mutations can enhance PDAC development. Double mutation of BRCA and KRAS in p53-intact cells cannot fully drive PDAC, but when p53 is also mutated, mice rapidly develop PDAC. Pancreatic cancer patients with BRCA2 mutations are found to be sensitive to DNA cross-linking agent therapy, and some conversion from sensitive to resistant is occasionally due to the secondary mutation that restores expression of wild-type BRCA2.[25,26]

p53 is one of the most frequently mutated human tumor suppressor genes that plays an important role in activating DNA repair, inhibiting autophagy, and promoting cell cycle arrest as well as apoptosis to limit transformation.[27-30] It is also frequently mutated in pancreatic adenocarcinoma. *p53* and its gene product TP53INP1 regulate the cycle though pre-transcriptional, transcriptional, and posttranscriptional actions.[31,32]

*p*16 is a tumor-suppressor protein also known as cyclin-dependent kinase inhibitor 2A (CKDN2A) $p16^{Ink4A}$ and multiple tumor suppressor-1 (MTS1). p16 proteins regulate cell cycle progression, apoptosis, and DNA repair, and the genes that encode p16 are lost in 80-95% of pancreatic cancer cases, which is observed in even the early stage of PanIN lesions.[8,33] P16 mutations, in combination with KRAS, p53, and SMAD4 mutations, have also been observed in advanced pancreatic cancer.[34-36] Although many efforts have been made in the field to understand how genetic mutations relate to pancreatic cancer, translating knowledge about genetic mutations to therapy and diagnosis is still ongoing.

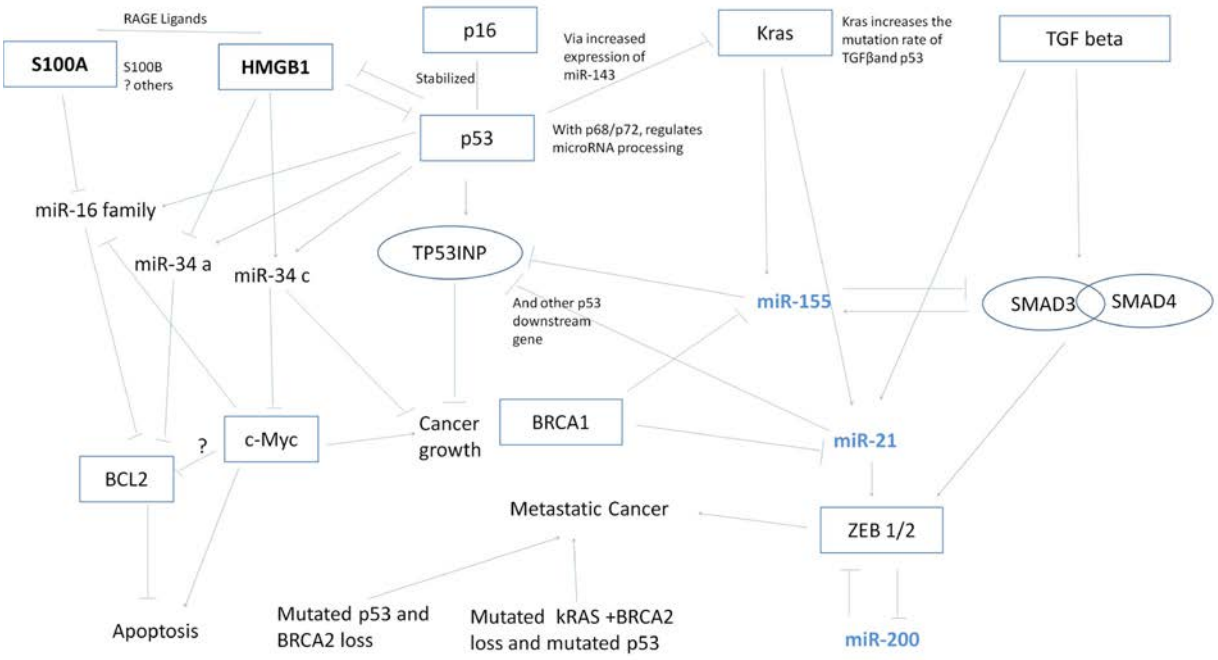


Figure 1-2: Predicted genetic lesions linking with pancreatic cancer miRNA markers

Predicted network linking pancreatic cancer miRNA markers, known pancreatic cancer genetic lesions, and DAMPs. miR-21, miR-155, and miR-200 family members are connected to known genetic lesions found in pancreatic cancers including mutant K-ras,BRCA1, TGFA, and DAMPs (HMGB1) and DAMP-receptors (RAGE) as well as the p53 pathway. Note that the role played by c-Myc in apoptosis regulation is paradoxical; under some conditions, c-Myc promotes proliferation, and under other condition c-Myc promotes apoptosis.221 c-Myc is crucial for apoptosis and requires p53 to activate apoptosis.

1.2.1.3 Stages of Disease Progression

Pancreatic carcinoma *in situ* (stage 0) is defined when abnormal cells are found within the pancreas ductal cells. The abnormal cells may become cancerous and spread into the nearby normal tissue. Stage 1 pancreatic cancer is defined when cancer has taken form and is only found in the pancreas. The five-year survival rate of stage 1A (tumor size less than 2cm) pancreatic cancer is ~14% and ~12% for stage 1B (tumor size greater than 2cm). When the cancer begins to spread to nearby tissue and organs and potentially spreads to lymph nodes near the pancreas, the cancer is defined as stage II. The five-year survival rate of stage IIA (cancer has spread to nearby

tissue and organs but not to the nearby lymph nodes) pancreatic cancer is ~7%, while that of stage IIB (cancer has spread to the lymph nodes as well) is ~5%. During stage III, cancer has spread to the major blood vessels near the pancreas and may have spread to nearby lymph nodes. Stage III pancreatic cancer has a ~3% five-year survival rate. The disease progresses to stage IV when the cancer has spread to distant organs such as the liver, lung, and peritoneal cavity. The cancer may also spread to organs and tissues near the pancreas or lymph nodes. The five-year survival rate of stage IV pancreatic cancer is ~1%.

1.2.1.4 Current Treatments

Pancreatic cancer treatment options depend on the stage of the disease and whether the tumor can be removed by surgery. When surgery is not an option, radiation, chemotherapy, and targeted therapy are the currently approved options. Other therapeutic strategies, such as immunotherapy, including transferring or modulating immune cells or administering cytokine to stimulate the immune response, are under investigation. Different options are available depending on the stage of the disease.

Currently, complete pancreatic surgical resection still remains the most effective treatment option, with a five-year survival rate of 7-34% compared to the median survival of three to 11 months for un-resectable cancer.[37] Unfortunately, only 10% of patients have the option to undergo curative surgery. Furthermore, even in experienced hands, pancreatic resection has a high morbidity rate (as high as 50%) Although techniques including gastroenteric reconstruction and application of somatostatin may improve morbidity, results are not promising.

Radiation damages cell DNA through waves or a stream of particles. When cell DNA is damaged, it loses the ability to proliferate. Over time, the cell dies; thus, radiation can be used to kill or shrink tumors. The cell cycle stage is crucial for effective radiation therapy, as

proliferative cells are more sensitive than resting cells. Radiation actively kills both normal and cancerous proliferating cells. This therapy thus maximizes the destruction of cancer cells while minimizing the destruction of normal cells, which is very important. Radiation takes days or even weeks of treatment before cancer cells start dying. Chemotherapy or targeted therapy in conjunction with radiation therapy can extend the survival time for patients with locally-spread pancreatic cancer from five to 10 months and from 11 months to nearly two years after surgery.

Chemotherapy can be used to treat pancreatic cancer. Gemcitabine and 5-fluorouracil (5-FU) are two common drugs used to treat the disease. Gemcitabine is a pyrimidine anti-metabolite that interferes with cell metabolism and growth.[38] It replaces pyrimidine deoxycytidine in DNA to prevent the DNA from being synthesized or repaired; thus, the cells cannot proliferate and eventually die. 5-FU, another anti-metabolite, works in a similar manner. Both gemcitabine and 5-FU can also affect normal proliferating cells, therefore affecting blood cells and increasing the risk of infection. Gemcitabine can be used in conjunction with other drugs like cisplatin or streptozotocin or radiation therapy. Gemcitabine can improve the median survival duration of five weeks; adjuvant chemotherapy with gemcitabine increases five-year survival from 10% to 20%.[39,40] Clinical trials show that combing gemcitabine with erlotinib (a tyrosine kinase inhibitor that acts on epidermal growth factor receptor) can slightly prolong median survival time from 5.91 months to 6.24 months with a 5% one year progression-free survival rate increase.[41] Other ongoing clinical trials for adjuvant (before surgery) and neoadjuvant (after surgery) therapy with gemcitabine are under investigation.

Immunotherapy, a strategy to enhance or suppress the immune response by activating immune cells, vaccines, or immunomodulators, can be used against pancreatic cancer. Immunomodulators including interleukins, cytokines, and chemokines have been used to

modulate the immune system to treat varies diseases. Interlukin-2 (IL-2), a T-cell growth factor, has been shown to be associated with an 8-10% complete response rate in renal clear cell carcinoma (RCC) and melanoma.[42] The mechanism by which IL-2 works against cancer is not fully understood but is likely due to enhancing the T-cell response to cancer. IL-2 is associated with side-effects and requires ICU care during administration. Unfortunately, IL-2 has not been approved for pancreatic cancer treatment because of poor efficacy thus, understanding the difference between RCC, melanoma, and pancreatic cancer may help improve its efficacy. Other interleukins (IL-12, IL-15, IL-24,), cytokines (interferons, G-CSF), and chemokines (CCL2, CXCL7) are also potential immunomodulator targets for pancreatic cancer treatment.[43,44 45-48]

The use of activating cytotoxic T lymphocyte (CTL) to induce the antitumor effect against pancreatic cancer is one of the aims of immunotherapy.[49] Dendritic cells (DCs) present antigens to CTLs via MHC class I (endogenously synthesized antigens) or MHC class II molecules (exogenous antigens) and activate CTLs to initiate a tumor-specific immune response.[50,51] The activated CTLs recognize MHC class I-peptide complexes in cancer and mediate cell death via effector molecules like granzyme B and perforin.[52,53] CTLs can also be activated by T-cell receptor mediated activation in which proinflammatory cytokines (INF-γ, TNF-γ, and TNF-α) secreted from Th1 cells activate DCs, which can regulate the survival and persistence of CTL memory cells.[54,55] The founding of tumor-associated antigens (TAAs) such as Wilms' tumor gene 1 (75%), mucin 1 (MUC1) (>85%), human telomerase reverse transcriptase (hTERT) (88%), mutated KRAS (73%), survivin (77%), carcinoembryonic antigen (CEA) (>90%), HER-2/neu (61.2%), and p53 (67%) expressed on pancreatic cancer cells are potential targets for immunotherapy.[56-63] Pancreatic cancer not only consists of cancer cells but also immune suppressive cells, including tumor-associated macrophages, regulatory T cells

(Tregs), tolerogenic DCs, and myeloid-derived suppressor cells (MDSCs). Furthermore, immune suppressive cytokines like TGF β, IL-10, and IL-6 are also presented in pancreatic cancer. Thus, a strategy to deliver TAAs to recruit and activate CTLs while overcoming the immunosuppressive environment in the meantime in pancreatic cancer is needed.

Coupled strategies to activate CTLs are used in clinical trial peptide vaccines, whole tumor cell vaccines, DC-based vaccines, and DNA-based vaccines. Peptide vaccines use antigenic protein fragments from TAAs and present them on the surfaces of antigen-presenting cells (APCs) to activate CTLs. Both short and long synthetic TAA peptides have been used to treat pancreatic cancer in clinical trials, and long synthetic peptides induce a long-term immunological memory response.[59,64-73] Because only a limited number of synthetic peptides are known, whole tumor cell vaccines generating TAAs from autologous whole tumor cells or allogeneic tumor cell lines, are alternative strategies. Whole tumor cell vaccine a) does not require prior knowledge about the antigens, b) can present multiple TAAs on MHC Class I or Class II molecules, and c) protects against tumor escape variants because of polyclonal antigen-specific CD4+ and CD8+ T cells. Autologous tumor cells vaccines have many technical drawbacks. First, only 10-15% of pancreatic cancer patients are eligible for surgery (autologous cell sources) and culturing sufficient numbers of cells with contaminations (bacteria, fungus, and fetal calf serum [FCS]) limits the clinical application. Using allogenic tumor cell line-generated TAAs solves the availability of cell numbers and well-characterized cell sources. Multiple clinical trials, using those testing allogeneic whole tumor cell vaccines, have shown an anti-tumor effect.[74-76] However, safety issues regarding the potential hazards of FCS and producing large batches of allogeneic whole tumor cells in good manufacturing practice-grade conditions remain challenging. Another approach uses DC-based vaccines to generate a CTL response

against tumor cells by using known tumor antigens, tumor cell lysate, apoptotic tumor cells, RNA derived from tumor antigens, and transfection with whole tumor cell nucleic acid.[77-82] Although DC-based vaccines have shown an anti-tumor immune response, further investigation to improve the efficacy of DC-based vaccines is needed due to the limited number of objective clinical responses in some cancer types.[83-86] However, some ongoing clinical trials on DC-based vaccine pancreatic cancer treatments (targeting MUC1, hTERT) show promising results with improved survival, reduced recurrence, and a few cases of complete remission.[68,87-91] Cell-based cancer vaccines can induce the antitumor immune response initially but can soon be attenuated by the host immune system, which recognizes them as foreign material. Presenting the TAAs and stimulatory factors through DNA vaccine can extend antigen expression, induce memory responses, process the antigen in both exogenous and endogenous pathways, and has characteristics of multiple epitopes. Indeed, pancreatic cancer murine models have shown that DNA vaccinations targeting MUC1 or survivin can induce an antitumor immune response.[92-94]

The benefits of using cancer vaccines are 1) they may prevent recurrence and metastasis after surgical resection, and 2) combining cancer vaccines with chemotherapy may also be synergetic. Drawbacks include 1) limited number of known synthesized short peptides when using peptide vaccines, 2) they may not be effective to treat cancer, which has downregulated tumor antigens and MHC class I molecules, 3) pancreatic cancer patients may have defective APCs that cannot stimulate CTLs, and 4) the immunosuppressive environment created by MDSCs or Tregs may affect CTL function.

Although many options, including surgery, chemotherapy, radiation therapy, immunotherapy, or adjuvant and neoadjuvant therapy, are available to treat pancreatic cancer, the five-year survival rate of pancreatic cancer remains approximately 5%. This is primarily due

to the difficulty of early diagnosis and that pancreatic cancer most often has already metastasized when symptoms are found.[95] Therefore, biomarkers that can effectively diagnose and monitor pancreatic cancer are also important to improve the pancreatic cancer survival rate.

Table 1-1: Individual Pancreatic Cancer Treatment Options

Stage	**Options**
Stage I Cancer has formed and is found in the pancreas only **Five-year Survival Rate:** Stage IA (tumor<2cm) ~14% Stage IIA(tumor>2cm) ~12%	Surgery with or without chemotherapy
	Surgery with chemotherapy & radiation therapy
	Clinical trial of combination chemotherapy
	Clinical trial of chemotherapy and targeted therapy, with or without chemoradiation
	Clinical trial of chemotherapy and/or radiation therapy before surgery
Stage II Cancer may have spread to nearby tissue and organs and may have spread to lymph nodes near the pancreas. **Five-year Survival Rate:** Stage IIA (not spread to lymph node) ~7% Stage IIB (spread to the lymph node) ~ 5%	Surgery with or without chemotherapy
	Surgery with chemotherapy & radiation therapy
	Clinical trial of combination chemotherapy
	Clinical trial of chemotherapy and targeted therapy, with or without chemoradiation
Stage III Cancer has spread to the major blood vessels near the pancreas and may have spread to nearby lymph nodes Five-year Survival Rate~3%	Palliative surgery or stent placement to bypass blocked areas in ducts or small intestine
	Chemotherapy (gemcitabine) with or without targeted therapy (erlotinib)
	Combination chemotherapy
	Chemoradiation followed by chemotherapy
	Chemotherapy followed by chemoradiation, for cancer that has not spread to other parts of the body
	Clinical trial of new anticancer therapies together with chemotherapy or chemoradiation
	Clinical trial of radiation therapy given during surgery or internal radiation therapy
Stage IV Cancer may be of any size and has spread to distant organs, such as the liver, lung, and peritoneal cavity. It may have also spread to organs and tissues near the pancreas or to lymph nodes. Five-year Survival Rate ~1%	Chemotherapy (gemcitabine) with or without targeted therapy (erlotinib)
	Combination chemotherapy
	Palliative treatments for pain, such as nerve blocks and other supportive care
	Palliative surgery or stent placement to bypass blocked areas in ducts or the small intestine
	Clinical trials of new anticancer agents with or without chemotherapy

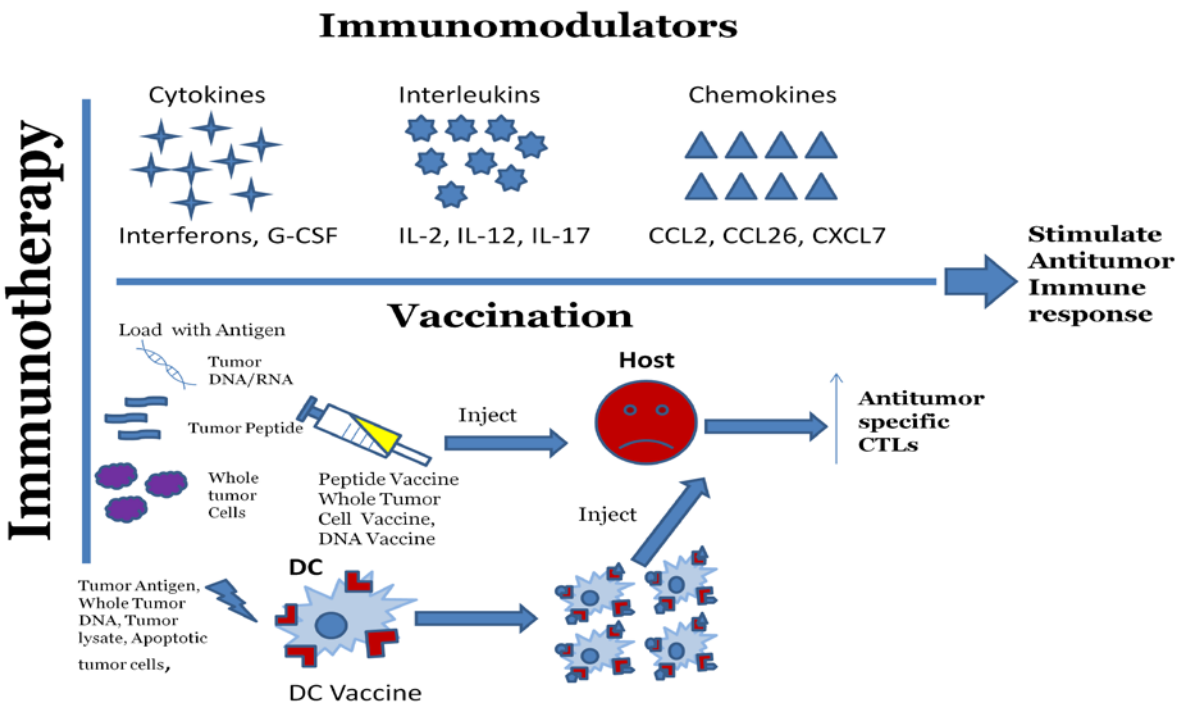


Figure 1-3: Schematic summary of current immunotherapy with immunomodulators

Schematic summary of current immunotherapy with immunomodulators (cytokines, interleukins, or chemokines) or vaccination (DNA/RNA, tumor antigen, whole tumor DNA, tumor lysate or apopototic tumor cells) to induce anti-tumor immune response to pancreatic cancer

1.2.2 Current Diagnosis and Unmet Clinical Need for Biomarkers

Although pancreatic cancer therapy development is advancing, the five-year pancreatic cancer survival rate remains low (3% for stage III, 1% for stage IV, and lower early stage survival rates). This rate is also low compared to that of other top 10 deadly cancers: lung, colon and rectal, breast, prostate, leukemia, non-Hodgkin lymphoma, liver and intrahepatic bile duct, ovarian, and esophageal, according to a American Cancer Society survey (Table 1.2). Although early diagnosis and surgery can increase the survival rate, some patients have reoccurrence, liver failure, or liver metastasis, depending on the tumor's aggressiveness and whether any microscopic tumor cells still remain in the patient after surgery. Thus, diagnostic techniques or

biomarkers to diagnose pancreatic cancer in the early stage and monitor the disease after treatment may help improve the survival rate.

Table 1-2: Five-year Survival Rate for the Top 10 Deadly Cancers in United States

Five- year Survival Rate for the Top 10 Deadly Cancers in United States with pancreatic cancer has the lowest survival rate in early stage**.**

Rank by Cause of Death	**Stage IA**	**Stage IB**	**Stage IIA**	**Stage IIB**	**Stage III**	**Stage IV**
Lung [non-small cell] (small cell)	[49%] (31%)	[45%]	[30%] (19%)	[31%]	[14%] (8%)	[1%] (1%)
Colon & Rectal	74%		67%	59%	73%-28%	6%
Breast Cancer	88%		81%	74%	67-49%	15%
Pancreas	14%	12%	7%	5%	3%	1%
Prostate	Local (100%)		Regional (100%)		Distant (29%)	
Leukemia	Cure rate Chronic myelogenous leukemia 60-80% Acute lymphocytic leukemia ~40% cure rate Acute myelogenous leukemia Children ~60-70%					
non-Hodgkin lymphoma	Cure rate ~67% depends on time of diagnosis					
Liver and intrahepatic Bile Duct	Localized 28%		Regional 10%		Distant 3%	
Ovarian	94%	91%	76%	67%	34%	18%
Esophageal	Localized 38%		Regional 20%		Distant 3%	

Many pancreatic cancer signs and symptoms (e.g. jaundice, abdominal or back pain, weight loss & poor appetite, digestive problems, gallbladder enlargement, blood clots or fatty tissue abnormalities, and diabetes) will alert the physician to conduct more detailed diagnostic exams and tests to determine if the patient has pancreatic cancer. Details about why pancreatic cancer will cause those signs and symptoms are beyond the scope of this dissertation. More and more evidence has shown that diabetic patients are at increased risk for developing pancreatic cancer.[96] Our laboratory has developed a spontaneous murine pancreatic cancer model to

investigate how pancreatic cancer is linked to diabetes with double KRAS and HMGB1 (Unpublished).

According to the American Cancer Association, when more than one sign or symptom is found, the physician will conduct a history & physical exam, imaging test, blood test, and biopsy to determine if the patient indeed has pancreatic cancer (Table 1.3). Although many exams and tests are available to diagnose pancreatic cancer, it quite often happens after the symptom or sign has developed, implying the cancer may have already spread. However, those diagnostic exams and tests do not give much information about therapy responsiveness or prognosis prediction. Therefore, a strategy to diagnose pancreatic cancer during the early stage and provide predictive therapy responsiveness and prognosis value is needed.

Because of the difficulties of early-stage pancreatic cancer diagnosis and the challenges of effectively treating late-stage pancreatic cancer, biomarkers that can help early disease detection, therapeutic outcome, or prognosis prediction are highly desirable. A biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention," as defined by the NIH Biomarker Working Group.[97] With the other word, a biomarker can either be used as a diagnostic, prognostic, or predictive tool for the disease.[98]

An ideal diagnostic biomarker would be non-invasive and inexpensive with a high degree of sensitivity and specificity that would allow its use as a routine test to detect premalignant lesions, early invasive but curable cancer, or relapse after clinical treatment.[98] A prognostic marker can be used to predict the patient's expected survival, aggressiveness of the disease, and risk of recurrence, which help the clinician determine whether to use surgery or systemic treatment or closely monitor disease recurrence for high-risk individuals.[98] A predictive

biomarker can be used to predict therapeutic outcome and help the clinician choose the most effective therapy or treatment strategy (surgery only, chemo/radiation/immune/therapy only, or adjuvant /neoadjuvant therapy) to optimize the treatment. Predictive biomarkers can facilitate a "personalized" treatment approach based on the specific tumor genotype or gene expression profile.[98]

Table 1-3: Current Diagnostic Exams and Tests for Pancreatic Cancer

Exams/Tests	**Procedure**
History and Physical (Symptoms)	Get information about pain, appetite, weight loss, fatigue, and other symptoms Check for abdomen masses or fluid buildup, check skin and eye sclera for signs of jaundice, gallbladder, and liver enlargement; check for lymph node swelling
Imaging Test (Tumor diagnosis)	Computed tomography (CT, CAT) scan Diagnose pancreatic cancer and determine the stage and whether it has spread to the lymph nodes. Magnetic Resonance Imaging (MRI) Similar to CT and CAT, but most physician prefer to use CT and CAT instead Positron Emission Tomography (PET) scan Useful to determine if the pancreatic cancer has spread Ultrasonography (ultrasound) Useful if symptoms are not clear Endoscopic retrograde cholangiopancreatography Useful to check the blockage of ducts and remove cells for biopsy
Blood Test (Tumor diagnosis)	Chemical-based Look at the levels of different kinds of bilirubin and decide whether jaundice is caused by liver disease or a blockage of bile flow (one of the symptoms of pancreatic cancer) Tumor Markers Elevated blood levels of CA19-9 and CEA may point to a diagnosis of exocrine pancreatic cancer. However, those tests may not always accurate (will be discussed in the following section).
Biopsy (Confirmation)	Fine Needle Aspiration (FNA) biopsy Insert a thin needle through skin and into the pancreas at the position where CT or ultrasonography detected the tumor Laparotomy or Laparoscopy A large incision through the wall of the abdomen to sample the biopsy or look at the pancreas with a microscope. If the surgeon confirms a tumor is present in the pancreas and has not spread, surgery is started immediately to remove the tumor. If the tumor has already spread, a small biopsy will be taken to confirm diagnosis.

Table 1-4: Current Biomarkers for Pancreatic Cancer

Category	Usage	Category
Diagnostic	Detect early pancreatic cancer and distinguish it from other pancreatic diseases or detect relapse after treatment	Diagnostic
Prognostic	Reveal the likelihood of the patient's disease aggressiveness (resectable/metastatic), survival rate/time, and risk of recurrence, which can help the physician decide treatment options	Prognostic
Predictive	Predict the clinical outcome of a specific therapy by assessing the genotype or gene expression profile of the tumor, which helps to optimize the therapeutic strategy for individual.	Predictive

1.2.2.1 Currently-Approved and Investigating Biomarkers

CA 19-9, a sialylated Lewis antigen, is the only FDA-approved biomarker for pancreatic cancer. CA 19-9 is produced by exocrine epithelial cells and normally absorbed into the surface of erythrocytes. About 5-10% of population is Le a and Le b (enzymes that produce the antigen) negative, to whom CA 19-9 cannot be applied.[99-102] CA19-9 is not an effective screening marker because it fails to distinguish between pancreatic cancer and other gastrointestinal complaints.[103].However, CA 19-9 can serve as a promising pancreatic cancer marker to distinguish the disease from other pancreatobiliary diseases with sensitivity, specificity, positive and negative predictive values, and accuracy (70%, 87%, 59%, 92%, and 84%, respectively); similar performance has also been shown in other studies. [104-106] CA 19-9 has limited prognostic value in localized pancreatic cancer, which has a low Coz proportional hazard ratio, does not correlate with survival, and is falsely elevated in other pancreatic diseases and biliary tract conditions.[107-110] Surgical procedures to remove the biliary obstruction make CA 19-9 a better

prognostic marker.[111] Although CA 19-9 is not a good prognostic marker for localized pancreatic cancer, studies have shown that it can help to determine whether the tumor is resectable or not.[112-114] On the other hand, CA 19-9 is a weak prognostic marker before treatment, but becomes informative after treatments (surgical resection, radiation, or chemotherapy). A CA 19-9 level drop from the baseline of 20-75% was associated with a median of survival of one year compared to less than three months.[115-121]

CEA and CA125, two other serum antigens, are also used to monitor the progression of the disease and therapeutic response; however, both CEA and CA125 are not FDA-approved for pancreatic cancer diagnostic application (they are approved for ovarian and colon cancer) because of their low sensitivities and specificities (30-60% and ~80%, respectively).[104,122,123] Ongoing investigations to identify new diagnostic, prognostic, and predictive biomarkers to better distinguish malignant and benign pancreatic disease forecast the nature of the disease and predict therapeutic outcome.

Potential diagnostic biomarkers using proteomics (71%, 91%), metabolomics (in urine 75%, 91%, in plasma 75%, 75%), miRNA (miR-21, 210, 155, 196a, 64%, 89%) and genotypic molecules (mutated KRAS in bile 94%, 89%, and in plasma 40-50% without specificity) as biomarkers yield sensitivity and specificity comparative to the current gold standard marker CA 19-19 (60-70%, 70-85%, respectively).[124-130]

Prognostic biomarkers to predict survival or likelihood of reoccurrence after resection have also been investigated. For instance, a study comparing gene expression profiles between patients with localized and metastatic pancreatic cancer discovered six genes (FOSB, KLF6, NFKBIZ, ATP4A, GSG1, and SIGLEC11) as a signature for poor survival.[131,132] Another tissue microarray study compared a short (<12 months after resection) and long survival group (>30

months after resection) with 13 putative pancreatic cancer biomarkers.[133] A three univariate biomarker panel (MUC1, MSLN, and MUC2) that is more predictive than the current routine four pathologic features (lymph node metastases, resection margin, tumor size, and histologic grade) were identified. Other studies that focused on identifying prognostic biomarkers to predict the reoccurrence pattern after surgery identified that loss of SMAD4 expression is associated with local failure and progression, but some contradicting results from other studies underscore the value of SMAD4 as a prognostic biomarkers.[98,115,134]

The decision to apply the right therapeutic strategy to treat pancreatic cancer can be facilitated by a well-defined predictive marker. Studies have shown that pancreatic cancer patients with DNA repair pathway mutations are more sensitive to DNA-damaging agents (e.g. mitomycin C and platinum-based agents) or PARP inhibitors (protein that is important for DNA repair).[135-139] Studies have found that patients with mutations in BRCA1/BRCA2 or PALB2 are more responsive to PARP inhibitors or mitomycin C therapies, respectively.[140,141] Other studies have also identified predictive biomarkers for gemcitabine, including hENT1, RRM1, ERCC1, and cytoplasmic expression of HuR.[142-144] HuR is found to be a promising predictive marker for gemcitabine responsiveness, in which it is independent of the tumor stage.

Currently, there is still an unmet clinical need to 1) identify early diagnostic markers to detect and treat early stage pancreatic cancer and monitor reoccurrence after resection, 2) discover prognostic markers to help select the best therapeutic option (e.g. resection, adjuvant or ne-adjuvant therapy) based on the survival and reoccurrence pattern, and 3) predictive markers to optimize therapy based on the drug resistance of individual patients. Most of the above studies explore potential biomarkers in pancreatic cancer tissue, serum, plasma, bile, and urine; we

believe that assessing patients' PBMC miRNA expression profile might reveal new biomarkers with diagnostic, prognostic, and predictive potential.

1.3 MICRORNA

MicroRNAs (miRNAs) are 18-22 nucleotide long, single stranded, non-coding RNAs that regulate important biological processes including cell differentiation, proliferation, and response to cellular stressors such as hypoxia, nutrient depletion, and traversion of the cell cycle by controlling protein expression within the cell. Many investigators have profiled cancer tissue and serum miRNAs to identify potential therapeutic targets, understand the pathways involved in tumorigenesis, and identify diagnostic tumor signatures. (Figure 1.4) In the setting of pancreatic cancer, obtaining pancreatic tissue is invasive and impractical for early diagnosis. Several groups have profiled miRNAs that are present in the blood as a means to diagnose tumor progression and predict prognosis/survival or drug resistance. Several miRNA signatures found in pancreatic tissue and the peripheral blood, as well as the pathways that are associated with pancreatic cancer, are reviewed here in detail.[5] In this dissertation, we identified and validated potential miRNA expression profiles differentially expressed in pancreatic cancer patients' PBMC compared to those of healthy individuals and explain how those miRNAs play a role in cancer progression.

1.3.1 miRNA Biogenesis and Mechanism to Regulate Gene Expression

miRNAs are transcribed by RNA polymerase II/III in the nucleus and primary miRNAs (pri-RNAs) are then processed by Drosha into hair-loop pre-miRNAs before they are exported to the cytoplasm by Exportin-5. In the cytoplasm, pre-miRNA is cleaved by Dicer into a mature single-stranded miRNA hairpin loop, which regulates its cognate targeted gene messenger RNA (mRNA) by two primary mechanisms. (Figure 1.5) miRNAs utilize the RNA-induced silencing complex (RISC) to regulate target genes by binding the 3' untranslated region (UTR). When miRNA is perfectly matched with the target mRNA, it will induce cleavage, thus inhibiting gene expression. When the miRNA is imperfectly matched, it will induce translational repression. Thus, the overall mRNA remains unchanged while gene expression is inhibited. miRNA can induce translational repression by: 1) translation initiation inhibition, 2) post-initiation inhibition, 3) mRNA decay in removal foci, and 4) mRNA storage in stress granules.[145] In brief, miRNA can induce translation initiation inhibition by repressing the 48S translational complex assembly, competing the m7G of mRNA binding site with eIF4E (miRNA binds to the Ago2 complex in order to bind to m7G) or blocking PolyA Binding Protein to affect translation initiation.[146-149] MiRNA can induce post-initiation inhibition by leading to higher rates of ribosome drop off, leading to immature termination during the elongation step.[150] Argonaute proteins are part of the catalytic components in RISC and are able to bind to small non-coding RNAs (including miRNAs, small interfering RNAS (siRNAs), and Piwi-interacting RNAs (piRNAs).[151,152] Some of the Argonaute proteins have endonuclease activity to enable degradation of perfectly complementary mRNA.[153] In eukaryotes, Argonaute proteins have been identified in high concentrations in regional foci within the cytoplasm known as p-bodies.[154,155] miRNA induces

sequestration of mRNA within P-bodies.[156] miRNA can also induce temporary storage of mRNA in stress granules which can either be degraded or de-repressed later within the cell.[157]

1.3.2 Role of miRNA in Cancer

miRNAs can negatively regulate multiple gene targets and play an important role in cell cycle, proliferation, differentiation, immune response and cancer development.[158] miRNA can either be tumor-suppressive or oncogenic depending on its down-stream targets. For example, the miR-15a/miR-16-1 target anti-apoptotic gene BCL2 serves as a tumor suppressor in tumor development; it is frequently deleted or downregulated in B-cell chronic lymphocytic leukemia.[159,160] Other tumor-suppressive miRs, including miR-143/miR-145 and the let-7 family, that negatively regulate cancer glycolysis rate-limiting enzyme and oncogene Ras, respectively, are frequently down-regulated or deleted in cancer.[161-165]

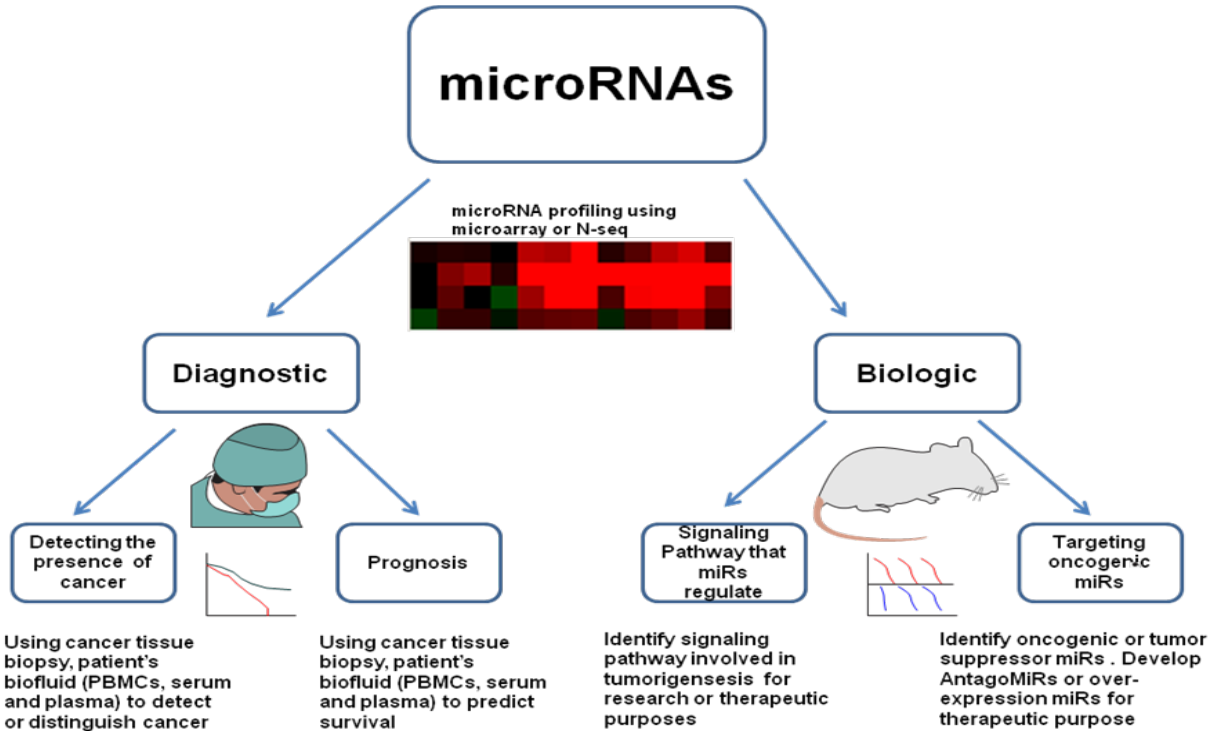


Figure 1-4: miRNA application in cancer research

miRNA application in cancer research. miRNAs can be used for both diagnostic and biologic studies. miRNA profiling usingmiR microarray, qRT-PCR, or RNA-seq can identify important miRNA expression changes at various disease stages. miRNA profiling (obtained from cancer tissue biopsies or patient's blood) can be used to detect the presence of tumor and help define prognosis. miRNA profiling is widely used to identify signaling pathways involved in tumorigenesis, to develop new therapeutic strategies, or to directly target the oncogenic MicroRNAs as therapies.

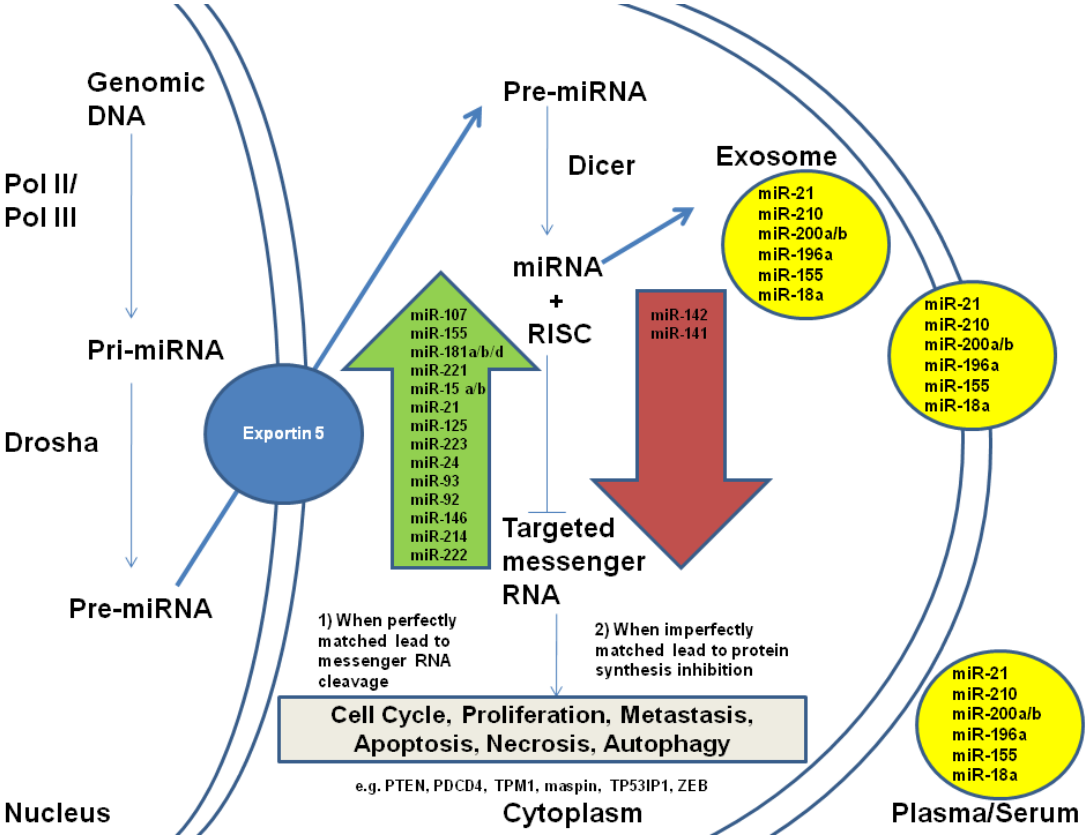


Figure 1-5: miRNAs that are distinctly different in pancreatic cancer tissues and blood

Primary miRNA (pri-miRNA) is transcribed in the nucleus and then processed by Drosha into pre-miRNA before being exported to the cytoplasm by Exportin 5. In the cytoplasm, the pre-miRNA, a hairpin/stem loop, is further processed by the enzyme Dicer, producing a double-stranded miRNA. One strand of the mature miRNA is then incorporated with RISC to regulate its gene targets. There are two distinct regulatory mechanisms: (1) mRNA cleavage if the miRNA is perfectly matched with its targets' 3' UTR and (2) protein synthesis inhibition if the miRNA is imperfectly matched with the targets' 3' UTR. The targeted genes' mRNA will be transported to P-bodies or stress granules and are thus inaccessible to the translational machinery. Some of the miRNAs found in tumor cells will, with necrosis, be released into the plasma/serum or alternatively released in exosomes.

1.3.3 miRNAs in Pancreatic Cancer

Many investigators have profiled cancer tissue and serum miRNAs to identify potential therapeutic targets, understand the pathways involved in tumorigenesis, and identify diagnostic tumor signatures. In the setting of pancreatic cancer, obtaining pancreatic tissue is invasive and impractical for early diagnosis. Several groups have profiled miRNAs that are present in the blood as a means to diagnose tumor progression and predict prognosis/survival or drug

resistance. The following section summarizes the current literature on pancreatic cancer miRNAs.

1.3.3.1 Origin of Blood miRNA

miRNAs can be isolated directly from the blood (PBMCs are especially sensitive to micro-environmental changes, including those arising in the setting of cancer), plasma, or serum. Studies of whole blood or PBMC miRNA expression to detect tumors (e.g. ovarian cancer and melanoma) are developing rapidly (BackgroundTable 5).[166,167] Circulating miRNAs are also normally present in the serum or plasma.[168] Many scenarios have been formulated to explain how miRNA can survive endogenous ribonucleases that are present within blood. These include miRNA binding to DNA for protection from RNases and DNases perhaps derived from exteriorized autophagosomes (exosomes).[169,170] The latter scenario appears to be the most likely mechanism that preserves miRs in plasma and serum.[168] Circulating miRs in the plasma and serum might originate from tumor-derived exosomes (e.g. miR-21, miR-106, miR-141, miR-14, miR-155, the mir-200 family, miR-203, miR-205, miR-214, etc.). Note that only the miR-21 and miR-18 families have been found to be up-regulated in more than two cancer types (Table 1.5). Perhaps blood miRNA-markers are more cancer type-specific than tissue miRNA markers.[171-174] The lack of appropriate endogenous controls (miRNAs that do not change with disease stage) limits the predictive power and further validation of the biological role of such circulating miRNAs is needed.

Table 1-5: microRNA in Cancer Patients' Blood

Type of Cancer	miRNAs Source	Down-regulated	Up-regulated	Compare with Cancer Tissue	Ref.
Pancreas	Serum, Plasma	miR-18a, miR-21, miR-210, miR-155, miR-196a, miR-200	N/A	miR-18a, miR-21, miR-155, miR-200, miR-196 is also over-expressed in pancreatic cancer tissue and cell line.	[127,175,176]
Lung	PBMCs	let-7 a,c,d,e,f,g, miR-15a, miR-20a, miR-98,miR-126, miR-195	let-7i, miR-19a, miR-22, miR-423-5p,	miR-126, let-7 family, miR-22, miR-19 expression in PBMCs are correlated with lung cancer tissue. MiR-20a is inversely correlated in lung cancer tissue.	[177]
Ovary	Whole Blood	Let 7f-1, miR-28-3p, miR-29a, mir-106b, miR-138-2, miR-146a, miR-181a, miR-181a-2, miR-192, miR-342-3p, miR-450-5p, miR-616, miR-628-5p,miR-1287	miR-16, miR-30c-1, miR-187, miR-191, miR-191, miR-383, miR-423-3p, miR-499-3p, miR-546-5p, miR-1181, miR-1228, miR-1253, miR-1254, miR-1289, miR-1908, miR-1915	miR-30c-1, miR-191, miR-155, miR-16, miR-106b, miR-146a, miR-29a, and miR-383 are connected to ovarian cancer while the other miRs are not reported to be connected to the ovarian cancer.	[167]
Gastric	Plasma	miR-21, miR-106b	Let-7a	Inverse relationship between plasma miRs and gastric cancer miRs expression level.	[178]
Acute Leukemia	Plasma	miR-92	N/A	Acute leukemia cell might in-take miR-92 with exosome thus decreasing the miR-92 concentration in plasma.	[179]
Oral	Plasma	N/A	miR-31	miR-31 is over-expressed in oral cancer tissue. miR-31 level decreased after surgical removal of cancer; it is very likely that the circulating miR-31 is tumor-derived.	[180]
Colorectal	Plasma	N/A	miR-29a, miR-92a	miR-92 is also found to be up-regulated in breast cancer serum sample and in other cancer tissue. MiR-29a is up-regulated in lung and advanced colorectal neoplasia and its role in cancer biology is still unclear.	[181]
Melanoma	PBMC	miR-452, miR216a, miR-17, miR-646, miR-217, miR-517, miR-593,let-7i,miR-330-3p, miR-767-5p, miR-20b, miR-509-3-5p, miR-519b-5p,	Let-7d, miR-18a, miR-22, miR-30a,e, miR-99a, miR-125a-5p, miR-142-3p, miR-145, miR-146a, miR-155-3p, miR-181a-2, miR-183, miR-186, miR-199a-5p, miR-328, miR-	miR-216a and miR-186 expression levels correlate with the melanoma tissue miR expression levels.	[166]

Table 1-5 (continued)

Melanoma (Continue)		miR-518e, miR-221, miR-214, miR-106b, miR-18b, miR-108, miR-20a	343-5p, miR-361-3p, miR-362-5p, miR-363-3p, miR-365, miR-378, miR-422a, miR-501-5p, miR-550, miR-584, miR-625, miR-664, miR-1249, miR-1280		

1.3.4 miRNA-profiling studies on pancreatic cancer patients' blood

Many studies have examined pancreatic cancer in the form of cancer-cell lines, mouse tumor, or human clinical specimens to identify miRNA signatures for diagnostic or therapeutic purposes (Table 1.6). Remarkably, perhaps reflecting the state of the science, the miRNA signatures found in these studies substantially differ from one another. This is, in part, due to the varied stages of pancreatic cancer examined as well as the heterogeneous patient populations. Importantly, it is also due to the differences between cancer cell lines and authentic primary tumors (tumor consisting of multiple cell types: blood components, endothelial cells, stromal cells, while cancer cell lines consist of only one cell type), the extraction method, comparison (tumor vs. normal surrounding tissue, tumor vs. normal healthy individual) and identification strategies (fold change threshold, pre-selected markers, clustering, etc.)

1.3.4.1 Comparing clinical specimens and cancer cell lines

For therapeutic purposes, it would be useful to identify pancreatic cancer miRNAs that are shared between clinical samples and cancer cell lines (cancer cell lines are more readily available for therapeutic target validation than clinical samples). One study compared the expression profiles of individual pancreatic cancer cell lines and clinical specimens using PCR (95 miRNA

primers). Eight miRNAs were found to be commonly expressed in both cell lines and clinical samples (miR-196a, mIR-190, miR-186, miR-221, miR-222, miR-200b, miR-15b, and miR-95).[182] When examining the clinical specimens, 20 miRNAs were over-expressed in all five specimens and eleven miRNAs were over-expressed in at least four specimens. The results suggest that although there are similarities between pancreatic cancer cell lines and clinical specimens, the miRNA expression patterns are not identical.

miRNA expression profiles in normal pancreatic tissue (referred to as pancreatic miRNome), PDAC, pancreatitis, and pancreatic cancer cell lines have been recently examined.[183] This study first created a pancreatic miRNome by clustering miRNAs that are highly expressed in pancreatic normal tissue compared to other tissues. The group used this miRNome as the parameter to measure miRNA expression changes in pancreatitis and PDAC miRNA. Twenty miRNAs were differentially expressed when comparing PDAC, chronic pancreatitis, and normal tissues. Twelve out of 20 miRNAs were also differentially expressed in cancer cell lines. Furthermore, two potential miRNA (miR-196a and miR-217) markers are over-expressed in both primary neoplastic ductal cells and in PDAC cell lines. A similar study found that 23 (15 over-expressed and eight under-expressed) miRNAs could be used to distinguish pancreatic cancer from pancreatitis with an extraordinary 93% accuracy.[182] These similar studies identified divergent sets of miRNAs, possibly due to the differences in comparison strategies and the patient populations utilized by the two groups. One method compared expression to normal tissue while the other group compared expression to a pancreatic tissue-specific gene expression file.

Pancreatic cancer-specific miRNAs are commonly expressed in both clinical specimens and pancreatic cancer cell lines, but the expression profiles are not identical to each other. Since

pancreatic tumors are indeed more than just pancreatic cancer cells, examining more stage- and cell type-specific miRNA profiles should provide a more refined result.

1.3.4.2 Comparing between pancreatic cancer stages and tumor types

Pancreatic cancer is a dynamic disease. Understanding the difference between the stages of pancreatic cancer utilizing miRNA profiles is very important. A murine RT2 pancreatic neuroendocrine tumor model study identified pancreatic cancer miRNA markers by stage.[184] The study identified primary tumor stage miRNA signatures and metastasis-specific miRNA signatures by comparing the normal islets with primary tumor, liver metastases, and tumor pools. They identified miRNA signatures for hyperproliferation and angiogenesis using flow cytometry to sort hyperproliferating islets and angiogenic islets. The result of the study provides more detail on tumor stage-specific and cell type-specific miRNA signatures in pancreatic tumors.

Two other studies compared pancreatic cancer tissue with adjacent tissue to identify miRNA markers.[185,186] One study identified 20 miRNAs that are differentially expressed in both pancreatic adenocarcinoma and cancer cell lines compared to normal pancreatic tissue miRNA. [185] The *in situ* result showed that miR-221 and miR-376a are localized to tumor cells, but not to benign pancreatic acini or stromal cells. Deregulation of miR-15a and upregulation of miR-214 are also potential pancreatic cancer markers.[186] Micro-sectioning to allow *in situ* hybridization on epithelial cells was also compared with matched normal pancreatic markers.[186] Micro-sectioning to allow *in situ hybridization* on epithelial cells was also compared with matched normal pancreatic tissues.[187] Ten miRNAs were differentially expressed and two miRNAs (miR-21, and miR-155) had the highest fold change, with miR-21 and miR-155 expression correlating with

precursor lesions. The results were congruent with murine RT2 studies demonstrating that miR-21 and miR-155 are over-expressed in hyperproliferating and angiogenic islets.

Nominally specific pancreatic cancer miRNAs could be shared with other cancer types. One study compared solid tumor samples' (breast, colon, lung, pancreas, prostate, stomach) miRNA expressions with those of normal tissues (stomach, lung) from patients or non-cancer individuals (for the breast, colon, pancreas, and prostate cancer specimens).[188] Twenty one miRNAs were shared among six individual solid cancer types. Twenty of the pancreatic cancer miRNAs were shared with more than one solid tumor type. Most of the targets of these 21 shared miRNAs are identifiable tumor suppressors and/or oncogenes. Seventeen miRNAs were up-regulated and three were down-regulated. A possible reason for the variation between individual clinical pancreatic cancer profiling studies might be attributable to the stage of the patient sample and the type of cell that makes up the tumor. Therefore, a more-refined classification of pancreatic cancer with cell type-specific isolation before miRNA profiling could be important for identifying suitable pancreatic miRNAs.

Another extensive study performed with human pancreatic cancer tissue identified miRNAs that are differentially expressed in individual patient groups.[189] Ten miRNAs (miR-10a, miR-21, miR-143, miR-145, miR-155, miR-222, miR-223, miR-224, and miR-373) were up-regulated while seven miRNAs (miR-148, miR-216, miR-217, miR-211, miR-345, miR-596, and miR-708) were down-regulated. The study also characterized some non-overlapping miRNAs: nine miRNAs to distinguish tumor stage, 16 miRs to distinguish tumor grade, four miRNAs distinguishing lymph node status, and miR-21 and miR-34a serving as survival-predictive miRNAs.

1.3.4.3 Identify prognostic, survival, and chemo-resistant markers

Since the current five-year survival rate for patients with pancreatic cancer is less than 5% and surgical resection remains the most effective therapy, identifying markers to predict survival and determine chemo-resistance may improve our ability to define subsets of pancreatic cancer patients most suitable for aggressive therapy. Some groups have combined clinicopathologic follow-up and miRNA expression data to identify useful biomarkers to help predict survival and clinical outcome. Two independent studies found that miR-21 is a potential marker for survival.[189,190] One group extracted RNA from fresh frozen samples while the other group used *in situ* hybridization to profile the miRNA. Both groups found that pancreatic cancer patients with high miR-21 expression had a low median survival time (13.7 months and 14.3 months) while patients with lower miR-21 expression had a longer median survival time (25.7 months and 23.1 months, respectively). The first group also identified potential markers for better prognosis (high expression of miR-29c, miR-30d, and miR-34a), and determined that patients who have high miR-21 expression are more effectively treated with chemotherapy than those who have lower miR-21 expression. Pancreatic cancer patients with high miR-196a expression in their serum are correlated with poor survival with 100% sensitivity and 75% specificity (6.1 month vs. 12 months for the low miR-196a expression group).[191] One group extracted RNA from fresh frozen samples while the other group used *in situ* hybridization to profile the miRNA. Both groups found that pancreatic cancer patients with high miR-21 expressions have a low median survival time (13.7 months and 14.3 months), while patients with lower miR-21 expressions have a longer median survival time (25.7 months and 23.1 months, respectively). The first group also identified potential markers for better prognosis (high expressions of miR-29c, miR-30d, and miR-34a) and determined that patients who had high miR-21 expressions were more effectively

treated with chemotherapy than those with lower miR-21 expressions. Pancreatic cancer patients with high miR-196a expressions in their serum were correlated with poor survival with 100% sensitivity and 75% specificity (6.1 months vs. 12 months for the low miR-196a expression group).[191] One study showed that patient tissue specimens with high expressions of miR-142-5p and miR-204 correlated with a better patient survival rate (45 months and 33 months vs. 16.3 months and 16.3 months for lower expression group) when receiving gemcitabine treatment. Patients whose tumors express higher levels of miR-125a and miR-34a seem to be more effectively treated by gemcitabine, although these findings did not reach statistical significance.[192] The miR-200 family and miR-21 are also predictive markers for an apparent increased benefit of chemotherapy.[193,194]

Table 1-6: Potential microRNA markers in pancreatic cancer tissue/cell line

a) Upregulated

miRNA	No. of studies	Validated Potential Targets	Biological Significance	References
miR-107	5	CDK6, DICER1, HIF-1 beta [117,195,196]	Proliferation, Cell Migration, Invasion, Suppressing Hypoxia Signaling	[182,185,187,188]
miR-155	5	TP53INP1, PU.1. SPCS1. RAST [197-200]	Suppressing Apoptosis, Inhibiting Tumor Suppressor	[182,183,185,187]
miR-181-a	5	TIMP3, TCL1 [201,202]	Inhibit Tumor Suppressor, Suppressing Oncogene	[183-185,187]
miR-181-a	5	TIMP3, TCL1 [201,202]	Inhibit Tumor Suppressor, Suppressing Oncogene	[184,185,187]
miR-221	5	DVL2, SOCS1, p57, PTEN, p27 [203-207]	Increase Cell Mobility, Inhibiting Tumor Suppressors	[182,185,188]
miR-15a,b and miR-16	4	Cyclin E, BCL2 [160,208]	Inhibit Tumor Suppressor, Inducing Apoptosis	[184-187]
miR-21	4	Big-h3, PTEN, PDCD4, TPM1, maspin [209-215]	Inhibit Tumor Suppressor, Suppress Apoptosis, Cell invasion	[182,183,185,187]
miR-125	4	Bcl1-2, p53 gene [216,217]	Suppressing Apoptosis	[182,185,188,218]
miR-223	4	C-myc, artn, LMO2-L/-S [219-221]	Repressing Estrogen receptor beta 1 expression, increase cell proliferation	[182,183,187,218]
miR-24	3	H2AX, FURIN, DND1, FAF1, DHFR, E2F2, MYC [222-228]	Cell Proliferation, Inducing Apoptosis	[182,185,188]

Table 1-6 (continued)

miR-93	3	Integrin beta [229]	Promoting tumor growth and angiogenesis	[182,183]
miR-181-d	3	TIMP3 [230]	Cell Invasion	[182-184]
miR-92	2	ERbeta1, p63 [231,232]	Repressing Estrogen receptor beta 1 expression, increasing cell proliferation	[184,185]
miR-146	2	Up-regulated by breast cancer metastasis suppressor 1 [233]	Suppressing breast cancer metastasis	[183,218]
miR-214	2	PTEN , ING4 [186,234]	Apoptosis, Chemotherapy resistance	[186,188]
miR-222	2	PUMA, AKT, p27Kip1 [235-240]	Cell Survival, Cell migration	[183,218]

b) Downregulated

miRNA	**No. of studies**	**Validated Potential Targets**	**Biological Significances**	**Reference**
miR-142	2	RAC1, LMO2-L/-S [221,241]	Suppressing Cell Invasion	[184,185]
miR-141	2	ZEB1 [242]	Inhibiting epithelial-mesenchymal transition (EMT)	[182,184]

c) Contradicting

miRNA	**No. of studies**	**Potential Targets**	**Biological Significance**	**Reference**
miR-145	2	OCT4, FSCN1, c-Myc [243-247]	Cell Proliferation, Tumor Suppressor function	[182,184,188]
miR-200	2	ZEB1, FAP1 [248-251]	Suppressing EMT, Suppressing Apoptosis inhibitor	[182,184]

1.3.5 miRNA-profiling studies in pancreatic cancer patients' blood

Tissue miRNA markers could do more to help us understand cancer biology, but also to advance therapeutic options in treating the disease. Such markers have clear limitations as early diagnostic tools for monitoring drug response and defining disease prognosis. First, limited solid tumor samples are available to scientists. Second, such an approach requires invasive procedures to obtain biopsies from solid tumors if they are identifiable. Thus, tissue is not as an ideal approach as an early stage diagnostic method (before symptoms develop). More importantly, it is

not practical to repetitively obtain solid tumor tissue miRNA to monitor disease progression. On the other hand, patients' blood is readily available. Blood samples can easily be obtained (pre/post treatment) and may be a more appropriate sample source to establish a miRNA-based biomarker for early diagnosis of cancer, prediction of drug responsiveness, and definition of prognosis. Studies have shown promising proof of concept to utilize cancer patients' blood miRNA profiles as diagnostic and prognostic tools in pancreatic cancer. miRNAs can be isolated from the PBMC, serum, or plasma components of blood specimens. Three individual studies found six miRNAs expressed in pancreatic cancer patients' serum and plasma as potential biomarkers. MiR-18a, miR-21, miR-210, miR-155, and miR-196a were over-expressed in the majority of the examined pancreatic cancer patients' plasma, with at least two-fold increases.[127,175,176] Sensitivity >40% and specificity > 70% (Table 1.7) can be achieved. When categorizing the patient population by age and sex, compared to healthy individuals, miR-200 a/b is over-expressed in primary pancreatic cancer and cancer cell lines, as well as pancreatic cancer patients' serum.[175] Sensitivities and specificities of 84.4% and 87.5%, respectively for miR-200a, and 71.1% and 96.9% for miR-200b were found. miR-18a (one of the miR17-92 gene cluster family) is upregulated in primary pancreatic cancer tissue and cancer cell lines.[176] MiR-18a expression in patients' serum was significantly reduced following surgical excision. Another study examined pancreatic cancer patients' serum and investigated whether or not miR-21, miR-155, miR-196a, miR-181a, miR-181b, miR-22, and miR-222, which are differentially expressed in cancer tissues, can serve as biomarkers.[191] Higher expression of miR-21, miR-155, and miR-196a are observed in pancreatic cancer patients' serum, but both miR-155 and miR-196a are also upregulated in chronic pancreatitis. The group also found that patients with higher miR-196a serum expressions have a lower median survival (6.1 months vs. 12 months). Since immune cells

respond to the cancer micro- and macro-environments, we hypothesize that in the presence of pancreatic tumor, miRNA expression in patient PBMCs will be altered. . In this dissertation, we discuss profiling pancreatic cancer patients' PBMC miRNA with TaqMan Low Density Array (TLDA) and comparing the profiles to those of age- and sex-match controls. We found miRNAs that are differentially expressed compared to those of healthy individuals.

Table 1-7: Potential Pancreatic Cancer Patients' Blood miRNA Markers

Type of Cancer/ Source	miRNA	Sensitivity	Specificity	Reference
Pancreatic Cancer Patients' Plasma	miR-21	46	89	[127]
	miR-155	42	42	
	miR-196a	53	78	
	miR-210	43	84	
Pancreatic Cancer Patients' Serum	miR-200 a	84.4	87.5	[175]
	miR-200 b	71.1	96.9	
Pancreatic Cancer Patients' Serum	miR-18a	N/A	N/A	[176]

1.3.6 microRNA125 Family

The microRNA-125 family (including miR-125a-5p, miR-125a-3p, miR-125b-1, and miR-125b-2) is encoded in several different chromosomal locations (miR-125b1,2 located on chromosomes 11 and 21, miR-125a-5p on chromosome 19, and miR-125a-3p on chromosome 12). MiR-125a-5p and miR-125b have similar sequences, with 87.5% base-paired identity, and share the same seeding sequence. (Figure 1.6)

miR-125b 1,2 and miR-125a-5p have the same seeding sequence and also play an important role in hematopoiesis.[252] MiR-125 is highly expressed in the long term hematopoietic stem cell (HSC) and is down-regulated as the cell differentiates into a committed progenitor cell.[253-255] While miR-125 is down-regulated as naïve T cells become activated, it is upregulated in activated macrophages.[256,257] Validated immune-related miR-125 targets are TNF-, IL-2RB, and IL-10RA. Besides playing a role in immune cells, miR-125 is also dysregulated in multiple human tumor types (stomach, colon, pancreas, bladder, ovary, gliomas, breast, and melanomas).[218,258-266] The MiR-125 family regulates cell survival, proliferation, and differentiation via upstream regulators of p53 (PPP1CA, PPP2CA, PRKRA, PLK3, PLAG1), p53 network pro-apoptotic genes (BAK1, PUMA, BMF, TRP53INP1, and KLF13), proliferative genes (DICER1, ST18, and SUV39H1), and inflammatory-related genes (IL-6, BLIMP-1, IRF-4).[252] In this dissertation, we focused on investigating how pancreatic cancer influences the expression of miR-125a-5p and how it plays a role in pancreatic cancer.

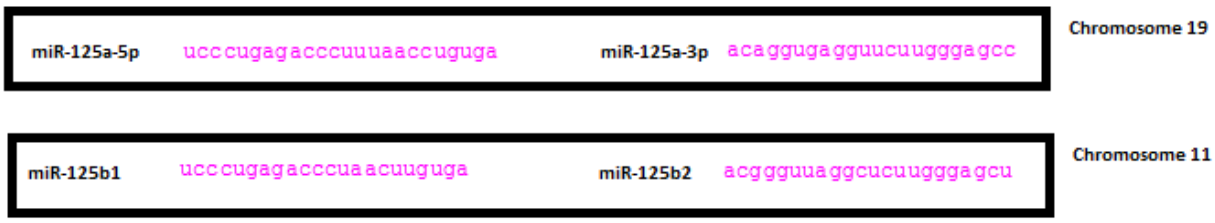


Figure 1-6: miR-125 Family Matured Sequences

miR-125a-5p, miR-125a-3p, miR-125b-1, miR-125b-2 are located in different chromosome but miR-125a-5p and miR-125b-1 shared identical seeding sequence.

1.4 PAMPS AND DAMPS

1.4.1 PAMPs

Pathogen-Associated Molecular Pattern (PAMPs) were initially called signal 0s and described as molecules recognized by receptors on antigen-presenting cells of the innate immune system.[267] PAMPs are derived from microbial components including microbial nucleic acid (unmethylated CpG), dsRNA, ssRNA, 5'-triphosphate RNA, lipoproteins, surface glycoproteins, and membrane component (peptidoglycans, lipoteichoic acid, lipopolysaccharide, and glycosylphosphatidylinositol).[268] PAMPs activate Toll-like receptors (TLRs) and other pattern recognition receptor (PRRs) and localize to the cell surface, the cytoplasm, and/or intracellular vesicles to signal the host to trigger pro-inflammatory and antimicrobial responses. PAMPs signal though NF-kB, AP-1, and IRFs to activate the adaptive immune response against microorganisms by regulating gene expression of cytokine, chemokines, cell adhesion molecules, and immuno-receptors.[269-273]

1.4.2 DAMPs

In the setting of sterile inflammation, damage-associated molecular pattern (DAMPs) are released from stressed or damaged tissues to activate antigen-presenting cells to distinguish between self and non-self.[274-277] DAMPs are cell derived molecules localized within the nucleus (HMBG1), cytoplasm (S100 proteins), exosomes (heat shock proteins), ECM (hyaluronic acid), plasma components (C3a, C4a, and C5a). DAMPs also include nucleic acids (ATP, uric acid, heparin sulfate, RNA, and DNA), and mitochondrial DNA.[278] DAMPs are recognized by and

interacted with TLRs and the receptor for advanced glycation end products (RAGE) to initiate immunity in response to trauma, ischemia, cancer, and other settings of tissue damage. DAMPs signal though MAPKs, NF-kB, and PI3K/AKT signaling pathways to regulate cell survival and cell death.[279] Increased DAMPs serum levels are associated with inflammatory diseases e.g. sepsis, arthritis, atherosclerosis, systemic lupus erythematosus, Crohn's disease, and cancer.[280]

1.4.3 Role of PAMPs/DAMPs in Cancer

PAMPs and DAMPs shared some common receptors (TLRs) to activate innate immunity and adaptive immunity in response to pathogens and tissue damage.[280] PAMPs and DAMPs act as double-edged swords in cancer in that they suppress or promote tumors. TLRs expressed on immune cells can activate innate and adaptive immune response. Bacterial toxins (later known as LPS) have an anti-tumor effect in soft tissue sarcoma patients, and signal via TLR4 to activate both innate and adaptive immune resposes against tumor.[281] A penicillin-killed and lyophilized preparation of a low-virulence strain of *Streptococcus pyogenes* was tested as an immunotherapeutic agent to stimulate TLR4 signaling with promising results.[282-285] TLR2/g agonist (*Mycobacterium bovis* BCG) or TLR2 knock-out studies showed that TLR2 and TLR4 signaling can treat or protect against superficial bladder tumors, colorectal tumors, or lung tumors.[286-288] Triggering TLR3, TLR9 with poly (I:C), CpG can induce apoptosis on tumor cells and vascular endothelium and promote B-cell differentiation and the anti-tumor T-cell response.[289-295] Although TLRs activation on immune cells can have an anti-tumor effect, TLRs also expressed on tumor cells/cell lines and tumor cells use TLRs signaling to produce pro-inflammatory cytokines, chemokines, and anti-apoptotic proteins to promote tumor progression.[296-303] Activating TLR signaling on tumor cells can promote proliferation, anti-

apoptosis, metastasis, ECM adhesion, production of immune-suppressive cytokines, and chemo-resistance.[299-301,304-326] Tumor cells release DAMP signaling though TLRs on tumor cells or immune cells to induce tumor growth, metastasis, and immune tolerance. Therefore, immunotherapy targeting TLRs with TLR agonists must specifically activate the innate immune response without activating TLR signaling on tumor cells.

1.4.4 DAMPs Hypothesis in Cancer

DAMPs are danger signal molecules. Normally sequestered within live cells, DAMPs are released from stressed/damaged/dying cells into the extracellular space and trigger significant host responses. There are three classes of DAMPs: 1) DAMPs exposed on the outer leaflet of the plasma membrane (e.g. chaperones); 2) DAMPs actively secreted or passively released extracellularly (e.g. ATP, uric acid, and HMGB1) and 3) DAMPs produced during cell death-associated end-stage degradation (e.g. mitochondrial components, nucleic acids, and nucleosomes).[327] DAMPs can recruit inflammatory cells to promote wound healing, tissue remodeling, angiogenesis, and modulation of the immune response and play an important role during cancer development.[328] Stress- (e.g. tissue damage, radiation, infection) induced genomic mutation in normal cells leads to cancer. During the early pre-cancer stage, DAMPs induce immune tolerance while they promote inflammation in the late pre-cancer stage. When the cancer becomes invasive, DAMPs become immunosuppressive, which leads to further tumor progression. This is the central theme of the DAMPs hypothesis: genomic damage in cancer is linked to inflammation via interaction with DAMPs. In this dissertation, we describe our investigation of how DAMPs from pancreatic cancer lysate and other immune stimuli change miR-125a-5p expression in human PBMCs. (Figure 1.7)

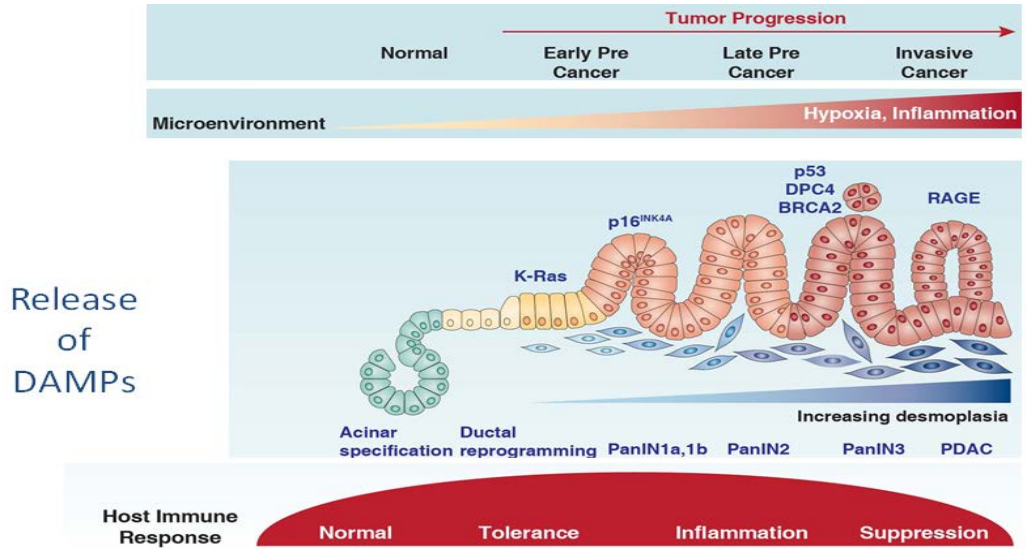


Figure 1-7: DAMPs Hypothesis

Stress (e.g. tissue damage, infection, and radiation) induce genetic mutations, and as more mutations accumulate, tumor cells progress into invasive cancer. As the tumor progresses, tumor cells release DAMPs that modulate immunity to facilitate tumor cell survival and metastasis. The central theme of the DAMPs hypothesis is that genetic damage in cancer is linked to inflammation via interaction of DAMPs.

1.5 IN-VITRO DAMPMIRS

In our previous publication (I was the second author of the paper), we used freeze-thaw cell lysate as DAMPs to stimulate PBMCs for 24 hours or 48 hours and demonstrated that microRNA expression profile in PBMCs will change in response to DAMPs.[4] We also validated the downstream target of one of the *in-vitro* DAMPmiRs (namely miR-34c), IKK-γ which plays an important role in regulating inflammation via the NF-kB signaling pathway.

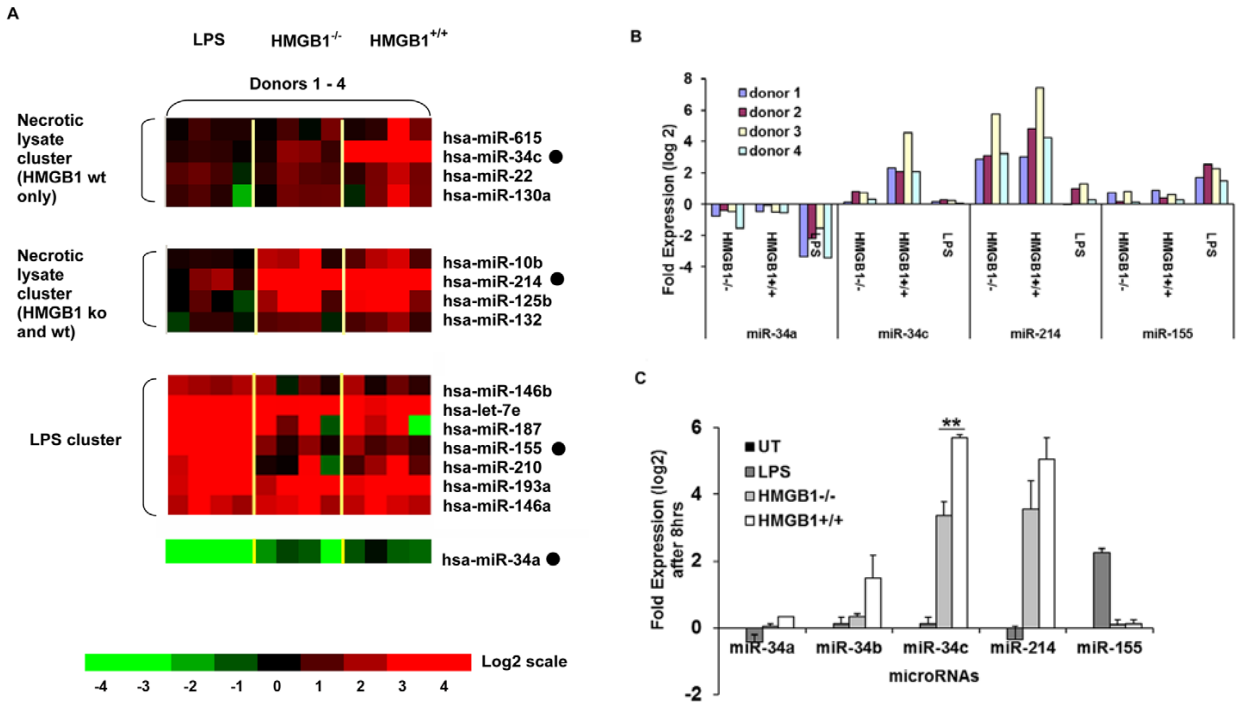
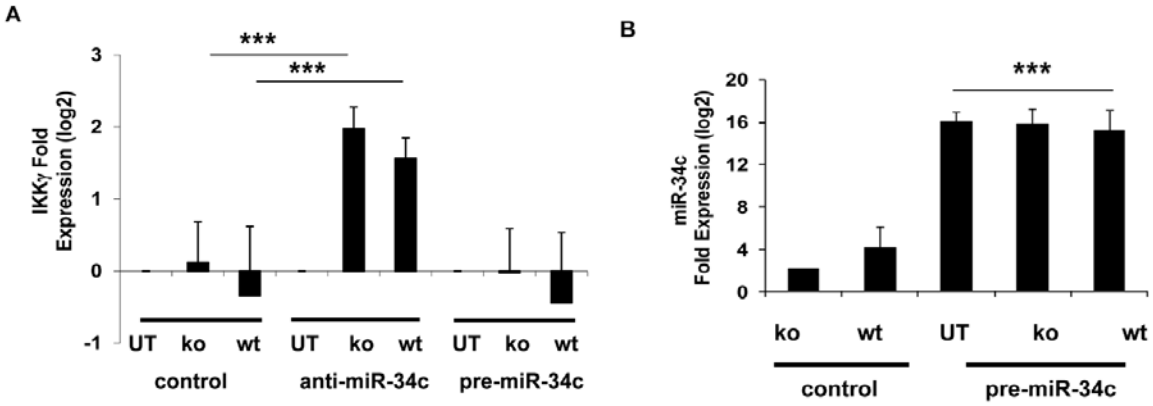


Figure 1-8: Hallmark of human PBMCs exposed to necrotic cell lysates

A: Hierarchical clustering or heat map of microRNA expression signatures (after real-time TaqMan RT-PCR array profiling) in donor PBMCs exposed to cell lysates and/or LPS. Total RNA extracted from PBMC cultures was run on microRNA TaqMan low-density arrays (TLDAs). B: Figure depicting changes in fold expression as log 2-transformed RQ (relative quantity) values of the statistically significant miRs (p values shown in from each of the four donors, after exposure to the respective conditions. All values were calculated from 22ddCt (RQ values) where the endogenous control was snoRNA U48. C: Differential expression of hsa-miR-34a, miR-34b, miR-34c and other miRs when donor PBMCs are exposed to HMGB1+/+ or HMGB12/2 lysates for eight hours.



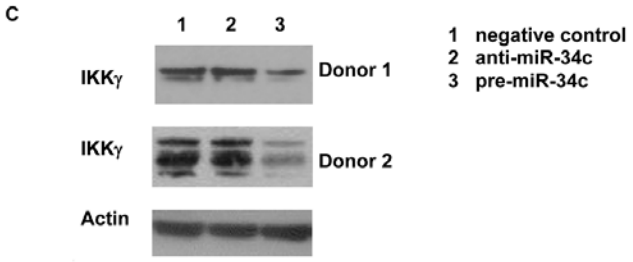


Figure 1-9: miR-34c regulates IKKγ mRNA and protein expression levels in PBMC

1.6 OVERVIEW OF THE DISSERTATION

We examined miRNA expression in human PBMCs from patients with pancreatic cancer and normal individuals. Our central hypothesis was that when tumors undergo necrotic death, DAMPs are passively released into the local micro-environment and progressively into the systemic circulation to initiate early innate and adaptive immune responses.[2,3] We previously found that miRNAs (miR-34c, miR-214, miR-146b, and the miR-125 family) are differentially expressed with DAMPs delivered to human PBMCs.[329] We hypothesized that the pancreatic tumor micro- and macroenvironments will be associated with PBMC miRNA expression changes in patients and play an important role in regulating immunity.

The outline for the upcoming chapters is as follows. First, in Chapter 2, we identify miRNAs differentially expressed in pancreatic cancer patients' PBMCs compared to normal healthy individuals (age- and sex-matched controls). Then we compare our *in vivo* data with our previous *in vitro* data to see if *in vitro* DAMP-miRs are the same as *in vivo* DAMP-miRs. We then selected the miR that is commonly differentially expressed in our *in vivo* and *in vitro* data to further analyze miR expression and determine if the miR expression correlated with any clinical measurement and outcome (e.g. CA19.9A, survival) in Chapter 3. Finally, we examine how

immunological stimuli affect miR expression and validate the downstream target of miRs and the miR functional role in tumor growth in Chapter 4. Figure 1.10 depicts an overview of the work described in the upcoming chapters.

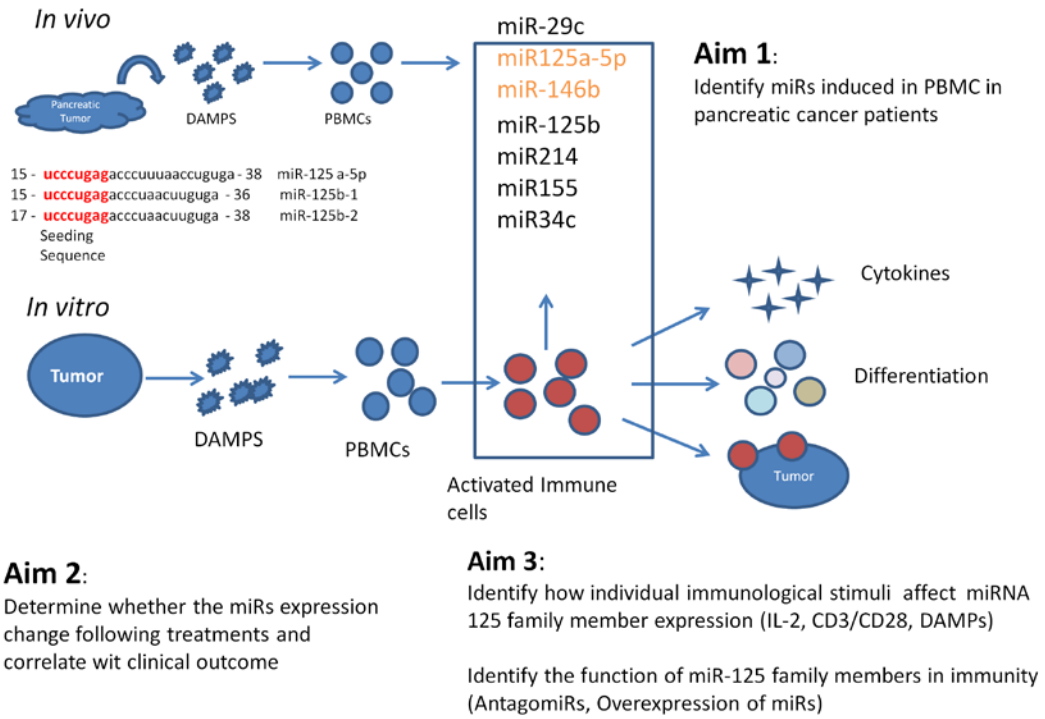


Figure 1-10: Overview of the Project

2.0 *IN VIVO* DAMPMIRS IN PANCREATIC CANCER PATIENTS' PBMC

2.1 CHAPTER OVERVIEW

Approximately 46,420 Americans are diagnosed with pancreatic cancer and 39,590 individuals die from the disease annually.[330] Pancreatic cancer is associated with a less than 5% five-year survival rate. Early diagnosis is rare and surgical treatment is most beneficial before the cancer becomes locally invasive or metastatic. There is a substantial unmet clinical need to develop early diagnostic reagents for identifying pancreatic cancer. Although CA19.9 is widely used to monitor therapy, it has proven to be detectable only late in disease and to be increased with pancreatitis.[7] Recently, miRNAs present within the tumor and blood have been found to be potential quantitative measures of tumor that may be identified earlier in disease. In this chapter, we discuss identifying potential *in vitro* DAMPmiRs from pancreatic cancer patients' PBMC.

2.2 RATIONALE

DAMP molecules (including HMBG1, S100, purine metabolites, heat shock protein, etc.) initiate and propagate the host immune response.[328] When tumors are stressed or undergo necrotic death, DAMPs are passively released into the local micro-environment and initiate early innate and adaptive immune responses via DAMP receptors.[2,3] DAMPs can also be released by activated immune cells via a noncanonical pathway. DAMPs induce cytokine production in immune cells, which is responsible for inflammation, tissue repair, and regeneration.[331] If those signals are uncontrolled, it may lead to cancer. HMBG1 can signal through RAGE and TLR2 and can increase neutrophil recruitment, increasing cytokine production in DCs.[332-334] HMGB1 is also actively released from macrophages.[335,336] S100 protein family member expression is increased in melanoma, non-small cell lung, gastric, breast, and pancreatic cancers, as well as lymphoma, and can recruit neutrophils and macrophages to tissue damage sites. Purine metabolites (including ATP, adenosine, and uric acid) in the extracellular space can also act as “danger” signals.[337] High ATP levels suppress immunity by blocking pro-inflammatory cytokine synthesis, while low levels enhance emigration and maturation of myeloid DCs, plasmacytoid DCs, macrophages, and natural killer cells.[338,339] Injured cells release uric acid, and at high concentrations (where it is high enough for precipitation), uric acid can activate DCs.[340]

In a previous publication, we demonstrated that some miRs (miR-34c, miR-214, the miR-125 family, and miR-146) are differentially expressed upon freeze-thaw lysate stimulation (DAMPs). We hypothesized that pancreatic cancer tumor will release DAMPs into the microenvironment, which would alter the microRNA expression in PBMCs.[4] This can be used to

distinguish between patient groups and healthy individuals. Some of the *in vitro* DAMPmiRs may also be altered in pancreatic cancer patient PBMC.

2.3 MATERIALS AND METHODS

2.3.1 Pancreatic Cancer Patient Sample Collection

We collected blood samples from 14 individuals (seven with pancreatic cancer and seven age- and sex-matched healthy individuals) and isolated their PBMCs as described previously.[4] In brief, each patient's blood sample was diluted with Roswell Park Memorial Institute medium (RPMI) (Thermo Scientific) culture media (1:1 ratio) and overlaid on Ficoll-Paque (GE Healthcare). The blood sample was then spun at 400g for 30 minutes. PBMCs were collected at the interface. PBMCs were transferred into a new tube and washed with RPMI. Red cell lysis buffer (0.15M ammonium chloride, 10mM potassium bicarbonate, 372mg sodium EDTA) was added to remove any remaining red blood cells in the PBMC. They were then washed with RPMI, spun down, and re-suspended in freezing media (10% DMSO, 90% fetal bovine serum [FBS]) at $1x10^7$ cells/ml and stored in the vapor phase of liquid nitrogen.

2.3.2 miRNA Isolation

Pancreatic cancer patients and healthy individuals' PBMC RNA was isolated using the miRNeasy mini Kit (Qiagen) and RNA integrity was checked using the Bioanalyzer 2100 (Agilent Technology). In brief, frozen pancreatic cancer patients' PBMC were thawed in a 37°C water bath and washed with RPMI. The PBMCs were then lysed by Qiazol for total RNA extraction (in no more than $3x10^6$ cells/ 700ul of Qiazol). Total RNA samples were then sent to the University of Pittsburgh Cancer Institute (UPCI) Genomic Core Facility for RNA integrity

(RIN) evaluation. Samples (with RIN higher than 8) were used for Taqman Low Density Array (TLDA) miRNA profiling.

2.3.3 miRNA Profiling

384-sample TaqMan low-density PCR arrays (Life Technologies) were used for microRNA expression profiling. In brief, 100ng of total RNA was reverse transcribed according to the instructions; then samples were diluted 62.5 fold and loaded onto 384-well TLDAs; the PCR program was used as the instructions recommended.

2.3.4 Statistical Analysis of miRNA Profiling Data

Raw Ct values from each sample were converted to RQ or 2^ΔΔCt values. In brief, ΔΔCt values were calculated from: (Ctpatient-Ctendog. Cont.) – (Ct healthy – Ctendog cont). The endogenous control was the small nucleolar RNA U48. Statistical analysis was performed using a Student T-test on the ΔΔCt value.

2.4 RESULTS

2.4.1 Three miRNAs are differentially expressed in patients' PBMC

To determine whether miRNA expression was differentially expressed in pancreatic cancer patients' PBMC, we compared miRNA expressions to those of age- and sex- matched healthy individuals. Total RNA was isolated and applied to TLDA array for microRNA profiling, and each array was designed to detect 384 specific human miRNAs. Relative quantification of miRNA expression was presented with respect to healthy individuals, and normalized to internal RNA control. Statistically significant miRNAs were defined by Student T-test with a p value of <0.05. From this analysis, three differentially-expressed miRNAs were revealed: namely, miR-125a-5p, miR-146b, and miR-29c. miR-146b and miR-29c were downregulated with an average fold change of 0.415 and 0.553, respectively. miR-125a-5p was upregulated with an average fold change of 2.007. (Figure 2.1)

2.4.2 *In vitro* DAMPmiRs were not expressed in patients' PBMC

We previously demonstrated that miR-34c and miR-214 are upregulated in PBMCs when treated with DAMPs (freeze-thaw lysate).[4] Surprisingly, miR-34c and miR-214 were expressed at only low levels in pancreatic cancer patients' PBMCs with CT>35. Furthermore, there was no statistically significant difference between the miR-34c and miR-214 of the pancreatic cancer

patients' group and the healthy individuals. Our *in vitro* control miR-155 was expressed in both the patient group and healthy individual group without apparent statistically significant difference. (Supplemental Figure 2.1)

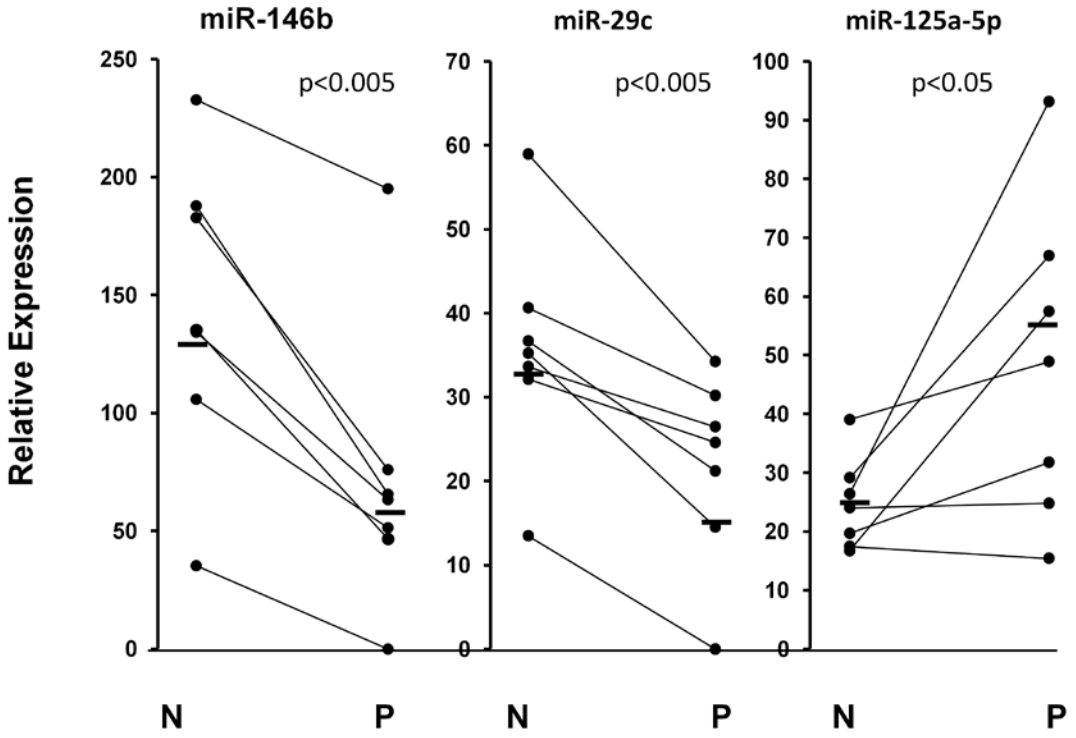
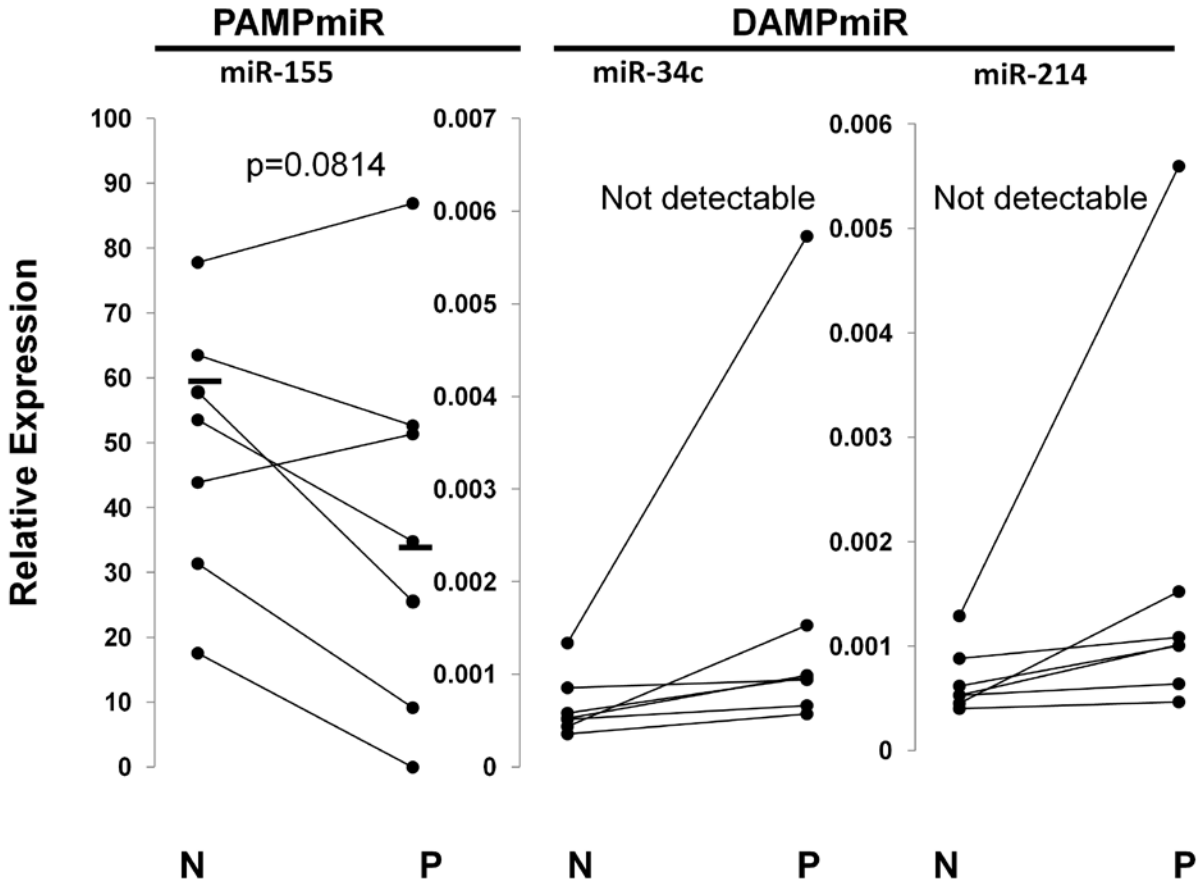


Figure 2-1: Three miRNAs are differentially expressed in patients' PBMC

Seven age- and sex-matched pancreatic cancer patients' PBMC(P) microRNA expressions were profiled with TaqMan Low Density microRNA microarray assay and compared with healthy individuals (N). Student paired T-test was performed and three microRNAs, miR-146b, miR-29c and miR-125a-5p, were found to be differentially expressed in pancreatic cancer patients' PBMC with an average fold change of 0.414 ($p<0.0012$), 0.553 ($p<0.0013$), and 2.006 ($p<0.0482$),respectively.



S. Figure 2-1: In vitro DAMPmiR and PAMPmiR expression in patients' PBMC

Seven age- and sex-matched pancreatic cancer patients' PBMC microRNA expressions were profiled with TaqMan Low Density microRNA microarray assay and compared with healthy individuals. Student paired T-test was performed previous identified DAMPmiRs (miR-34c, miR-214) were not expressed and PAMPmiRs expression (miR-155) was not significantly differences between two groups.

	Paired T-test
hsa-miR-146b-4373178	0.001175999
hsa-miR-29c-4373289	0.001283537
hsa-miR-31-4373190	0.033598107
hsa-miR-125b-4373148	0.044586009
hsa-miR-125a-4373149	0.048200037
hsa-miR-19b-4373098	0.067655481
hsa-miR-423-4373015	0.071202339
hsa-miR-342-4373040	0.073928298
hsa-miR-130a-4373145	0.07648661
hsa-miR-126-4378064	0.077731023
hsa-miR-221-4373077	0.078323332
hsa-miR-151-4373179	0.081144541
hsa-miR-155-4373124	0.081435667
hsa-miR-484-4381032	0.082376606
hsa-miR-27b-4373068	0.085844208
hsa-miR-486-4378096	0.0913668
hsa-miR-30e-5p-4373058	0.108685183
hsa-miR-27a-4373287	0.11058007
hsa-miR-29a-4373065	0.114729257

Normal		Pancreatic Cancer Patient	
Gender	**Age**	**Gender**	**Age**
M	69	M	69
M	59	M	60
M	73	M	75
M	79	M	79
F	72	F	71
F	76	F	76
F	61	F	60

S. Figure 2-2: Patient Information and TLDA array

2.5 DISCUSSION

Pancreatic cancer is associated with several defined genetic mutations or losses (e.g. TGF β/SMAD4 signaling pathway loss, KRAS and BRCA mutations, p53 loss or mutation, and $p16^{INK4A}$ alterations).[341] Pancreatic cancer is not only a genetic disorder but also a disease associated with profound changes in the host within stromal and endothelial cells as well as recruited inflammatory cells.[342-346] DAMPs including HMBG1, heat-shock protein, S100 molecules, ATP, DNA, and uric acid are released into the micro-environment when cancer cells die or under conditions of stress, acting as immmunostimulatory or immunomodulatory factors.[347-352] In our previous *in vitro* studies, we used freeze-thaw cell lysates to mimic DAMPs released from tumor cells and found that several miRNA genes, including miR-34c, miR-214, miR-210, miR-125, miR-146b, and miR-10b in human PBMC were involved in the inflammatory response to damaged cells. We identified additional miRNAs, including miR-125a-5p, miR-146b, and miR-29c, in human circulating PBMC that respond to the pancreatic cancer micro-environment. miR-125a-5p and miR-146b were also involved in the inflammatory response to damaged cell lysates. Surprisingly, expression of other previously identified miRNAs such as miR-34c, miR-214, miR-210, and miR-10b were low in PBMC and not statistically significant. Differences in response to the pancreatic cancer micro-environment might be observed only within the tumor or draining lymph nodes themselves and not in the peripheral blood.

The miR-125 family of miRs plays a crucial role in solid tumors, hematological malignancies, autoimmune disease, immune system development, and immunological host defense.[353] The miR-125 family exerts both tumor-suppressor and tumor-promoting functions depending upon the cell context.[218,262,263,354-365] miR-125 is down-regulated in ovarian, bladder,

and breast cancer, as well as, hepatocellular carcinoma, melanoma, cutaneous squamous cell carcinoma, and osteosarcoma. miR-125 inhibits lung cancer formation and hepatocellular carcinoma proliferation and metastasis and targets ETS1 (via the ERBB2/ERBB3 pathway), MUCI (to promote DNA damage-induced apoptosis), matrix metalloproteinase 11, vascular endothelial growth factor A, Mcl-1, and IL6R. In contrast to the above cancers, miR-125 is up-regulated in pancreatic, prostate, and oligo-dendroglial cancers, as well as others. miR-125 promotes breast cancer growth (through targeting STARD13), enhances invasive potential in urothelial carcinomas, and suppresses Bmf-dependent apoptosis in human glioblastoma multiform cells.[366] [367,368]

Besides modifying solid tumors, miR-125 also plays an important role in hematological malignancies, autoimmune disease, immune system development, and immunological host defense. Translocation of miR-125b is associated with B-cell acute lymphoblastic leukemia, myelodysplasia, and acute myeloid leukemia.[65,369,370] Over-expression of miR-125b is found in megakaryoblastic and acute promyelocytic leukemia.[371,372] miR-125a-5p is down-regulated in systemic lupus erythematosus (SLE). In that setting it negatively regulates the inflammatory response by targeting RANTES.[373] miR-125a-5p is up-regulated in ischemic stroke patients' macrophages following exposure to oxidized low density lipoprotein.[374] miR-125b is also associated with eosinophilic chronic rhinosinusitis, Alzheimer's disease, and Myotonic Dystrophy Type 2.[375,376] miR-125b regulates HSC homeostasis and increases HSC engraftment and self-renewal in experimental transplantation models.[377,378] miR-125b is up-regulated during B-lineage differentiation but is down-regulated during T cell differentiation, suggesting it targets BLIMP-1, IRF-4, and other T-cell differentiation genes.[379-381] miR-125a-5p is also down-regulated upon differentiation of hematopoietic stem cells (HSCs) and enhances HSC numbers,

possibly via targeting the pro-apoptotic gene BAK1.[377,382,383] Unlike miR-125b, which is crucial in naïve T-cells, miR-125a-5p plays a role in activating T-cells via targeting KLF13 to reduce RANTES expression.[373] Our results show that miR-125a-5p is up-regulated when treating PBMC with cell lysate *in vitro* and is also up-regulated in pancreatic cancer patients' PBMC *in vivo*. We hypothesized that pancreatic tumors release DAMPs into the micro-environment, causing the miRNA expression changes that we observed in patient PBMC.

miR-146b is involved in the inflammatory response and oncogenesis. It is up-regulated in inflammatory diseases (e.g. osteoarthritis and rheumatoid arthritis [RA]).[384-386] miR-146 plays a crucial role in the TLR-signaling feedback loop by targeting tumor necrosis factor receptor-associated family (TRAF) 6 and interlukein-1 receptor-associated kinase. miR-146 expression can be induced by LPS, TNF-α, and IL-1β stimulation.[387] miR-146 plays a role in Th1/Th2/naive T-cell balance.[388-390] miR-146 is up-regulated in autoimmune diseases including RA, with Th1 cells dominating over Th2 , while it is down-regulated in patients with systemic lupus erythematosus (SLE)[56]. Furthermore, low levels of miR-146 result in higher levels of IFN-γ and IFN-α implying a role in regulating interferon production in PBMC to fine-tune the immune response.[391] Our results show that miR-146b is expressed in healthy PBMC *in vitro* but surprisingly is down-regulated in pancreatic cancer patients' PBMC. Our *in vitro* findings demonstrate that healthy individual PBMC treated with freeze-thaw lysate release a higher level of TNFγ which in turn up-regulates miR-146 expression.[387] Furthermore, increase of IL-10 in pancreatic cancer patients' plasma may indirectly inhibit miR-146 expression.[392,393] It is also possible that the TLR-signaling pathway activating miR-146 transcription is mutated or blocked, causing an inappropriate chronic inflammatory response leading to cancer. Indeed, loss of miR-146 and miR-145 expressions causes inappropriate activation of TIRAP and TRAF6 innate

immune signaling pathways in myelodysplastic syndrome (one of the most common hematopoietic malignancies).[394]

The miR-29c family, including miR-29a, miR-29b, and miR-29c, is associated with cell differentiation, senescence, proliferation, apoptosis, epigenetic modulation, and immune regulation. The miR-29 family is negatively regulated by c-myc, hedgehog signaling, NFκB, and Yin Yang 1 (YY1). They are up-regulated by p53 and Wnt signaling, forming a feedback loop with TGFβ.[395-402] The miR-29 family is down-regulated in various tumors, including chronic lymphocytic leukemia, endometrial carcino-sarcoma, lung cancer, breast cancer, cervical carcinomas, melanoma, nasopharyngeal carcinomas, hepatocellular carcinoma, and mantle cell lymphoma.[162,403-409] The miR-29 family negatively regulates cell proliferation, DNA synthesis, senescence, and differentiation by targeting CDK6, Ppm1d phosphatase, B-Myb, HDAC4, TGF-β3, CVR2A andYY1.[398,402,406,410-412] miR-29 inhibits apoptosis by targeting Mcl-1, Tcl-1, p85a, and CDC42, while positively regulating pro-apoptotic genes like BIM and PDCD4.[408,410,413-415] miR29 regulates metastasis by targeting the extracellular matrix protein encoding genes and the epithelial-mesenchymal transition (EMT)-related TGFβ-signaling pathway when oncogenic Ras signaling is not involved.[404,407,416] miR-29 regulates epigenetics by targeting DNA methyltransferase and methylation-related genes (e.g. Sp1, $p15^{INK4b}$, and ESR1) while upregulating demethylating genes.[405,410,412,417-421] miR-29 can upregulate tumor suppressor genes including fragile histidine triad protein (FHIT) and WW domain-containing oxidoreductase (WWOX) by reducing the methylation of their promoter region. miR-29 can reduce IFN-□-production (by targeting T-bet and Eomes), enhance natural killer cells' effect (via targeting B7-H3), and alter helper T-cell differentiation, thus interfering with the immune response. Knocking down miR-29 in mice leads to a more potent Th1 response and higher IFN-γ-producing cell

numbers.[422-424] Here we show that miR-29 is down-regulated in pancreatic cancer patients' PBMC but does not change when stimulated by DAMPs (freeze-thaw lysate). The NFκB signaling pathway was not activated in PBMCs following freeze-thaw lysate stimulation, nor were other pathways regulated by miR-29, e.g. hedgehog signaling, CEBPA, c-myc, p53, and TGFβ. These targets were not present in freeze-thaw lysate. Interestingsly, IL-6, IL-1b, IL-10, and IL-13 are increased in pancreatic cancer patients' plasma. Down-regulation of miR-29 correlates with an increase of IL-6 and TNF-α production in patient DCs[392,425] suggesting that miR-29 down-regulation in pancreatic patients' PBMCs may be secondary to changes in cytokine levels.

Previously, we identified miR-34c and miR-214 as *in vitro* DAMPmiRs. They are not expressed at a detectable level in pancreatic cancer patients' circulating PBMC. It is possible that tumor infiltrating lymphocytes in tumor might be better at demonstrating these changes than circulating PBMCs. Therefore, it will be interesting to compare the expression profiles of infiltrated lymphocytes and circulating PBMC in the future. Furthermore, the PBMCs we used to identify the *in vitro* DAMPmiRs came from healthy donors and pancreatic cancer patients' PBMC may be impaired.

Since the miR-125 family plays an important role in solid tumors, hematopoietic development, and the immune response, it is possible that some factors released from pancreatic tumor modulate miR-125a-p expression in patients' circulating PBMC. Because miR-125a-5p is up-regulated in both *in vivo* and *in vitro* samples, we chose miR-125a-5p for further analysis.

3.0 *IN VIVO* DAMPMIR CHANGES IN RESPONSE TO TREATMENT

3.1 CHAPTER OVERVIEW

In the previous chapter, we identified three microRNAs (miR-125a-5p, miR-146b, and miR-29c) that are differentially expressed in pancreatic cancer patients' PBMC compared to age- and sex-matched healthy individuals. We also compared the *in vivo* DAMPmiRs to our *in vitro* data and found that only miR-125a-5p and miR-146b are differentially expressed both *in vitro* and *in vivo*. However, how those microRNA expressions change in response to treatment remains unknown. In this chapter, we examine how miR-125a-5p expression changed following chemotherapy and surgery. We also examine whether there is any correlation between miR-125a-5p expression and measurement (e.g. CA19.9, HMGB1 release, LC3) or clinical outcome (survival). The purpose of this chapter is to evaluate the potential of miR-125a-5p as a prognostic, diagnostic, and predictive marker.

3.2 RATIONALE

Approximately 43,140 Americans are diagnosed with pancreatic cancer and 36,800 individuals die from the disease annually.[330] Five-year survival for pancreatic cancer is low (<5%) because of late-stage diagnosis, lack of effective treatment protocols, early recurrence, and lack of clinically useful biomarker(s) for early diagnosis.[426] Although CA19.9 is the most acceptable biomarker for pancreatic cancer, it has limitations such as poor specificity, lack of expression in the Lewis-negative population, and a high false positive rate for patients with obstructive jaundice.[95] A biomarker for early diagnosis, prognosis, and predictive treatment outcome will help improve the survival rate of pancreatic cancer. Over the last two decades, many novel pancreatic cancer biomarkers have been discovered, such as pancreatic juice biomarkers, salivary biomarkers, stool biomarkers, serum biomarkers (growth factor, immunoglobulin, secreted proteins, antigen), and genetic markers (DNA, mRNA, and miRNA), which have shown promising or even superior results compared to CA 19-9 (by using them alone or combination with CA19.9 or CEA).[427] However, further validation for reproducibility and clinical utility is required before using these biomarkers to manage pancreatic cancer. In chapter 2, we identified three microRNAs that are differentially expressed in pancreatic cancer patients' PBMCs. In chapter 3, we will discuss the expression change in miR-125a-5p following chemotherapy and surgery and determine whether there is any correlation between miR-125a-5p expression, CA19.9 level, and clinical outcome.

3.3 MATERIALS AND METHODS

3.3.1 Pancreatic Cancer Patients' Samples Collection

We collected blood samples at individual time points from 14 pancreatic cancer patients undergoing neoadjuvant chemotherapy on UPCI #09-122, approved by the University of Pittsburgh IRB. Samples were initially collected on day 29 following two cycles of chemotherapy and then again one month following surgical extirpation. PBMC were isolated as described in our previous publication.[4] In brief, each patient's blood sample was diluted with Roswell Park Memorial Institute medium (RPMI) (Thermo Scientific) culture media (1:1 ratio) and overlaid on Ficoll-Paque (GE Healthcare). The blood sample was then spun at 400g for 30 minutes. PBMC were collected at the interface. PBMCs were transferred into a new tube and washed with RPMI. Red cell lysis buffer (0.15M ammonium chloride, 10mM potassium bicarbonate, 372mg sodium EDTA) was added to remove any remaining red blood cells in the PBMC. They were then washed with RPMI, spun down, and re-suspended in freezing media (10% DMSO, 90% fetal bovine serum [FBS]) at $1x10^7$ cells/ml and stored in the vapor phase of liquid nitrogen.

3.3.2 miRNA Isolation

Pancreatic cancer patients' PBMC RNA was isolated with miRNeasy mini Kit (Qiagen) and RIN was checked using a Bioanalyzer 2100 (Agilent Technology). In brief, frozen pancreatic cancer patients' PBMC were thawed in a 37°C water bath and washed with RPMI. The PBMCs were then lysed by Qiazol for total RNA extraction (in no more than $3x10^6$ cells/ 700ul of Qiazol).

Total RNA samples were then sent to the University of Pittsburgh Cancer Institute (UPCI) Genomic Core Facility for RNA integrity (RIN) evaluation. Samples (with RIN higher than 8) were used for Taqman Low Density Array (TLDA) miRNA profiling.

3.3.3 miRNA Expression Assay

TaqMan MicroRNA Array (Life Technologies) was used for miRNA expression profiling. In brief, 20ng of total RNA was reverse transcribed according to the instructions, then samples qt-PCR with specific Taqman primer and run in StepOne (Life Technologies). Real-Time PCR was carried out with the recommended protocol.

3.3.4 CA19.9, Survival, LC3, HMGB1 ELISA

Serum CA19.9 (measured by UPMC Patient Care Service with a Chemiluminescence Immunoassay) and HMGB1 levels (IBL International Corp) and PBMC LC3 puncta using an ArrayScan II (Themo Scientific). Survival data were collected from patients enrolled on clinical trial UPCI # 09-122 in our laboratory, approved by the University of Pittsburgh IRB.

3.3.5 Statistical Analysis of miRNA Profiling Data

Raw Ct values from each sample were converted to RQ or 2^ΔΔCt values. In brief, ΔΔCt values were calculated from: (Ctpatient-Ctendog. Cont.) – (Ct healthy – Ctendog cont). The endogenous control was the small nucleolar RNA U48. Statistical analysis was performed using a Student T-test on the ΔΔCt value.

3.4 RESULTS

3.4.1 miR-125a-5p Expression is Downregulated after Chemotherapy

There was significant downregulation in miR-125a-5p expression in pancreatic cancer patients' PBMCs after receiving chemotherapy. (Figure 3.1) The average fold change was 0.8177 ($p<0.05$) However, miR-125a-5p expression was upregulated significantly following surgical excision, demonstrating the dynamism of this marker in the peripheral blood mononuclear cells. The average fold change was 1.814 ($p<0.05$). We were able to collect PBMC samples from two of the patients three months following surgical excision; their miR-125a-5p expression was again downregulated three months after their surgical procedurewhen patients continue to receive chemotherapy, but more samples are needed to validate this result.

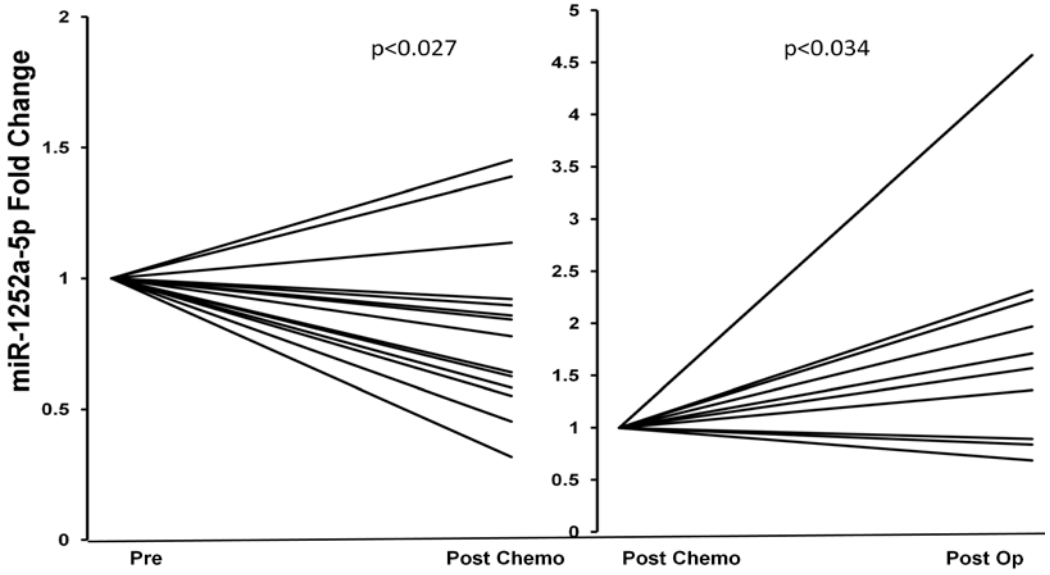
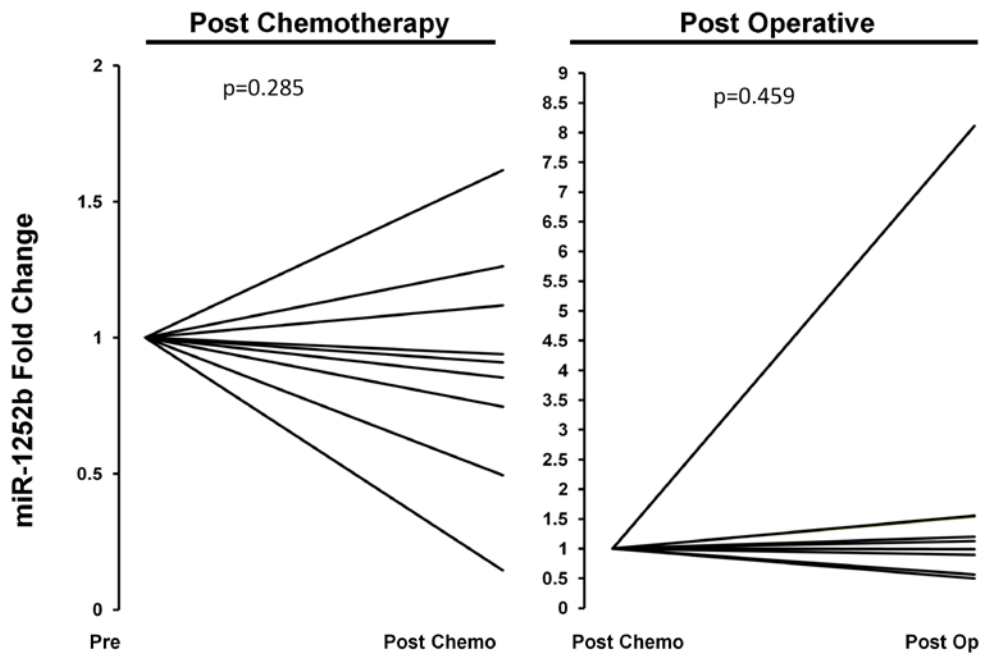


Figure 3-1: Dynamic Changes in miRNA following Neoadjuvant Treatment

Patients who are diagnosed with early resectable pancreatic cancer received two doses of gemcitabine chemotherapy (on day 1 and day 15) along with daily oral hydroxychloroquine, undergoing surgical excision on day 29. Blood samples were drawn before any treatment (day 0, day 29 prior to surgery, and 30 days after surgery). miR-125a-5p expression was downregulated after patients received chemotherapy (average fold change 0.817, $p<0.027$) and upregulated following surgical excision (average 1.814, $p<0.034$)

3.4.2 miR-125b Expression Did not Correlate Directly with Treatment

No significant differences were found in miR-125b (a closely-related family member of miR-125a-5p) following treatment (neither chemotherapy nor surgery). (Supplementary Figure 3.1)



S. Figure 3-1: miR-125b Did Not Change Significantly Following Treatments

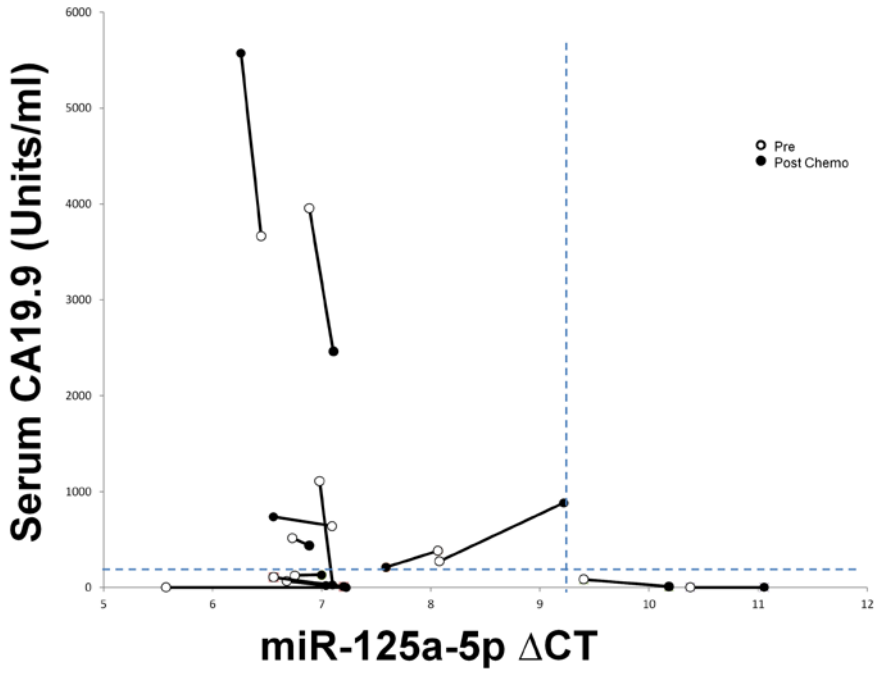
Patients who are diagnosed with early resectable pancreatic cancer received two doses of gemcitabine chemotherapy (on day 1 and day 15) along with daily oral hydroxychloroquine, undergoing surgical excision on day 29. Blood samples were drawn before any treatment (day 0, day 29 prior to surgery, and 30 days after surgery). miR-125b (a closely-related family member of miR-125a-5p) did not change significantly following chemotherapy or surgery.

3.4.3 Correlation Between miR-125a-5p and Other Putative Markers

Serum HMBG1 levels were low (with one exception, patient 11) and did not change significantly after chemotherapy ($p>0.05$), although most patients' serum HMGB1 levels fell following chemotherapy (13/16). Basal miR-125a-5p before chemotherapy or serum HMBG1 level did not correlate directly with survival. When excluding one outlier, there was a significant difference between serum HMBG1 levels following chemotherapy; a bigger sample size is needed to confirm this observation (pre-treatment average 9.26ng/ml, post-chemotherapy average 5.26ng/ml, $p<0.005$) and we are now performing a randomized prospective clinical trial to examine this. No correlation was found between changes in the serum HMBG1 level, miR-125a-

5p expression, and serum CA19.9 level, although all decreased. (Figure 3.2 and Supplementary Figure 3.2)

a)



b)

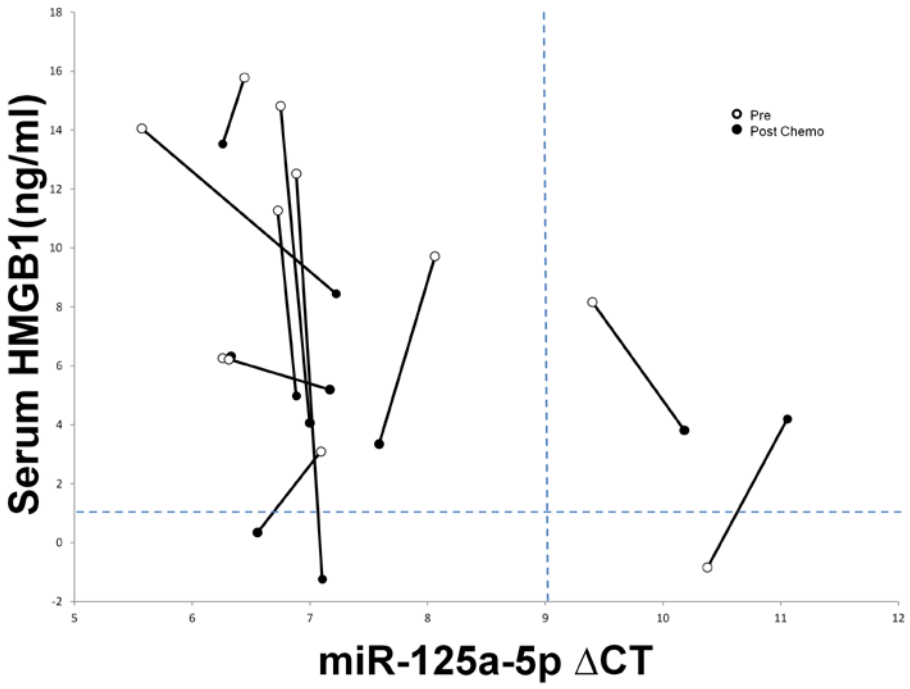
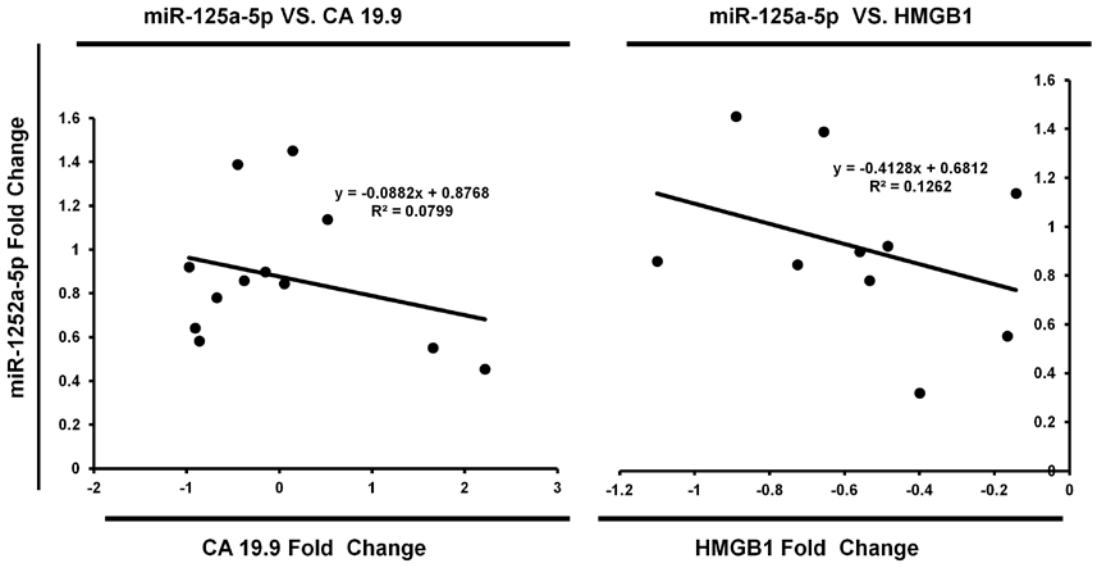


Figure 3-2: Correlation of miR-125a-5p with Other Putative Biomarkers

Serum CA19.9 and b) HMGB1 level was measured by ELISA and compared with the miR-125a-5p expression level for each patient. Fisher's Exact Test was performed using GraphPad 2X2 contingency table and there was no correlation between miR-125a-5p expression level and serum CA19.9 or serum HMGB1 level. The lower limits of normal are <1.4ng/ml and the reference range of serum CA 19-9 is less than 37 U/mL. Shown for miRNA to the dotted line to the left are 'detectable and increasing levels of miRNA (with smaller ΔCT values) and above the dotted line for detectable levels of CA 19-9 and HMGB1 respectively; open circles prior to therapy and closed circles, following.



S. Figure 3-2: Correlation between Changes in miR-125a-5p and Putative Markers

Patients' serum CA19.9 and HMGB1 level was measured by chemiluminescence immunoassay and ELISA respectively and compared with the miR-125a-5p expression of all patients. There was no apparent correlation between miR-125a-5p expression and serum CA19.9/HMGB1 levels.

3.4.4 Correlation between Survival and miR-125a-5p

We analyzed miR-125a-5p expression in PBMCs and serum HMBG1 level results to determine whether there was a correlation between miR-125a-5p and survival. There was no direct correlation between miR-125a-5p expression changes or serum HMBG1 level changes in disease-free survival (DFS) or overall survival. However, decreases in serum CA19.9 (the current gold standard for pancreatic cancer management as a marker) showed a correlation with both overall and disease-free survival. (Figure 3.3 and Supplementary Figure 3.3) The CA19.9 responders (serum CA19.9 level decreased by more than 50%) had longer median survival (overall survival 20.0225 months, DFS 17.67 months) than the CA19.9 non-responders (overall survival 9.82 months, DFS 5.74 months) ($p<0.05$ for both overall survival and DFS).

3.4.5 PBMC LC3 Puncta did not correlate with miR-125a-5p Expression

We also measured patients' PBMCs LC3 puncta (measurement of autophagy) to determine if it correlated with better survival or miR-125a-5p expression level. There was no correlation between miR-125a-5p expression change and autophagy or survival. (Supplementary Figure 3.4)

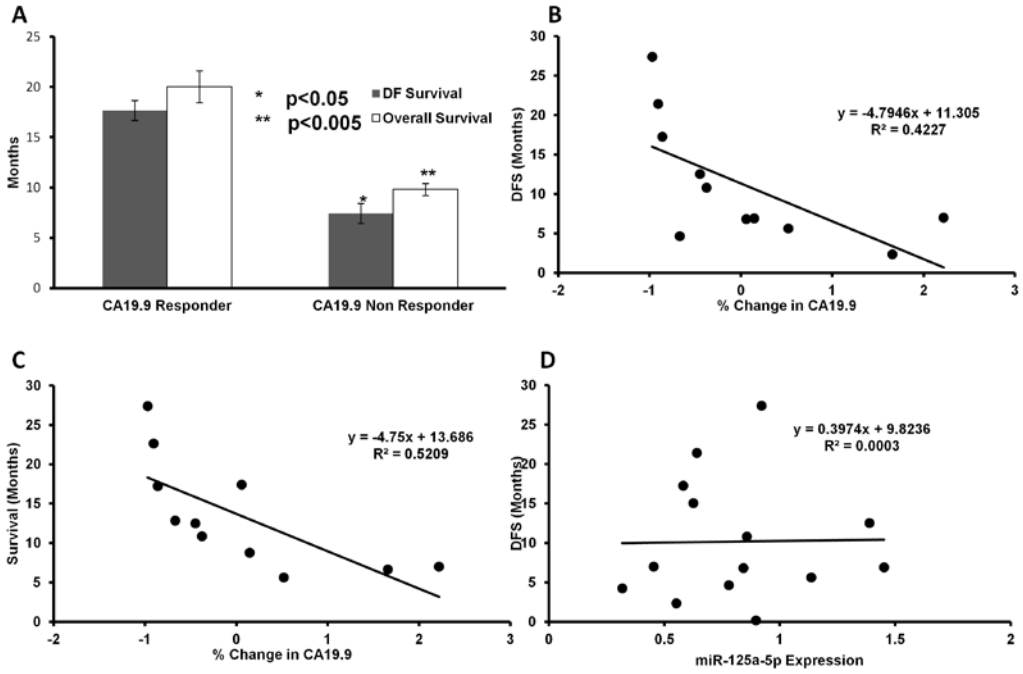
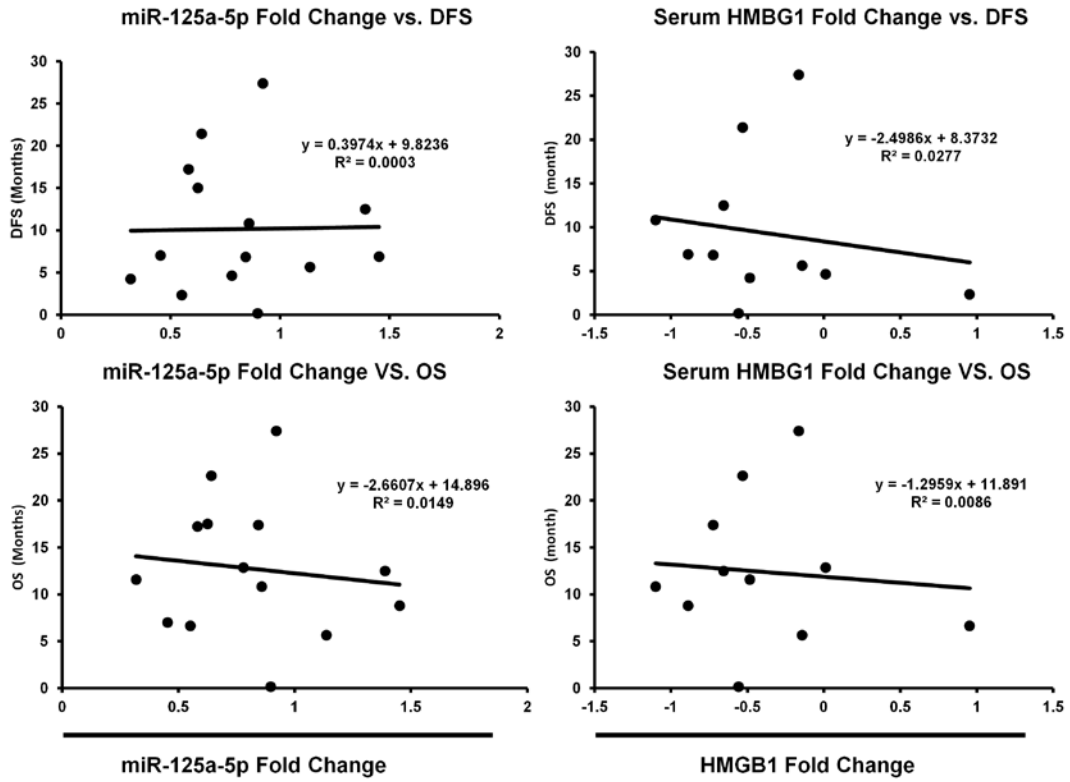


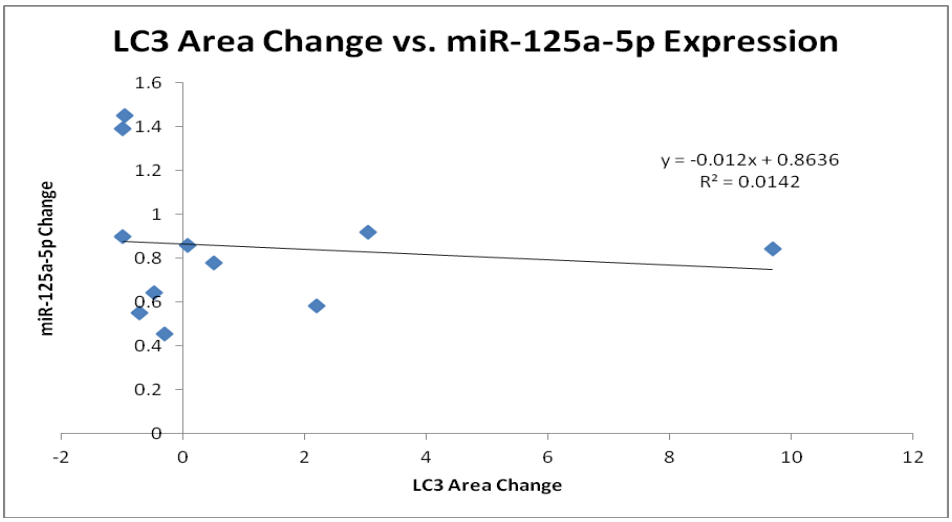
Figure 3-3: miR-125a-5p did not correlate with Survival

CA19.9 responder is defined by a decrease of serum CA19.9 of more than 50% following chemotherapy. A) The median DFS for the CA19.9 responder group was 15.66 months and 5.74 months for the non-responder group. The median OS for the CA19.9 responder group was 20.02 months and 9.82 months for the non-responder group. B) and C) shows the linear regression of CA19.9 change vs. DSF and OS. D) There was no correlation between miR-125a-5p expression change after chemotherapy and survival.



S. Figure 3-3: miR-125a-5p and Serum HMGB1 did not correlate with DSF or OS

Changes in miR-125a-5p expression (A and B) or change in serum HMGB1(C and D) after chemotherapy did not correlate with disease-free survival or overall survival with linear regression



S. Figure 3-4: Change in miR-125a-5p expression did not correlate with LC3 puncta

Change in miR-125a-5p expression did not correlate with change in LC3 puncta after chemotherapy with linear regression.

3.5 DISCUSSION

In chapter 2, we identified three *in vivo* DAMPmiRs (miR-125a-5p, miR-146b, and miR-29c) that are differentially expressed in pancreatic cancer patient's PBMCs. In this chapter we examined how miR-125a-5p expression changed in patients' PBMCs after treatments (chemotherapy and surgery) and determined whether the expression change correlated with any clinical outcome (CA19.9 response and survival), serum HMBG1 release, and autophagy.

We found that miR-125a-5p expression in PBMCs was down-regulated after pancreatic cancer patients received chemotherapy and autophagy inhibition with hydroxychloroquine and up-regulated immediately following surgical excision. The initial down-regulation of miR-125a-5p could have been due to the response to chemotherapy, reduction in tumor size following chemotherapy, reduction of autophagy, or activation of an effective immune response. Serum CA19.9 levels (pre-treatment and post-treatment) is a developing prognostic biomarker for pancreatic cancer. Our results from this study and other studies from our research group show that the CA19.9 response to chemotherapy (reduction in serum CA19.9 after treatment) is associated with improved survival.[117-121] Serum CA19.9 level is accepted as a measure of pancreatic cancer tumor burden. The down-regulation of miR-125a-5p did not correlate with serum CA19.9 level. Although serum CA19.9 increased in some of our patients' samples following chemotherapy, implying their tumor burden increased after the treatment this does not necessary indicate that the short course of chemotherapy completely failed to activate the immune system or induce some tumor cell death. In addition, this was relatively short term treatment. We believe it is likely that chemotherapy-induced cell death releases both CA19.9 as well as DAMPs into the circulation. The DAMPs released from apoptotic cells, unlike necrotic cells, may be immuno-tolerizing and not capable of up-regulating miR-125a-5p expression.

Therefore, measuring the percentage of apoptotic cells and necrotic cells before and after chemotherapy following surgical excision could be useful. Some chemotherapeutic agents induce immune activation. Indeed, gemcitabine can induce naïve T-cell activation.[428,429] miR-125b (one or the miR-125 family) is downregulated in active T-cells.[381,429]

We previously showed that miR-125a-5p is up-regulated by DAMP simulation *in vitro* and is up-regulated in pancreatic cancer patients (compared to healthy individual) *in vivo*. We measured serum HMBG1 levels pre- and post- chemotherapy and the change in serum HMGB1 release did not correlate with miR-125a-5p expression change. Since HMGB1 was not the only DAMP in our *in vitro* freeze-thaw lysate and in patients' samples, miR-125a-5p expression level may correlate with other DAMPs, e.g. ATP, uric acid, chaperones, mitochondrial components, nucleic acids, and nucleosomes. Therefore, measuring the serum level of other DAMPs might give us a better picture of whether factors released from tumor regulate miR-125a-5p expression level.

We showed that miR-125a-5p is up-regulated by DAMP simulation *in vitro* and is up-regulated in pancreatic cancer patients (when compared with healthy individuals) *in vivo*. We measured serum HMBG1 levels pre- and post- chemotherapy and the change in serum HMGB1 release did not directly correlate with miR-125a-5p expression changes. Since HMGB1 was not the only DAMP in our *in vitro* freeze-thaw lysate and in patients' samples, miR-125a-5p expression level may correlate with other DAMPs alone or in combination with HMGB1. These would include ATP, uric acid, chaperones, mitochondrial components, nucleic acids, and nucleosomes. Therefore, measuring the serum level of other DAMPs might give us a better picture of whether factors released from tumor regulate miR-125a-5p expression level.

We hypothesized that tumor burden should initially decrease following surgery and that miR-125a-5p should be further down-regulated following surgical excision. However, surprisingly, miR-125a-5p was up-regulated immediately following surgical extirpation (within one month). This could possibly be due to postoperative wound healing or immune alterations, indeed, postoperative immune-suppression and soluble IL-2R level decreases have been observed following operative procedures in other studies.[430] Those signals may temporarily dominate miR-125a-5p regulation. Our ongoing investigations shows a trend: miR-125a-5p is further decreased (three months after surgery) when the patient continues chemotherapy following operation (data not shown).

MiR-125a-5p is down-regulated after chemotherapy in pancreatic cancer patients' PBMCs. However, miR-125a-5p expression does not correlate with serum CA19.9 or survival. Therefore, further validation (e.g. increase sample size) is needed to determine whether miR-125a-5p may serve as a prognostic or predictive marker for pancreatic cancer management or not. In chapter 4, we will identify immunological stimuli that can regulate miR-125a-5p expression and examine the role of miR-125a-5p in immunity.

4.0 EVALUATE THE FUNCTION OF MIR-125A-5P

4.1 CHAPTER OVERVIEW

In previous chapters, we identified three microRNAs (miR-125a-5p, miR-146b, and miR-29c) that are differentially expressed in pancreatic cancer patients' PBMCs compared to healthy individuals and observed that miR-125a-5p expression is downregulated following chemotherapy but upregulated following surgery. We found that miR-125a-5p expression does not correlate with serum CA19.9 level (tumor burden indicator, chemotherapy response marker), serum HMBG1 (DAMPs), LC3 puncta (autophagy in PBMCs), or survival (prognostic measurement). In this chapter, we examine how immunological stimuli affect miR-125a-5p expression and its downstream targets mRNA and protein expression. We also examine if miR-125a-5p plays a role in proliferation and anti-tumor effects.

4.2 RATIONALE

In chapter 3, we observed that miR-125a-5p is downregulated following chemotherapy, but the decrease did not correlate with decrease in tumor burden (serum CA19.9 level), decrease in DAMPs (HMGB1), or improve prognosis (improve median disease-free survival and overall survival). Following surgery, when tumor burden supposedly decreases significantly compared to post-chemotherapy, miR-125a-5p expression is upregulated. In a previous study, we used cell lysate generated from mouse embryonic fibroblasts as DAMPs and found that miR-125a-5p expression did not correlate with serum HMGB1 level in pancreatic cancer patients following chemotherapy. It is possible that DAMPs released from pancreatic tumor cells have different effects on PBMCs or some other factor can also regulate miR-125a-5p expression. Studies have shown that gemcitabine can activate naïve T-cells and post-surgery stress can alter cytokines (e.g. soluble IL-2). Therefore, in this chapter we examine how IL-2, CD3/CD28 activation, and tumor cell lysate alter miR-125a-5p expression. We also validate the potential downstream miR-125a-5p targets IL-2RB, IL-10RA, and IL-6R. Finally, we evaluate if miR-125a-5p plays a role in PBMC proliferation and anti-tumor effects.

4.3 MATERIALS AND METHODS

4.3.1 PBMCs Isolation

Buffy coats were obtained from the UPMC blood bank and diluted with RPMI (Thermo Scientific) culture media (1:1 ratio) and and overlaid on Ficoll-Paque (GE Healthcare). The blood sample was then spun at 400g for 30 minutes. PBMCs were collected at the interface. PBMCs were transferred into a new tube and washed with RPMI. Red cell lysis buffer (0.15M ammonium chloride, 10mM potassium bicarbonate, 372mg sodium EDTA) was added to remove any remaining red blood cells in the PBMC. Then the PBMCs were washed with RPMI and spun down and resuspended in RPMI complete media (RPMI, 10%FCS and 1% pen-strep) in 1X10^6 cells/ml for cell culture.

4.3.2 Preparation of Tumor Cell Lsyate

Sub-confluent Panc 2.0.3 cells were re-suspended in ice-cold 1X PBS, with 1 mM PMSF and centrifuged at 10,000g for 1 min at 4^{o} C. The cells were re-suspended in cold non-denaturing lysis buffer (600 mM KC1, 20mM Tris-CI, pH 7.8 and 20% (v/v) Glycerol) at a concentration of about 100X10^{6} cells/1.3ml of lysis buffer, supplemented with 1 mM PMSF(Invitrogen, Inc), protease inhibitors cocktails (Invitrogen, Inc) and 1 mM dithiotreitol (Invitrogen, Inc). The sample was dropped into liquid nitrogen until completely frozen and placed on ice to thaw slowly. When thawed, the sample was briefly vortexed at maximum speed. This was repeated three times. At this point, all cells were nonviable as determined by Trypan blue staining. The cell suspension was centrifuged at 10,000 g at 4^{o} C for 10 minutes to pellet debris, and

supernatants aliquoted into small volumes and frozen at -80^{o} C. For an equivalent of 3X10^{5} cells/ml of cell lysates in culture, about 7 ul of the supernatant was added to 2 ml of PBMCs culture.

4.3.3 Cell Culture and Stimulation

PBMC were cultured in RPMI completed medium (RPMI, 10% FCS (Gemini Bioproduct Inc) 1% pen-strep (Invitrogen Inc) at 37^{o} C, 5% CO2. Cells were treated with 6000UI/ml of recombinant IL-2 (Prometheus Inc.), 1:1 ratio of antiCD3/CD28 Dynabeads (Life Technology), or 7ul per 2 ml of tumor cell lysate for 48 hrs. In separate experiments, PBMC were stimulated with IL-2, or tumor cell lysate for 24hrs, 48hrs and 72hrs.

Lenti-virus Transfection

miR-125a-5p lentivirus and control lentivirus was generated and purchased from UPCI Lentiviral Core Facility. THP-1 and Jurkat cells are a kind gift from Dr. Hideho Okada's laboratory at the University of Pittsburgh. THP-1 is transfected with MOI 10:1 viral particle and Jurkat cell with MOI 1:1 viral particle at 32^{o} C, 5% CO2 overnight. We then pelleted the cells and removed media the following day and cultured the cells at 37^{o} C for 7 days. GFP positive cells were sorted on day 8 and continued in culture as transfected THP -1 and Jurkat cells

4.3.4 RNA Isolation

PBMCs and transfected THP-1 or Jurkat cells' RNA were isolated with a miRNeasy mini Kit (Qiagen) and RNA integrity was checked using a Bioanalyzer 2100 (Agilent Technology). In brief, frozen pancreatic cancer patients' PBMC were thawed in a 37°C water bath and washed

with RPMI. The PBMCs were then lysed by Qiazol for total RNA extraction (in no more than $3x10^6$ cells/ 700ul of Qiazol). Total RNA samples were then sent to the University of Pittsburgh Cancer Institute (UPCI) Genomic Core Facility for RNA integrity (RIN) evaluation.

4.3.5 miRNA and gene expression Taqman Assay

From the returned results we selected IL-2RB, IL-6R and IL-10RA for additional investigation. Taqman MiRNA Array (miR-125a-5p, RNU48) and Gene Expression (IL-2RB, IL-10RA, IL-6R, ERBB2, alpha-actin) assays (Life Technologies) were used for analysis. In brief, 20ng of total RNA was reverse transcribed according to instructions for miRNA array and 100ng of total RNA was reverse transcribed according to instruction for the gene expression array. Samples underwent qt-PCR with specific taqman primers and run on the StepOne (Life Technologies Inc) Real-Time PCR system with the recommended protocols.

Conditioned Median Preparation

0.3X10^6 of THP-1 cells (miR-125a-5p over-expressing or control vector) and 0.5X10^6 Jurkat cells (miR-125a-5p over-expressing or control vector) were cultured in T-75 culture flask in RPMI complete media for two days. The conditioned media was then stored in aliquots at -80 °C until use.

4.3.6 Western blotting

Downstream targets of miR-125a-5p proteins expression level were evaluated by western blotting. Samples were lysed in lysis buffer (20 nM Tris base, 150mM NaCl, 1 mMEDTA, 1 mM

EGTA, 2 mM Na_3VO_4, 1% NP-40, 10% Glycerol, pH 7.4) and probed for human IL-2Rb, IL-10RA with specific antibodies (Sigma-Aldrich).

4.3.7 Cell Cycle

Transfected THP-1 and Jurkat cells were centrifuged at 800 rpm for five minutes and supernatant was then removed. Samples were fixed with 75% ethanol for four hours. Then samples were centrifuged at 1500 rpm and the cells were re-suspended in 400 ul of PBS (w/ 20ul of 1X RNase A) at 37°C for 30 minutes. 50ug//ml propidium iodide were added to the samples and kept at 4°C for two hours. Samples were then run and analyzed with Accuri C6 and its software package.

4.3.8 CCK8 Proliferation

Panc 2.0.3 cells were seeded in a 96-well plate (5000 cells/well, at 37°C, 5% CO2) for 24 hours with normal RPMI complete media, RPMI complete media with LPS (100ng/ml), RPMI complete media with ionomycin (1ug/ml) +PMA (10nM), or conditioned media for 24 hours. 10 ul of CCK-8 solution (Dojindo Molecular Technologies, MD) was added to each well of the plate. The plate was incubated for two hours and the absorbance was measured at 450 nm using a micro plate reader.

4.3.9 Potential target screening

The miR-Ontology Database from Ferro Lab (http://ferrolab.dmi.unict.it) was used to screen for potential downstream targets of miR-125a-5p. The database used targetScan, miRanda, and

Pictar for analysis. The database sorted the results into three categories (associated broad phenotypes, associated functions, and associate processes). From the returned results, we selected IL-2RB, IL-6R, and IL-10RA for future investigation.

4.3.10 Statistical analysis of microRNA profiling data

The raw Ct values from each sample were converted to RQ or 2^ΔΔCt values. In brief, ΔΔCt values were calculated from: (Ctpatient-Ctendog. Cont.) – (Ct healthy – Ctendog cont). The endogenous control was the small nucleolar RNA U48. Statistical analysis was performed using Student T-test on ΔΔCt value.

4.4 RESULTS

4.4.1 MiR-125a-5p expression altered upon immunological stimulation

To determine whether cytokines, T-cell activation/maturing signals, or DAMPs derived from pancreatic cancer cells were associated with miR-125a-5p expression changes in PBMC, healthy donor PBMC from several individuals were isolated from buffy coat and stimulated with IL-2, CD3/CD28 ligation, tumor cell lysate, or placed solely in culture media for 48 hours. Expression of miR-125a-5p was downregulated when cells were treated with IL-2 and anti-CD3/CD28, but was upregulated when treated with Panc 2.03 tumor cell lysate (Figure 4.1). miR-125a-5p expression level remained upregulated at 72 hours without any additional tumor cell lysate, while miR-125a-5p expression gradually returned to a normal level at 72 hours without additional anti-CD3/CD28 (Supplementary Figure 4.1).

4.4.2 DAMPs Increase miR-125a-5p Expression Level with Dose

To determine whether miR-125a-5p expression changes induced by DAMPs is dose-dependent, PBMC were treated with increasing doses of DAMPs (untreated, 1X, 2X). Expression of miR-125-a-5p increased more as tumor cell lysate quantity increased. The mRNA expression levels of the potential downstream targets IL-2RB, IL6R, and IL10RA were repressed as the miR-125a-5p expression level increased. ERBB2 is a validated downstream target of miR-125a-5p (Figure 4.2).

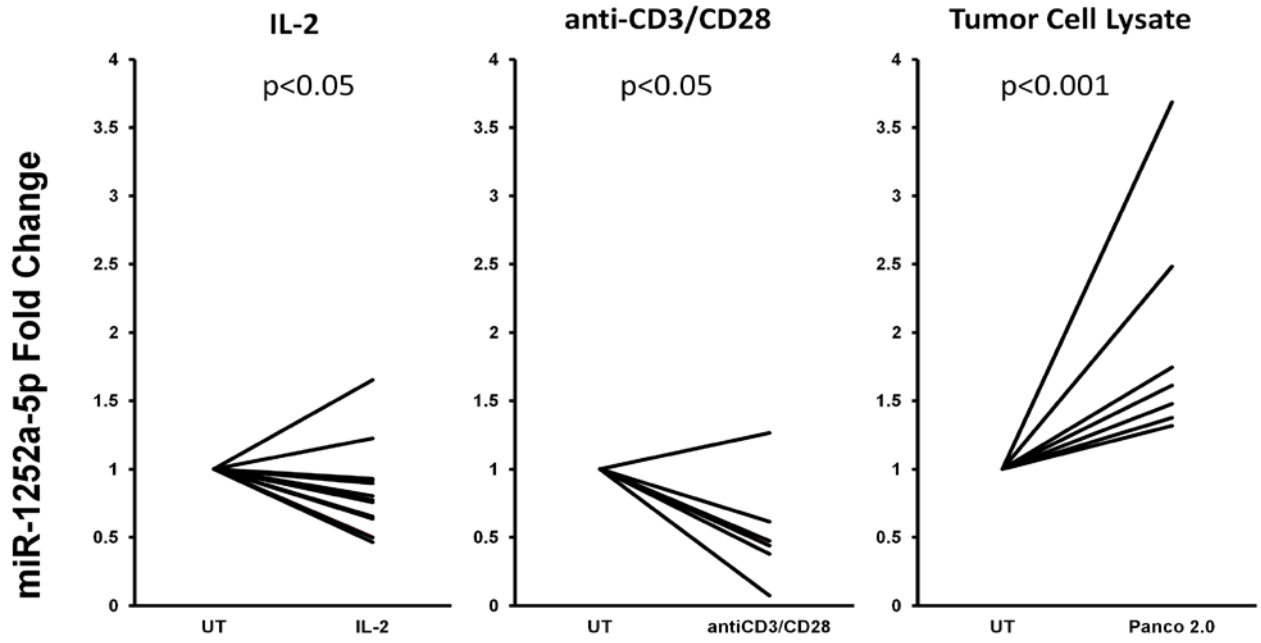
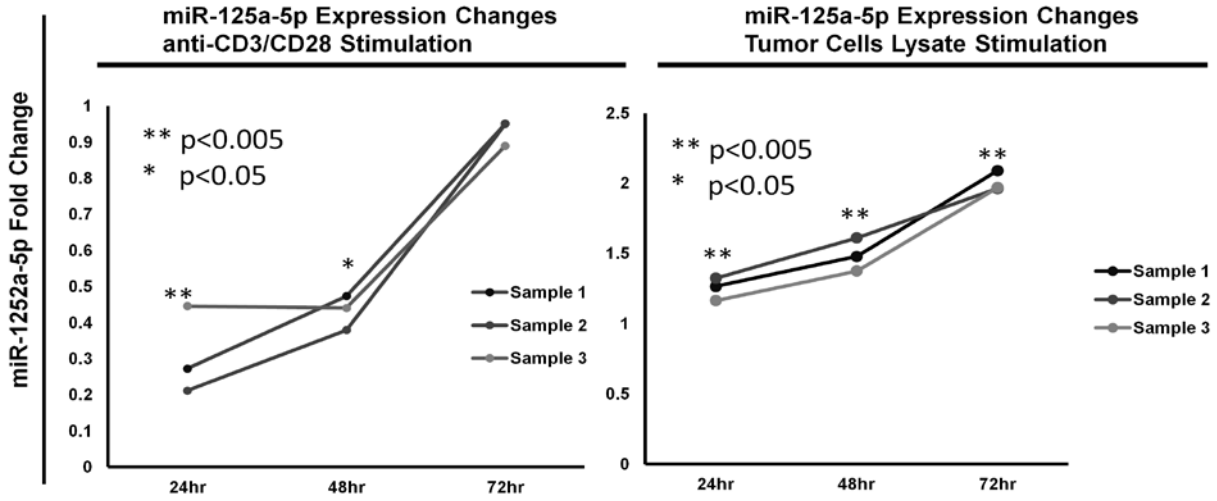


Figure 4-1: Immunological stimuli altered miR-125a-5p expression

Healthy individual PBMC were stimulated by IL-2 (6000UI/ml), anti-CD3/CD28 dynabeads (1:1 bead to cell ratio), or tumor cell lysate ($3x10^5$ cells/ml of cell lysate) for 48 hrs. miR-125a-5p expression is down-regulated with IL-2 or anti-CD3/CD28 stimulation compared to the untreated (average fold change 0.825, and 0.528, $p<0.0257$, $p<0.0331$ respectively) while it is up-regulated with tumor cell lysate (average fold change 1.958, $p<0.005164$)



S. Figure 4-1: miR-125a-5p expression change after stimulation after 72hours

miR-125a-5p expression in PBMCs treated with anti-CD3/CD28 dynabeads (1:1 cells to beads) was initially down-regulated and returned back to normal level after 72 hrs. miR-125a-5p expression in PBMCs treated with tumor cell lysate ($3X10^5$ cells/ml of cell lysates) continued to be up-regulated at 72hrs.

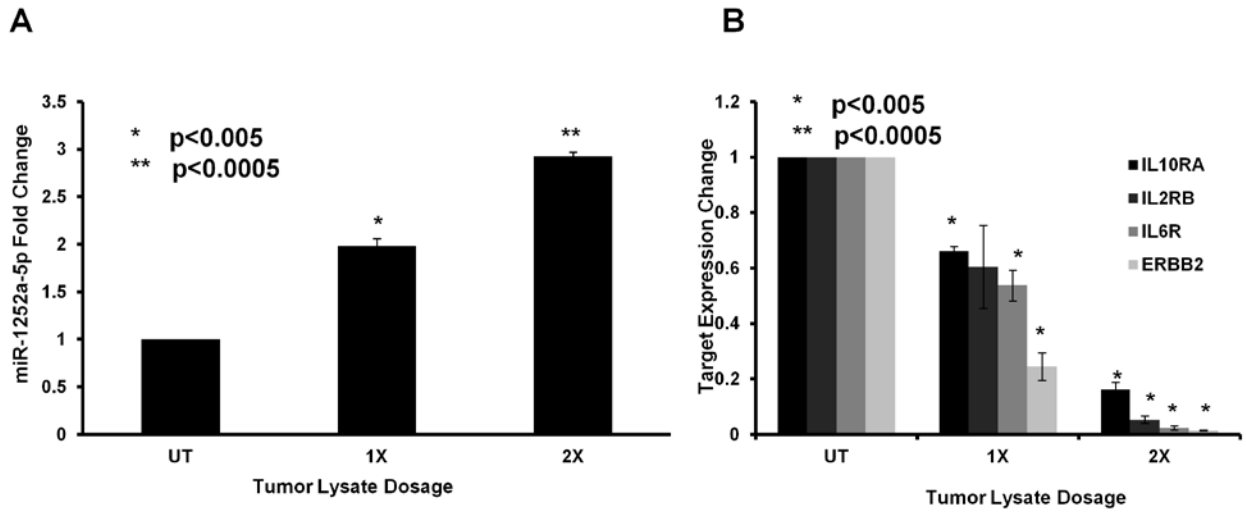


Figure 4-2: Tumor Cell Lysate Upregulate miR-125a-5p Expression in a Dose-Dependently

ERBB2, IL-6R, IL10RA as downstream targets were diminished at 1X ($p<0.04$) of tumor lysate and at 2X ($p<0.05$) of tumor cell lysate. ($p<0.00286$, $p<0.0473$, $p=0.0505$, $p<0.0281$ respectively)

4.4.3 IL-10RA and IL-2RB are Potential Functional Targets of miR-125a-5p

IL-6R, IL-10RA, and IL-2RB are computational targets of miR-125a-5p in the miR-Ontology Database from Ferro Lab (http://ferrolab.dmi.unict.it). We transfected THP-1 and Jurkat cells with lenti-virus to over-express miR-125a-5p (Supplementary Figure 4.2) and positively selected the GFP-positive cells to examine protein expression of the downstream targets. IL-10RA was downregulated in both THP-1 and Jurkat miR-125a-5p over-expressing cells, while IL-2RB was downregulated in Jurkat miR-125a-5p over-expressing cells, but not in THP-2 miR-125a-5p over-expressing cells (Figure 4.3).

4.4.4 miR-125a-5p over-expression does not affect immune cell cell cycle

To determine whether miR-125a-5p expression affects the cell cycle in immune cells, THP-1 and Jurkat cells' DNA content were accessed by propidium iodide staining and analyzed by flow cytometry (Supplementary Figure 4.3). No significant difference was found between the percentage of cells in G0/G1, S phase, or G2/M.

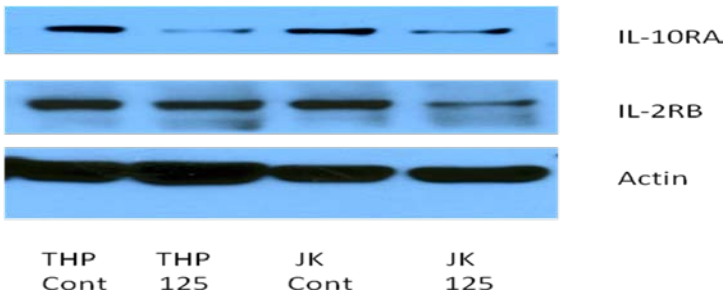
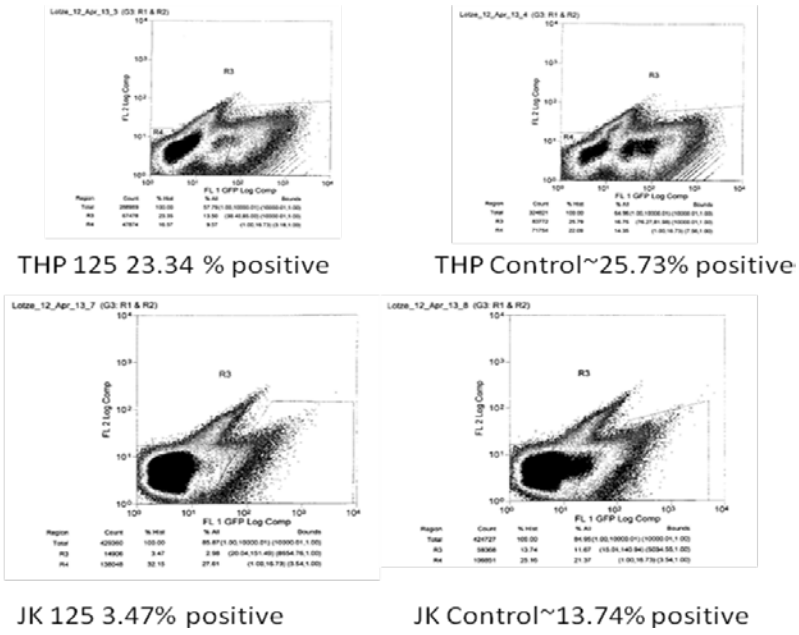
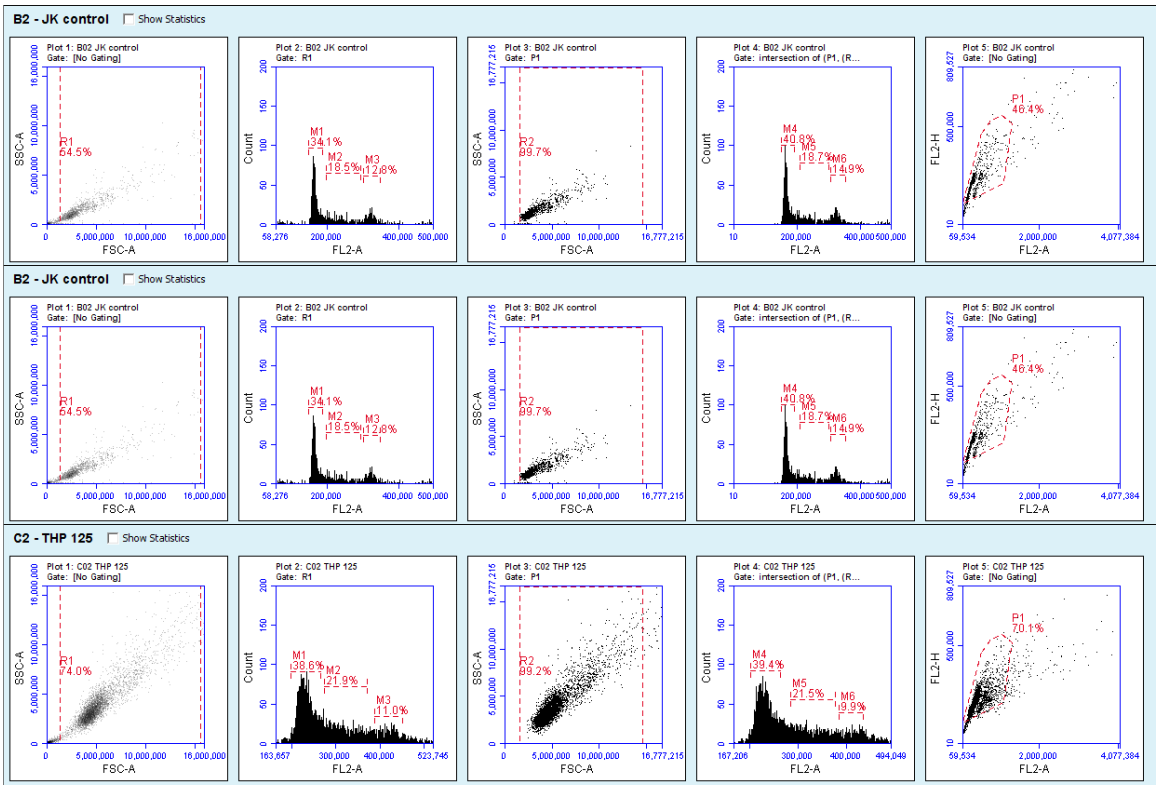


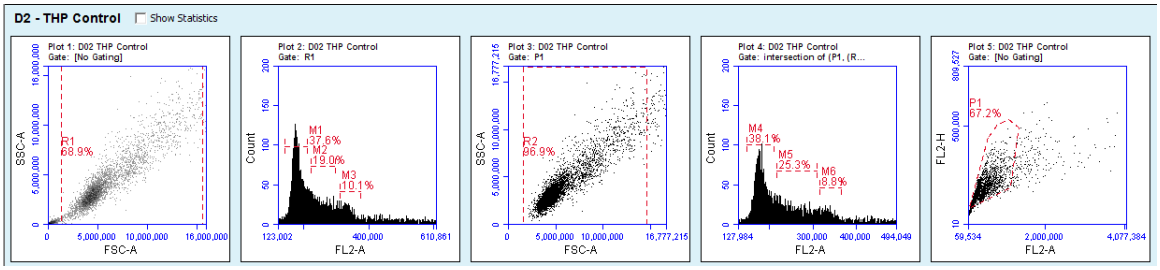
Figure 4-3: miR-125a-5p inhibits IL-10RA and IL-2RB protein expression level

10RA protein expression was decreased in both THP 125 and JK 125 over-expressing cells compared to controls. IL-2RB protein expression is decreased in JK 125 over-expressing cells, but not significantly different in THP 125 over-expressing cells.



S. Figure 4-2: Positive Selection



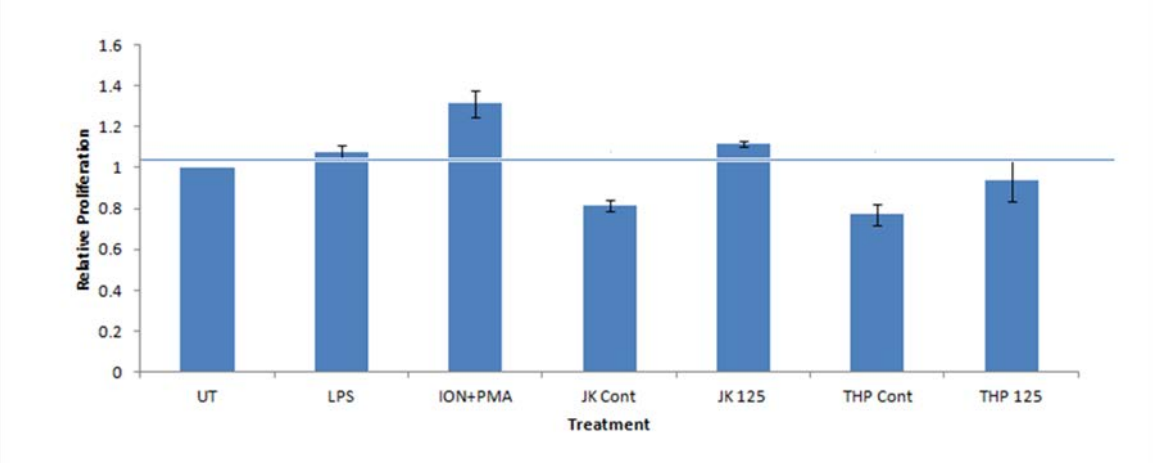


S. Figure 4-3: miR-125a-5p over-expression does not affect immune cell cycle

No differences were found between the percentage of population in G0/G1, S phase, or G2/M in miR-125a-5p over-expressing THP-1/Jurkat cells compared to the control vector.

4.4.5 Conditioned Media has no effect on Panc 2.03 Cell proliferation

To determine whether conditioned media from THP-1 or Jurkat miR-125a-5p over-expressing cells had any effect on tumor cell proliferation, Panc 2.03 cells were cultured with conditioned media and proliferation was assessed by CCK8 assay (Figure 4.4). There are not statistical significant different on their proliferation with ANOV analysis.



S. Figure 4-4: Conditioned Media has no effect on Panc2.03 proliferation

Panc 2.03 cells were cultured with RPMl complete media, RPMI complete+LPS (100ng/ml), RPMI complete+ionomycin(1ug/ml)+PMA(10nM), JK cont conditioned media, JK 125 conditioned media, THP cont conditioned media, THP 125 conditioned media for 24 hrs. There is no differences between their proliferations.

4.5 DISCUSSION

In chapter 3, we observed that miR-125a-5p was downregulated in pancreatic cancer patients' PBMCs following chemotherapy and was upregulated following surgery. In this chapter, we demonstrated that stimulating PBMCs by anti-CD3/CD28 dynabeads or IL-2 leads to miR-125a-5p downregulation, while stimulating PBMCs with Panc 2.03 tumor cell lysate leads to miR-125a-5p upregulation. The literature shows that gemcitabine can activate T-cells and reduce soluble IL-2 level after surgery; our *in-vitro* data implied that the reason why miR-125a-5p was downregulated in pancreatic cancer patients' PBMCs following gemcitabine administration may be due to T-cell activation. MiR-125a-5p upregulation following surgery may be due to a decrease in soluble IL-2R. Thus, analyzing the PBMC subpopulation, activation stage, and serum cytokine level (especially IL-2) following treatment and then investigating correlation with miR-125a-5p in the future may answer these questions.

In chapter 3, we found no correlation between miR-125a-5p expression or change in expression with serum HMBG1 level (or change in serum HMGB1). Although HMBG1 is one of the major DAMPs, it is possible that it is not directly involved in the miR-125a-5p pathway. Our *in-vitro* results in this chapter showed that tumor cell lysate (a complex form of DAMP) upregulated miR-125a-5p expression, implying that other DAMPs may regulate miR-125a-5p expression.

We also validated that miR-125a-5p can downregulate IL-6R, IL-10RA, and IL-2RB mRNA levels. IL-10RA and IL2-RB have been regulated by miR-125b (another closely-related miR-125 family member) in naïve CD4+ T-cells, but we are the first group to validate miR-125a-5p downstream IL-10RA, IL-2RB, and IL-6R mRNA levels in PBMCs. The level of miR-125a-5p upregulation is dose-dependent with tumor cell lysate and correlates with

downregulation of its downstream target mRNA level. We have also shown that over-expressing miR-125a-5p in Jurkat cells with lentiviral vector downregulated IL-2RB and IL-10RA protein expression.

5.0 SUMMARY AND FUTURE DIRECTION

Approximately 46,420 Americans are diagnosed with pancreatic cancer and 39,590 individuals die from the disease annually. Pancreatic cancer is associated with a low five-year survival rate compared to the other top 10 deadly cancers. Early stage five-year survival is below 14% (Stage I ~12-14%, Stage II ~5-7%) compared to small-cell lung cancer (Stage I ~49%, Stage II ~31%). The five-year survival rate decreases substantially as it progress to Stage III (~3%) and Stage IV (<1%). Therefore, useful biomarkers for early stage diagnosis can significantly improve survival rate. Beside late stage detection, lack of effective treatment protocols and locoregional recurrence also contribute to the challenge of pancreatic cancer management.[427] Currently, CA 19-9 is the most widely-used biomarker in pancreatic cancer diagnosis and prognosis, but it has limitations, including lack of expression in the Lewis-negative group (5-10% population) and interference from obstructive jaundice.[95] Because of the unmet clinical need for more ideal pancreatic cancer biomarkers, extensive research to discover body fluid markers (pancreatic juice, saliva, stools), cytokine or cytokine inhibitor, antigen, protein peptide genetic markers, and miRNA are ongoing and have yielded some promising nascent results. Further validation is required before applying the findings to clinical use.[427]

In this dissertation, we used PBMC to identify potential miRNA markers to distinguish between pancreatic cancer patients and healthy individuals and see if the miRNA markers correlated with treatment (chemotherapy, surgery), CA 19-9 level, DAMPs (HMGB1 in our

case), and clinical outcome (disease-free survival and overall survival). Furthermore, we identified immunological stimuli that can alter miRNA marker expression and validated the potential downstream targets miRNA markers.

In Aim 1, we found that miR-125a-5p, miR-146b and miR-29c were differentially expressed in pancreatic cancer patients' PBMC, however previously identified *in-vitro* DAMPmiRs (miR-34c and miR-214) were not expressed in pancreatic cancer patients' PBMCs. The *in-vitro* DAMPmiRs may be differentially expressed in the infiltrated lymphocyte population but not in the circulating PBMC. Therefore, future work to answer this question and follow up for Aim 1 include miRNA profiling of pancreatic cancer patients' infiltrated lymphocyte and comparing the expression profile with circulating PBMC of tumor patients/ healthy individuals, and splenocytes of healthy individual lymph nodes.

In Aim 2, we tried to see whether the miRNA markers that we identified in Aim 1 would change in pancreatic cancer patients' PBMC following treatments. Because we have limited access to samples (with the same amount of blood drawn in Aim 1, we were only able to allocate 1/6 for total RNA isolation and microRNA expression analysis) and resources, we selected miR-125a-5p for further investigation because its potential downstream targets (IL-10RA, IL-2RB, IL-6R) play a role in immunity. We found that miR-125a-5p expression change following treatment and did not correlate with serum CA 19-9 level or HMGB1 level. We believed that this may be due to gemcitabine's ability to activate T-ells and reducte soluble IL-2R following operation. In Aim 3, we showed that T-cell activation signaling (anti-CD3/CD28) and IL-2 can downregulate miR-125a-5p expression. We used serum HMGB1 as the measure of DAMPs; we would expect that miR-125a-5p expression would correlate with serum HMGB1 level. However, there is disconnection between serum HMBG1 level and miR-125a-5p

expression. Data from Aim 3 showed that miR-125a-5p expression is up-regulated by Panc 2.03 lysate *in vitro*; therefore it is possible that other DAMPs regulate miR-125a-5p expression in patients' PBMCs. In Aim 2, we saw that miR-125a-5p did not correlate with the change in CA 19-9 level (an indicator of chemotherapy responsiveness and tumor burden) following chemotherapy; this could be due to the effect of gemcitabine on T-cell activation. Therefore future work includes measuring other DAMPs (e.g uric acid, ATP, IL-1β, S100) following chemotherapy, measuring soluble IL-2R and other cytokine level following surgery and determining if they are associated with miR-125a-5p expression. Furthermore, to validate if miR-125a-5p is useful to monitor reoccurrence following surgery, longer-term studies on miR-125a-5p is required. Finally, due to the limited access to the samples, we were not able to measure miR-146b and miR-29c expression. Including those miRNAs in the future studies would be beneficial.

In Aim 3, we found that T-cell activation and maturing signal (via anti-CD3/CD28, and IL-2 stimulation) lead to a decrease in miR-125a-5p expression in PBMC and tumor cell lysate (complex DAMPS) up-regulated miR-125a-5p expression. We also validated that miR-125a-5p inhibits potential downstream targets' mRNA expression (IL-2RB, IL-10RA, IL-6R) and confirmed that it downregulated IL-10RA and IL-2R protein expression in miR-125a-5p Jurkat cells. The future work for Aim 3 includes: identifying individual DAMP components that can regulate miR-125a-5p expression which can help us pre-select molecules to measure for Aim 2 future work; determining miR-125a-5p expression levels in PBMC subpopulation after stimulation, as they might lead to future therapeutic targets; and more direct validate the downstream target by using luciferase reporter system. Figure 5.1 summarize the dissertation.

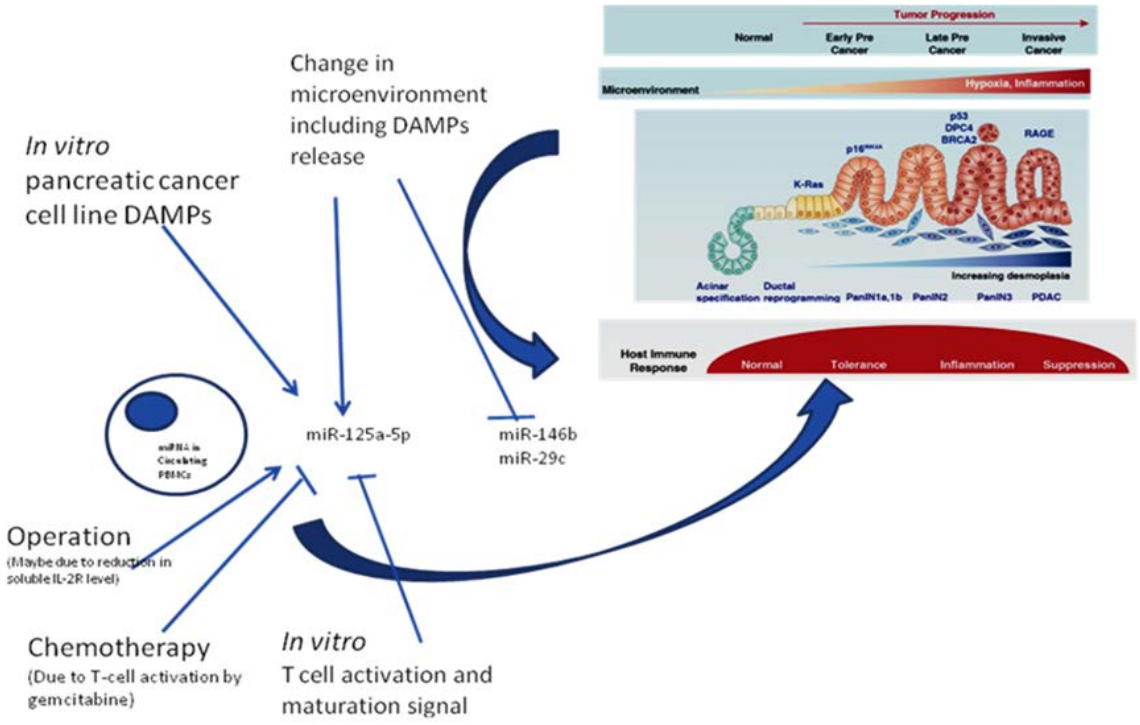


Figure 5-1: Summary of the dissertation

6.0 SIGNIFICANCE OF THIS WORK

This dissertation work has been used to apply grant from Coulter Foundation and entered Student Healthcare Entrepreneurship Competition in 2011 and 2012, Randall Family Big Idea Competition in 2013 hold by University of Pittsburgh. The following were the applications.

6.1 COULTER FOUNDATION APPLICATION

6.1.1 Abstract

Interleukin 2 can cure a small subset (<10%) of patients with advanced melanoma and kidney cancer. It is associated We will develop the premier strategy for identifying factors associated with miR regulation of responsiveness allowing: 1) Diagnostic strategies useful for more effectively identifying and only treating those patients; 2) Means to target these miRs or upstream/downstream pathways to increase responses in these diseases; and 3) Expand the target treatment population to include other epithelial malignancies such as lung, breast, prostate, colon, and pancreatic cancers.

6.1.2 Funding Outcome

The application made it to the semi-final around but not funded. Two reviewers gave an A, one reviewer gave a B, and one gave a C because the idea was in nascent stage.

6.2 STUDENT HEALTHCARE ENTREPRENEURSHIP COMPETITION

6.2.1 Abstract (2011)

Renal Cell Carcinoma (RCC) and melanoma cause more than 21,000 deaths with annually 110,000 new cases each year in the US (58,240 new cases and 13,040 deaths for RCC; 68,130 new cases and 8,700 deaths for melanoma). Survival for patients with stage III RCC is 22% and melanoma, 25-60%. For those with Stage IV disease, survival is measured in months. Interleukin 2 can cure a small subset (<10%) of patients with advanced melanoma and kidney cancer. Who responds and why they respond is still obscure. We will develop the premier strategy for identifying factors associated with responsiveness, specifically the role of microRNA (miR) regulation allowing: 1) Quantitative diagnostic strategies useful for more effectively identifying and only treating those patients who will benefit from the disease; 2) Means to target these miRs or upstream/downstream pathways to increase the number of responses in these diseases; and 3) Expand the target treatment population to include other epithelial malignancies such as patients with lung, breast, prostate, colon, and pancreatic cancers.

6.2.2 Funding Outcome

The application made it to the semi-final around but not funded. The reviewers believed that the idea was in nascent stage and not ready for commercialization.

6.3 STUDENT HEALTHCARE ENTREPRENEURSHIP COMPETITION

6.3.1 Abstract (2012)

Approximately 43,140 Americans are diagnosed with pancreatic cancer and 36,800 individuals die from the disease annually. Pancreatic cancer is associated with less than a 5% five year survival rate. Early diagnosis is rare and surgical treatment is most beneficial before the cancer becomes locally invasive or metastatic. There is a substantial unmet clinical need to develop early diagnostic reagents for identifying pancreatic cancer. Although CA19.9 is widely used to monitor therapy, it has proven to be detectable only late in disease and to be increased with pancreatitis. Recently miRNAs (miRNAs) present within the tumor and in the blood are potential quantitative measures of tumor that may be identified earlier in disease. We will develop a microRNA based non-invasive strategy to detect pancreatic cancer. The strategy allowing: 1) Quantitative diagnostic strategies for identifying pancreatic cancer patient; 2) Means to target these miRs or theirs regulated pathway as a therapeutic approach; and 3) Apply the strategy to other epithelial malignancies population e.g. lung, breast, prostate, colon, and pancreatic cancers patients.

6.3.2 Funding Outcome

The application made it to the semi-final around but not funded. The reviewers believed that the idea was in nascent stage and not ready for commercialization.

6.4 RANDALL FAMILY BIG IDEA COMPETITION

6.4.1 Abstract (2013)

We are developing a strategy to using miR-125a-5p expression level in pancreatic cancer patients' peripheral blood mononuclear cells to distinguish the disease population from normal population. From your preliminary data, we have shown that miR-125a-5p is up-regulated in the disease population with statistical significant. ($P<0.05$) The strategy can also apply to predict and monitor the chemotherapy outcome, supported by our preliminary result that patients who received the chemotherapy have miR-125a-5p expression decrease. Furthermore, the identified miRNA marker can also serve as a therapeutic target to enhance treatment efficiency as we are currently validating the functional target of the miR-125a-5p.

6.4.2 Funding Outcome

The application made it to the semi-final around and oral presentation but not funded.

APPENDIX A

PUBLICATIONS

Sebnem Unlu, **Siuwah Tang**, Ena Wang, Ivan Martinez, Daolin Tang, Macro E. Bianchi, Herbert J. Zeh III, Michael t. Lotze, "Damage Associated Molecular pattern Molecule-Induced microRNAs (DAMPmiRs) in Human Peripheral Blood Mononuclear Cells." *PLoSOne*. 2012;7(6)e38899.

Tang S, Bonaroti J, Unlu S, Liang X, Tang D, Zeh HJ, Lotze MT, "Sweating the small stuff: microRNA and genetic change define pancreatic cancer." *Pancreas*. 2013 Jul;24(5):740-59.

Tang S, Lotze MT, "The power of negative thinking: which cells limit tumor immunity?" *Clin Cancer Res.* 2012 Oct 1: 18(19):5157-9.

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