MIR-198 IN PANCREATIC CANCER PROGRESSION AND MANAGEMENT

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
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To my wife Janet and my children Annabelle and Nathan

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LIST OF ABBREVIATIONS

cDNA	Complimentary deoxyribonucleic acid
CT	Cycle threshold
HRP	Horseradish peroxidase
KRAS	Kirsten rat sarcoma viral oncogene homolog
miRNA	Micro-RNA
mRNA	Messenger RNA
qRT-PCR	Real-time quantitative reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid

Abstract of Thesis Presented to the Graduate School
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Pancreatic cancer is the fourth most deadly cancer in the US. Most patients present with inoperable disease that can only be treated with chemotherapeutic agents like gemcitabine. However, the vast majority of patients with pancreatic cancer ultimately die largely due to resistance to current chemotherapeutic strategies. The molecular mechanisms underlying chemoresistance in pancreatic cancer are unclear. Also, there is currently no clinically useful biomarker for predicting response to chemotherapy, screening, diagnosis, and for monitoring response to chemotherapy. Moreover, there is an urgent need for novel therapeutic approaches. Micro-RNAs (miRNAs) are non-coding RNAs that are known to play an important role in carcinogenesis. Micro-RNA 198 (miR-198) was recently discovered to be significantly down-regulated in hepatocellular carcinoma and to directly target c-Met to inhibit invasion and migration of hepatocellular carcinoma cells. However, the role of miR-198 in other cancers is unclear. Although c-Met has been implicated in chemoresistance in pancreatic cancer, the role of miR-198 in pancreatic cancer chemoresistance is unknown. This project used a combination of molecular and functional studies in both cell culture models and patient clinical samples to examine the role of miR-198 in

pancreatic cancer. Data obtained show that miR-198 silencing is a mechanism of gemcitabine-resistance in a human pancreatic cancer model and that miR-198 may be a useful non-invasive biomarker in human pancreatic cancer. Therefore, we conclude that miR-198 may be useful in preventing or overcoming chemoresistance in pancreatic cancer and for monitoring progression in a non-invasive fashion.

CHAPTER 1
INTRODUCTION

Pancreatic Cancer

Pancreatic cancer is a very deadly disease. Pancreatic ductal adenocarcinoma is the most common form.[1] Therefore, it is the subject of this research project. Consequently, the use of the term “pancreatic cancer” in this thesis applies primarily to pancreatic ductal adenocarcinoma.

It has been estimated that over 45,000 Americans will be diagnosed with pancreatic cancer in 2013 and that over 38,000 (about 85%) of them will die from the disease.[2] Currently, pancreatic cancer is the fourth most common cause of cancer-related deaths in the United States with overall five-year survival rate of about 5%.[1, 3] Consequently, pancreatic cancer has the worst prognosis among all solid tumors. [4] About 80% of all pancreatic cancer patients present with disease that is surgically unresectable.[5] Patients with pancreatic cancer tend to present late because their initial symptoms are typically non-specific. Ultimately, many of these patients present with metastatic disease. In the 20% able to have surgery with curative intent, the prognosis is still dismal with 5-year survival being only 10-15%. Chemotherapy with gemcitabine is currently the most common treatment modality for most patients.[6, 7] This is due to its efficacy in alleviating disease-related symptoms as well as data from a randomized clinical trial.[8] However, up to 94% of patients are either unresponsive or develop resistance to chemotherapy.[8-11] Unfortunately, the underlying mechanisms for resistance to chemotherapy are still unclear.

Since the 1980s serum CA 19-9 has been used as a clinical biomarker and for prognostication in pancreatic cancer.[12] Specifically, it is used in evaluating patients

suspected of having a disease of the pancreas. It is a serum biomarker that is often elevated in patients with pancreatic cancer. However, it is known to have many flaws. For example, it has been shown to be ineffective for screening for pancreatic cancer.[13] Its effectiveness as a prognostic marker is also questionable.[14-18] Unfortunately, up until the present time effective non-invasive strategies for early detection, diagnosis, and prognostication of pancreatic cancer are not available. This is certainly an area of urgent need.

Almost all cases of human pancreatic cancer are characterized by constitutively active Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations.[1, 19] KRAS is an oncogene which encodes a small GTPase transductor protein. The KRAS protein regulates cell division by relaying signals from outside the cell to the cell nucleus.[20] It is known that KRAS mutations occur early in the development of pancreatic cancer.[1] However, efforts to target KRAS for therapy in pancreatic cancer have largely failed.[21] Moreover, multiple studies suggest that KRAS mutation alone may not be sufficient to promote progression of pancreatic cancer.[22, 23] Therefore, it is likely that other currently unknown mechanisms are involved.

miR-198 in Cancer

Micro-RNAs (miRNAs) are small non-coding RNAs. In animal cells, they typically inhibit initiation of translation of their target mRNAs by binding to the 3' untranslated region of the target mRNA.[24] Research has now established that miRNAs play an important role in carcinogenesis[25] In pancreatic cancer recent studies[26-28] have shown that some miRNAs are aberrantly expressed in pancreatic cancer and may play a role in the development and progression of pancreatic cancer by down-regulating pro-

tumorigenic genes.[27] However, further work is required to identify specific miRNAs that may have beneficial clinical use in specific cancers such as pancreatic cancer.

The proto-oncogene, c-Met, is overexpressed in human pancreatic cancer and is involved in growth and invasion of pancreatic cancer.[29-32] c-Met is the receptor for hepatocyte growth factor. Recent studies have suggested that chemoresistance in pancreatic cancer may be due to overexpression of c-Met.[33] c-Met was shown to be directly regulated by miR-198 in hepatocellular carcinoma.[34] miR-198 is located in exon 11 of the 3'-untranslated region of FSTL1[35] and binds to the 3' untranslated region of c-Met resulting in decreased protein expression and downstream signaling.[34] A recent study showed that miR-198 is significantly down-regulated in hepatocellular carcinoma in comparison to normal liver tissue by more than 5-fold, thus suggesting that loss of miR-198 expression may have a tumorigenic effect and that miR-198 may be a tumor suppressor.[36] A more recent study has further shown that miR-198 directly targets c-Met to inhibit migration and invasion of hepatocellular carcinoma cells.[34] However, the biological and functional role of miR-198 in pancreatic cancer is still unclear.

Two recently published studies have made preliminary observations. Schultz *et al*. studied formalin-fixed and paraffin-embedded tissue samples of 170 patients with pancreatic ductal adenocarcinoma and compared them with 23 patients with chronic pancreatitis and 28 individuals with normal pancreas.[37] They performed miRNA profiling using RNA extracted from the tissue samples and a commercially available miRNA microarray. Their data suggest that miR-198 is expressed at lower levels in normal pancreas than pancreatic ductal adenocarcinoma tissues. However, the reverse was found to be true in the studies performed and reported by Marin-Muller *et al.*[28]

Marin-Muller *et al*. studied pancreatic adenocarcinoma cell lines, patient pancreatic adenocarcinoma samples and mouse models of pancreatic adenocarcinoma. They consistently found that miR-198 is down-regulated in pancreatic adenocarcinoma. Furthermore, they reported that re-expression of miR-198 in pancreatic cancer cells leads to reduced tumor growth, reduced tumor metastasis and increased survival, thus showing that miR-198 has tumor suppressor activity in pancreatic adenocarcinoma. Therefore, significant controversy exists regarding the precise biological and functional role of miR-198 in pancreatic cancer. Consequently, there is a compelling need for additional research into the role of miR-198 in pancreatic cancer. These further studies may conclusively determine the role of miR-198 in pancreatic cancer and they may reveal novel insights into miR-198's potential clinical applications in the management of pancreatic cancer.

This research project has, therefore, focused on investigating the role of miR-198 in resistance to chemotherapy in pancreatic cancer and the potential of using miR-198 in the clinical setting for non-invasive early detection, diagnosis, and monitoring of therapy. Our studies employed the use of a combination of cellular experimental models and analysis of human clinical samples.

CHAPTER 2
MATERIALS AND METHODS

Cell Culture, Cell Lines and Transfection Experiments

The L3.6pl human pancreatic ductal adenocarcinoma cell line was obtained from the laboratory of Jose G. Trevino, MD, at the University of Florida and cultured as previously described.[38-41] The L3.6pl cell line was established from a liver metastatic deposit that resulted from implantation of the COLO 357 human pancreatic cancer cell line into the pancreas of nude mice.[42, 43] The L3.6pl cell line is responsive to the cytotoxic effect of gemcitabine. For this project, gemcitabine-resistant L3.6pl cells were also obtained from Dr. Trevino's lab. The gemcitabine-resistant L3.6pl subline was established and maintained as previously described.[41, 44, 45]

Ten thousand gemcitabine-responsive L3.6pl cells were cultured in 6-well plates until 70% confluent. 50nM of a specific oligonucleotide inhibitor or mimic of miR-198 was transfected into the cells as appropriate and following manufacturer's instructions. Control cells were transfected with equivalent concentration of a non-specific control oligonucleotide. After 6 hours incubation, medium was changed to complete culture medium and cells were cultured for a further 24 hours before RNA extraction, real-time quantitative polymerase chain reaction (qRT-PCR), apoptosis, and cell viability assays were performed. All oligonucleotides and transfection reagents used for transfection were purchased from Qiagen (Valencia, CA).

Cell line RNA Extraction, cDNA Synthesis and Real-time Polymerase Chain Reaction

Total RNA was extracted from cell lines using TRIzol reagent (Life Technologies, NY). cDNA synthesis and qRT-PCR were performed as previously described.[45-46] The proprietary primers used for amplification were obtained from Qiagen (Valencia, CA).

qRT-PCR was performed using SYBR Green. Reactions were conducted in a 96-well spectrofluorometric thermal cycler (Applied Biosystems, CA).

Protein Extraction and Western Blotting

Gemcitabine-responsive and gemcitabine-resistant L3.6pl cells were grown in a monolayer in cell culture dishes. Total protein was extracted from the cells as previously described.[47] Western blotting was performed as previously described.[47] The specific primary antibodies used were anti-HGF (sc-13087), anti-c-Met (sc-161) and anti-phosphorylated c-Met (sc-34085). All primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoreactive proteins were visualized by incubating in horseradish peroxidase (HRP)-conjugated secondary antibodies. Chemiluminescence was detected by incubating in an equal-parts mixture of the SuperSignal West Pico stable peroxide solution and luminol/enhancer solution (Pierce, IL) and visualized using an image processing machine. Densitometry was performed using Adobe Photoshop CS5 software from Adobe Systems (San Jose, CA).

Assessment of Apoptosis and Cell Viability

For assessment of apoptosis, cells were seeded into 6-well plates in cell culture medium. Transfection experiments were performed as described above. Cells were also treated with gemcitabine as appropriate. Cell culture medium was removed and replaced with fresh cell culture medium containing Hoechst 33258 (1 µg/ml). Cells were then further cultured at 37°C for 10 to 30 minutes. Cells with chromatin condensation were visualized and photographed using a digital fluorescence microscope (Olympus) 30 minutes after addition of the staining solution. Chromatin condensation is the most characteristic feature of apoptosis. Apoptotic cell death was assessed by counting the number of apoptotic cells with condensed nuclei in six randomly selected areas.

For cell viability assays, cells were seeded at 5×10^4 cells per well in 48-well plates in cell culture medium for 24 hours. After 24 hours, transfection experiments were performed as described above. Cells were subsequently treated with gemcitabine. Cell viability after 24 hours was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as previously described.[48]

Analysis of miR-198 Expression in Human Plasma Samples

Blood samples were obtained from seven pancreatic cancer patients recruited through the medical oncology clinic of the University of Florida (UF) following a UF Institutional Review Board (IRB)-approved protocol and informed consent. Blood samples from four normal healthy subjects were obtained through the Gainesville LifeSouth Community Blood Center following a UF IRB-approved protocol. Plasma samples were obtained by centrifuging blood samples at 2500 revolutions per minute for five minutes and collecting the supernatant for further analysis.

Fifty microliters (50 μl) of plasma sample was centrifuged at high speed for five minutes. The supernatant was collected, mixed with 750 μL of TRIzol® LS reagent (Life Technologies, NY). Total RNA extraction was accomplished by further processing using chloroform, isopropanol and 75% ethanol and following the manufacturer's instructions. cDNA synthesis was performed using 0.6 μg of total RNA and miScript II RT kit (Qiagen, CA) following manufacturer's instructions. Real-time quantitative polymerase chain reaction (qRT-PCR) was performed using the miScript SYBR green PCR kit (Qiagen, CA) and proprietary specific miR-198 and U6 snRNA primers (both from Qiagen) following the manufacturer's protocol. Reactions were performed in the StepOne Plus 96-well spectrophotometric thermal cycler (ABI Biosystems, CA).

Statistical Analysis

qRT-PCR was performed in triplicates and normalized to GAPDH or U6 snRNA. Apoptosis and cell viability experiments were performed in quadruplicates and at least 3 times. Results are expressed as mean ± SEM. Effects were compared with controls. Paired *t* tests were used to analyze the significance of differences between experiments and controls. $P < 0.05$ was considered significant. For human plasma miR-198 analysis, absolute CT values of miR-198 expression were divided by the corresponding absolute CT value for U6 snRNA expression. Since higher CT values indicate lower expression, inverse of the mir-198/U6 snRNA CT ratios were used as a measure of relative miR-198 expression levels. Mean and standard deviation were compared. Box plots were constructed to compare relative miR-198 expression levels.

CHAPTER 3
RESULTS

c-Met is Overexpressed in Gemcitabine-resistant L3.6pl Pancreatic Cancer Cells

To determine involvement of c-Met in gemcitabine resistance in our cellular model system, we compared c-Met gene and protein expression profiles in both the gemcitabine-sensitive and gemcitabine-resistant L3.6pl sublines using real-time quantitative polymerase chain reaction (qRT-PCR) and western blotting, respectively. We also analyzed the expression profile of phosphorylated c-Met (p-c-Met) and hepatocyte growth factor (HGF) by the gemcitabine-sensitive and gemcitabine-resistant subclones. Our data presented in Figure 3-1 show that gemcitabine-resistant cells have increased gene and protein expression of c-Met in comparison to gemcitabine-sensitive cells. Furthermore, gemcitabine-resistant cells have increased phosphorylation of c-Met in comparison to gemcitabine-sensitive cells. However, there was no noticeable difference in HGF expression between gemcitabine-sensitive and gemcitabine-resistant cells.

miR-198 Expression is Lost in Gemcitabine-resistant L3.6pl Pancreatic Cancer Cells

Next, we proceeded to investigate whether c-Met overexpression in the gemcitabine-resistant pancreatic cancer cells is related to miR-198. To do this, we used qRT-PCR to perform analysis of miR-198 expression in both the gemcitabine-sensitive and gemcitabine-resistant cells. Our data presented in Figure 3-2 show that gemcitabine-resistant pancreatic cancer cells have significantly decreased expression of miR-198 in comparison to gemcitabine-sensitive pancreatic cancer cells.

miR-198 Regulates Apoptosis and Cell Viability in Gemcitabine-sensitive L3.6pl Pancreatic Cancer Cells

To determine a direct causal role for miR-198 in resistance to gemcitabine by pancreatic cancer cells, we evaluated the effect of specific silencing of miR-198 expression in gemcitabine-sensitive pancreatic cancer cells by transfection of a specific oligonucleotide inhibitor of miR-198. Our data presented in Figure 3-3 show that silencing of miR-198 expression significantly inhibits gemcitabine-induced apoptosis in gemcitabine-sensitive pancreatic cancer cells as determined by Hoechst staining analysis. Therefore, we have direct evidence that miR-198 silencing may be a mechanism for resistance to gemcitabine by pancreatic cancer cells.

To further establish the role of miR-198 in resistance to gemcitabine in pancreatic cancer, we assessed the effect of loss of miR-198 expression on viability of gemcitabine-responsive pancreatic cancer cells. We observed that inhibition of miR-198 expression with a specific oligonucleotide inhibitor of miR-198 significantly promotes viability of pancreatic cancer cells ($P = 0.02$). Our data also suggest that inhibition of miR-198 expression may protect pancreatic cancer cells from gemcitabine-induced loss of cell viability although this effect did not reach statistical significance ($P = 0.1$). These data are graphically presented in Figure 3-4.

Because miR-198 silencing inhibited gemcitabine-induced apoptosis, we hypothesized that miR-198 may have apoptotic effects on pancreatic cancer cells. To test this hypothesis, L3.6pl human pancreatic cancer cells were transfected with a synthetic oligonucleotide mimic of miR-198. For experimental controls, cells seeded in separate wells were also treated with either DMSO alone, gemcitabine alone or transfection reagent alone. Subsequently, Hoechst staining analysis was performed and

apoptotic cells counted in four randomly selected high power fields. The data presented in Figure 3-5 show that miR-198 mimic induced significant apoptosis in the L3.6pl pancreatic cancer cells in comparison to cells treated with transfection reagent alone. This suggests that miR-198 may directly have apoptotic effects on pancreatic cancer cells.

Further, we explored the cytotoxic effects of miR-198 by assessing the effects of transfection of the oligonucleotide mimic of miR-198 on viability of the L3.6pl human pancreatic cancer cells using MTT assays. Our data presented in Figure 3-6 show that either 50 nM miR-198 mimic or 0.1 μM gemcitabine on their own inhibited cell viability. However, treatment of cells transfected with 50 nM miR-198 mimic and with 0.1 μM gemcitabine resulted in significantly increased inhibition of cell viability than either alone (P = 0.004).

Plasma miR-198 Expression in Pancreatic Cancer

RNA extraction from plasma samples of all human subjects yielded total RNA concentration of between 52 and 2293 ng/μl. The means of the resulting ratios from dividing miR-198 CT values with U6 snRNA CT values were generally lower for normal healthy subjects than for pancreatic cancer patients. Accordingly, the relative plasma miR-198 expression as determined by the inverse of the miR-198/U6 snRNA CT ratios was lower in pancreatic cancer patients than in normal healthy subjects, but did not reach statistical significance. It should be noted that the data included in the box plot for pancreatic cancer patients include a couple of outliers. The data reaches statistical significance with exclusion of these outliers .The data are represented as box plots in Figure3-7.

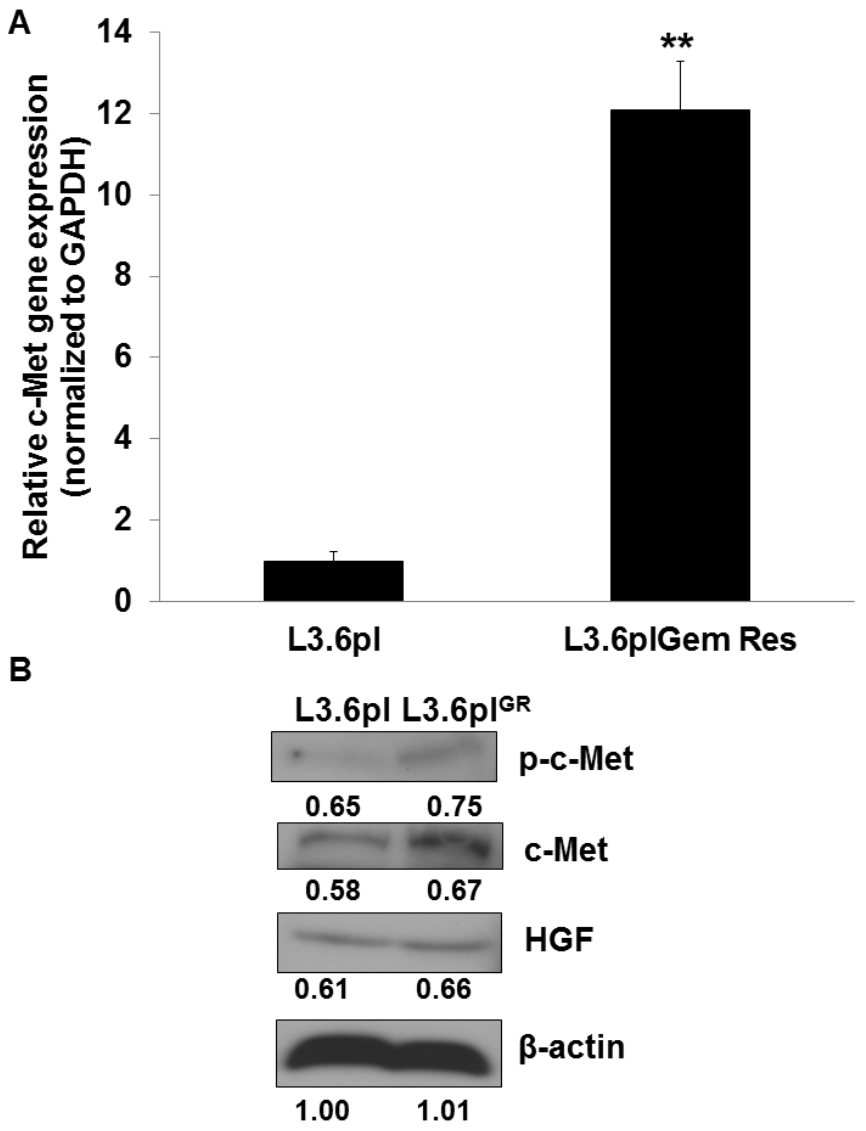

Figure 3-1. c-Met is overexpressed in gemcitabine-resistant L3.6pl pancreatic cancer cells. A. Total RNA was extracted from gemcitabine-responsive and gemcitabine-resistant subclones of the L3.6pl human pancreatic adenocarcinoma cell line. Gene expression of c-Met was analyzed using real-time polymerase chain reaction and specific primers. Gemcitabine-resistant pancreatic cancer cells have increased c-Met gene expression. Results are presented as mean ± standard error of the mean; ** $P < 0.01$; $N = 3$. B. Total protein was extracted from gemcitabine-responsive and gemcitabine-resistant subclones of the L3.6pl human pancreatic adenocarcinoma cell line. Protein expression of c-Met, phosphorylated c-Met and HGF were analyzed using western blotting and specific antibodies. Gemcitabine-resistant pancreatic cancer cells have increased c-Met protein expression and phosphorylation, but not HGF expression. Densitometry was performed using Adobe Photoshop CS5 software from Adobe Systems (San Jose, CA).

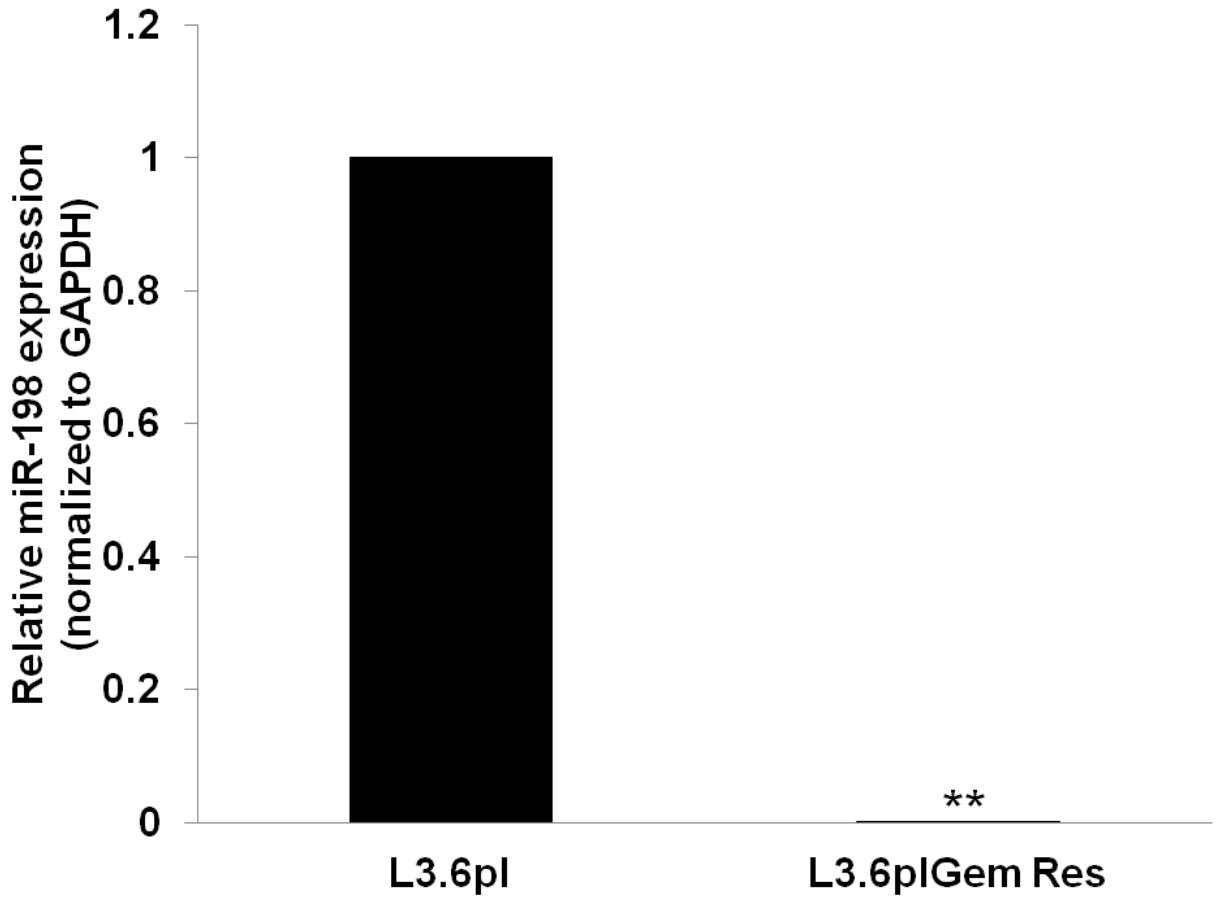

Figure 3-2. miR-198 expression is lost in gemcitabine-resistant L3.6pl pancreatic cancer cells. Total RNA was extracted from gemcitabine-responsive and gemcitabine-resistant subclones of the L3.6pl human pancreatic adenocarcinoma cell line. miR-198 expression was analyzed using real-time polymerase chain reaction and specific primers. Gemcitabine-resistant pancreatic cancer cells have significantly decreased miR-198 expression in comparison to gemcitabine-sensitive pancreatic cancer cells as determined by qPCR. Data presented as mean ± SEM; ** $P < 0.01$; N = 3.

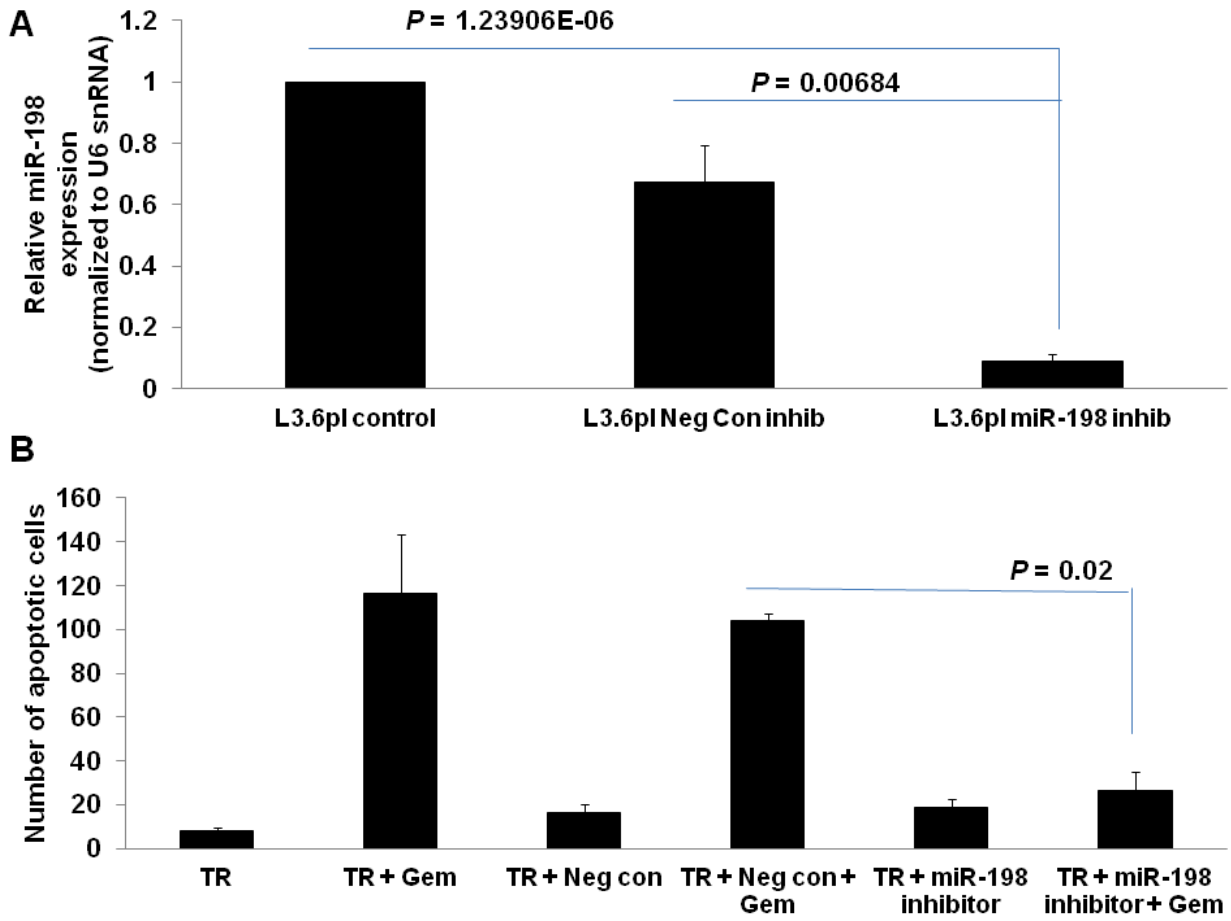

Figure 3-3. Loss of miR-198 expression has anti-apoptotic effect on gemcitabine-sensitive L3.6pl pancreatic cancer cells. A. Transfection of a specific oligonucleotide inhibitor of miR-198 into gemcitabine-sensitive pancreatic cancer cells significantly decreased miR-198 expression. B. Transfection of a specific oligonucleotide inhibitor of miR-198 into gemcitabine-sensitive pancreatic cancer cells significantly inhibited gemcitabine-induced apoptosis as determined by Hoechst staining analysis. Data presented as mean ± SEM; N = 3. TR: transfection reagent alone; Gem: gemcitabine; Neg Con: negative control.

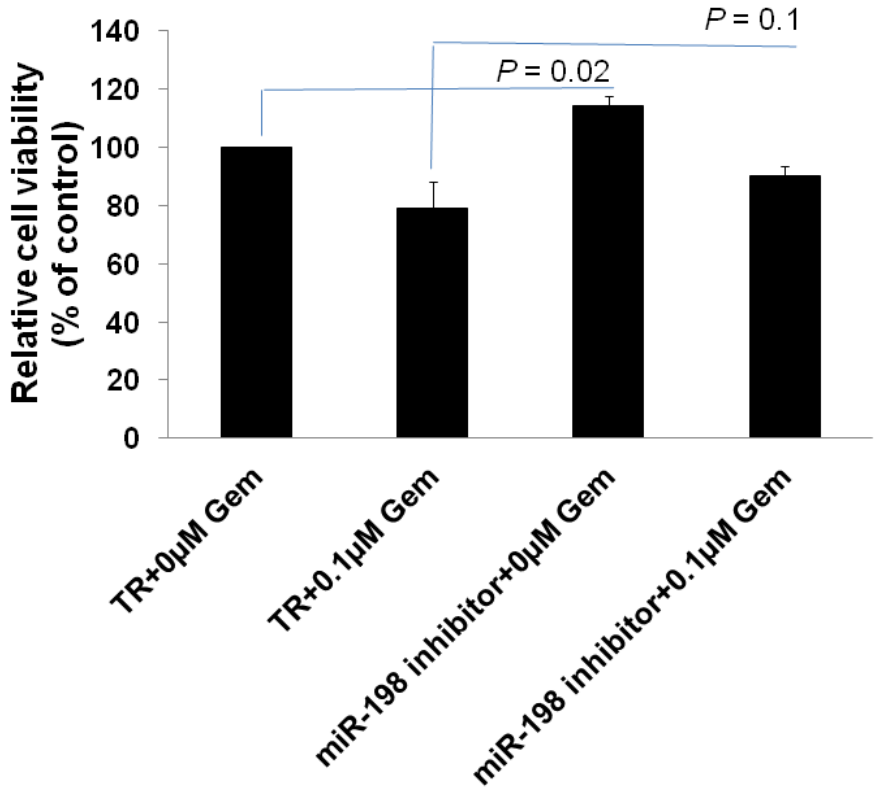

Figure 3-4. Loss of miR-198 expression promotes viability of gemcitabine-sensitive L3.6pl pancreatic cancer cells. Transfection of a specific oligonucleotide inhibitor of miR-198 into gemcitabine-sensitive pancreatic cancer cells promoted viability of cells and protected cells from gemcitabine-induced loss of cell viability as determined by MTT assays. Data presented as mean ± SEM; N = 3. TR: transfection reagent alone; Gem: gemcitabine.

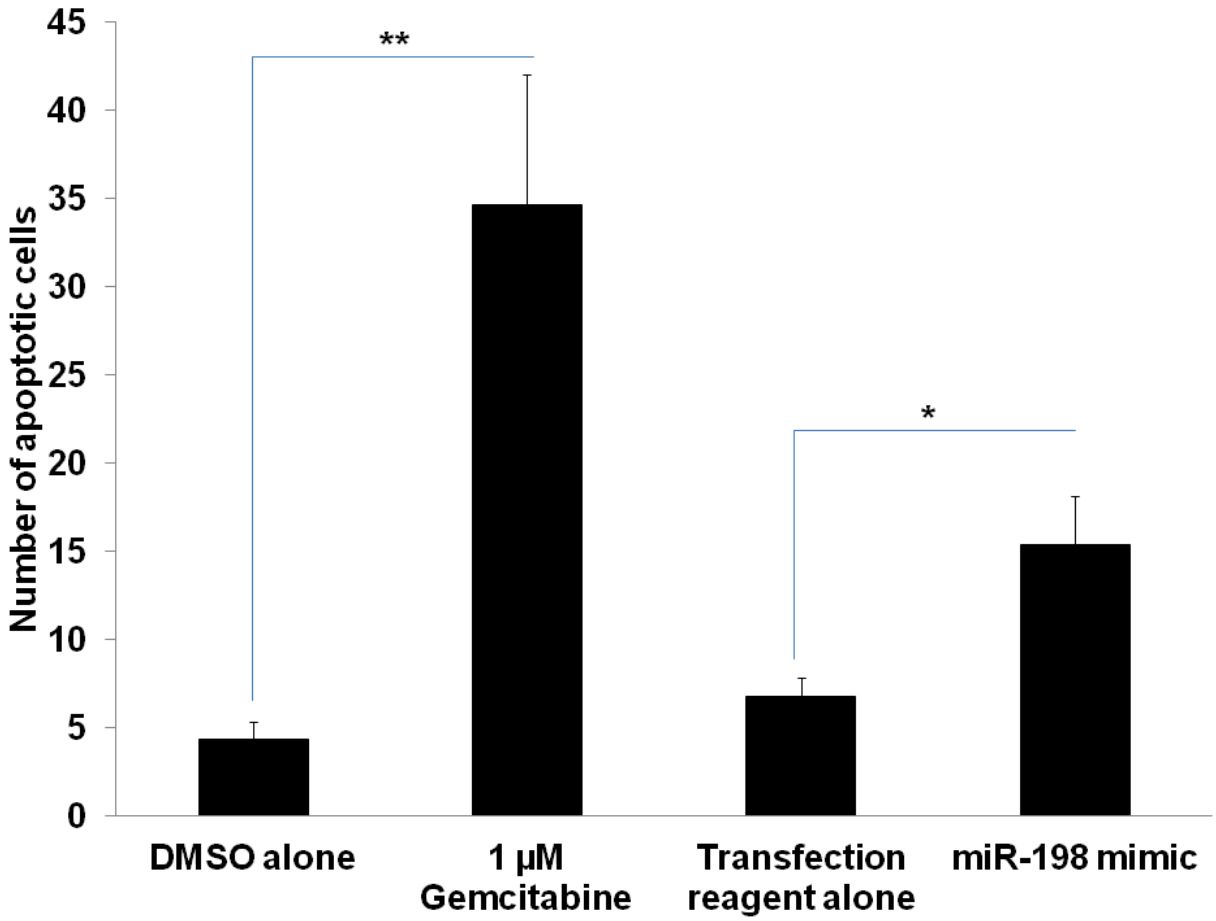

Figure 3-5. miR-198 mimic induced apoptosis in gemcitabine-sensitive L3.6pl pancreatic cancer cells. 1µM gemcitabine induced significant apoptosis in L3.6pl cells in comparison to cells treated with DMSO alone. Furthermore, transfection of a specific oligonucleotide mimic of miR-198 into L3.6pl pancreatic cancer cells significantly induced apoptosis in comparison to cells treated with transfection reagent alone. Morphological evidence of apoptosis was determined by Hoechst staining. Apoptotic cells were counted in 4 randomly selected high power fields. Data presented as mean ± SEM; * $P < 0.05$; ** $P < 0.01$; N = 3.

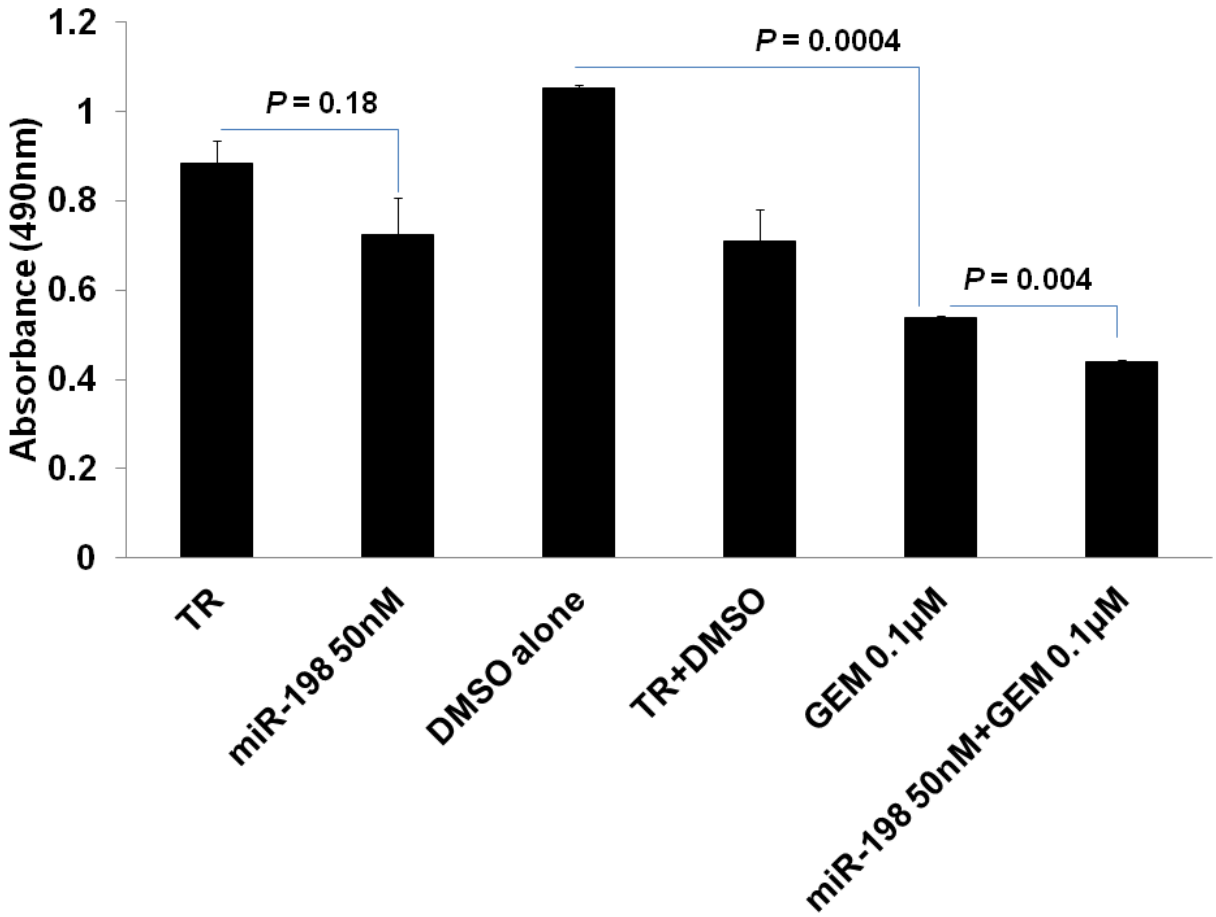

Figure 3-6. miR-198 mimic inhibited viability of gemcitabine-sensitive L3.6pl pancreatic cancer cells. Transfection of 50nM miR-198 mimic inhibited viability of pancreatic cancer cells. 0.1μM gemcitabine also inhibited viability of pancreatic cancer cells. However, treatment of pancreatic cancer cells transfected with miR-198 mimic with 0.1μM gemcitabine led to further significant inhibition of cell viability greater than that induced by 50nM miR-198 mimic or 0.1μM gemcitabine alone. Cell viability was assessed with MTT assays. Data presented as mean ± SEM. TR, transfection reagent alone; GEM, gemcitabine.

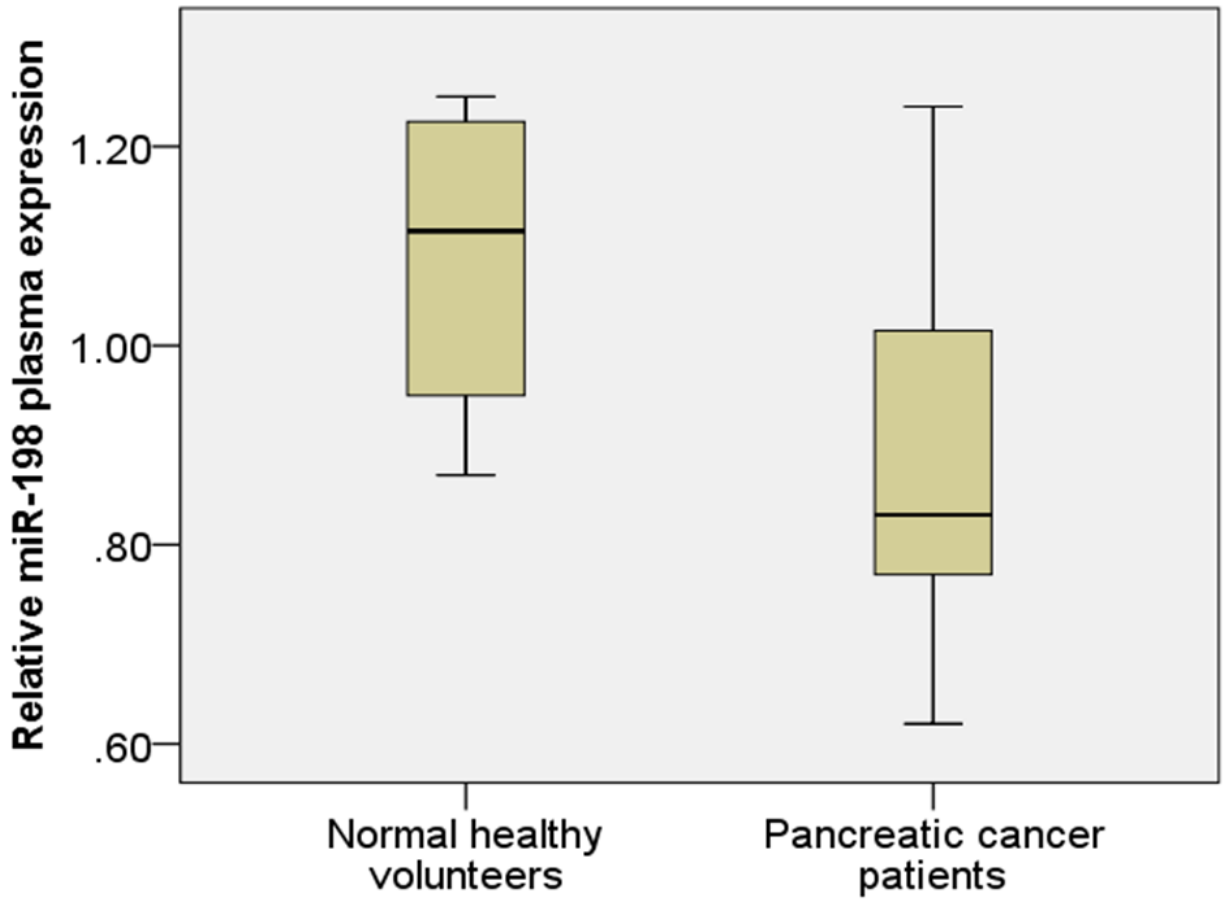

Figure 3-7. Plasma expression of miR-198 is reduced in pancreatic cancer patients. Total RNA was extracted from the plasma of normal healthy volunteers and pancreatic cancer patients. Plasma expression of miR-198 was assessed using real-time quantitative polymerase chain reaction and specific primers. Data was normalized with U6 snRNA expression for internal control. The inverse of mean CT values was used for graphical presentation of data. Data presented as mean ± standard deviation. The bars at the top and bottom indicate maximum and minimum, respectively. The top and bottom of the box indicate third quartile and first quartile, respectively. The line in the middle of the box indicates the median.

CHAPTER 4
DISCUSSION

In this study we have examined, for the first time, the role of miR-198 in resistance to gemcitabine cytotoxicity by pancreatic cancer cells. We have also examined, for the first time, the expression pattern of miR-198 in the plasma of normal healthy humans in comparison to pancreatic cancer patients. Our studies showed that loss of miR-198 expression may be protective against gemcitabine-induced apoptosis and loss of cell viability. It is possible that the underlying mechanism involves upregulation and increased phosphorylation of c-Met leading to increased cell viability. We also observed that miR-198 has pro-apoptotic and cytotoxic effects on pancreatic cancer cells. Furthermore, our studies showed that plasma miR-198 expression is lower in pancreatic cancer patients than normal healthy people. Therefore, plasma miR-198 may be a useful non-invasive biomarker for early detection and diagnosis in pancreatic cancer.

The role of miRNAs as important regulators of carcinogenesis is now established [49-51]. Specific miRNAs may act as tumor suppressors by inhibiting expression of oncogenic target mRNAs. Conversely, specific miRNAs may also be oncogenic by inhibiting expression of tumor suppressor target miRNAs.

The specific role of miR-198 in pancreatic cancer has not been clearly described in the published literature. To our knowledge, the role of miR-198 in cancer has been previously described only in eight previous publications.[28, 34, 37, 52-56] All eight studies were published between 2011 and 2013. Of the eight, only two investigated the role of miR-198 in pancreatic cancer. Unfortunately, the data from these two previous studies are not consistent.

Schultz *et al*. suggested from their publication that the expression of miR-198 is less in normal pancreas in comparison to pancreatic ductal adenocarcinoma tissues.[37] In contrast, Marin-Muller *et al*.[28] more recently reported that miR-198 is down-regulated in pancreatic adenocarcinoma cell lines, human pancreatic adenocarcinoma tissues and in mouse models of pancreatic adenocarcinoma. Furthermore, they demonstrated using their experimental systems that miR-198 has tumor suppressor activity in pancreatic adenocarcinoma. Although the data and conclusions from these two previous studies are conflicting, it is noteworthy that Marin-Muller *et al*.'s study that shows miR-198 is down-regulated and has tumor suppressor activity in pancreatic cancer is likely a superior study. Firstly, they used multiple experimental systems and methodologies. Secondly, unlike Schulz *et al*.'s study that was solely descriptive, their study was both descriptive and mechanistic. Our current study has also been both descriptive and mechanistic. We have also used multiple experimental systems and methodologies. Although the specific experiments we have performed and the model systems we have used are completely different from those used by Marin-Muller *et al*., it is very interesting to note that our data strongly agree with theirs.

Furthermore, our current study has shown for the first time that miR-198 can be easily detected in human plasma, and that there is potential that miR-198 can be useful clinically for non-invasive early detection and diagnosis of pancreatic cancer. To our knowledge, the potential clinical role of plasma miR-198 expression has not been previously reported in the published literature. The only previous study of the potential usefulness of cell-free miR-198 was in lung cancer. Han *et al*. compared the expression of miR-198 in benign pleural effusion versus lung adenocarcinoma-associated

malignant pleural effusion[55] in a total of 107 patients. They found that out of all 160 miRNAs their assay could examine, miR-198 was significantly down-regulated in lung adenocarcinoma-associated malignant pleural effusion in comparison to benign pleural effusion. Their analysis also revealed that expression of miR-198 in pleural effusion can be used to clinically distinguish between patients with benign pleural effusion and those with pleural effusion from lung adenocarcinoma. Therefore, our study further strengthens the fact that cell-free miRNA is detectable and may be very useful clinically in non-invasive detection and diagnosis of cancers.

The clinical component of our study involved only 11 human participants: four normal healthy volunteers and seven patients with pancreatic cancer. We acknowledge that this number is relatively small. Ideally, we would want to use a larger number of both normal healthy participants and participants with pancreatic cancer. In addition, we would also want to include among the participants with pancreatic cancer both individuals that are responsive and those resistant to gemcitabine. This will enable us to get an indication as to whether plasma miR-198 expression can be used to monitor response to treatment of pancreatic cancer with gemcitabine. Fortunately, the preliminary data we now have from this current study will enable us to perform a power analysis to determine the exact numbers of participants we need to recruit for a larger study that may prove to be definitive. Also, the mechanism for decreased plasma miR-198 expression in pancreatic cancer patients in comparison to normal healthy control participants is currently unclear. Studies of other miRNAs suggest that an exosome-mediated mechanism may be involved.[57] Future studies should investigate this potential mechanism experimentally.

In conclusion, our study is the first to examine the role of miR-198 in resistance to gemcitabine by pancreatic cancer cells and it is the first study to evaluate the plasma expression profile of miR-198 in pancreatic cancer patients in comparison to normal healthy individuals. Our experiments showed that loss of miR-198 may be a novel mechanism for resistance to gemcitabine in pancreatic cancer, that miR-198 may have therapeutic efficacy in pancreatic cancer and that plasma miR-198 expression may be a potential novel plasma biomarker for use in non-invasive early detection and diagnosis in pancreatic cancer.

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BIOGRAPHICAL SKETCH

Olorunseun Olatunji Ogunwobi was admitted to study medicine at the prestigious University of Ibadan, Ibadan, Nigeria. The following year he was awarded the Shell Petroleum Development Corporation Scholarship (National Merit Category) for the entire duration of his medical training. He successfully obtained his medical degree and was awarded an international student scholarship to study Biomedical Science at the University of Hull, Hull, United Kingdom. He was awarded the Master of Science degree in Biomedical Science and immediately received the Norfolk and Norwich University Hospital Bicentenary studentship to commence his PhD training under the supervision of Dr. Ian Beales of the School of Medicine, University of East Anglia. Shortly thereafter he received additional support for his research from the Institute of Biomedical Science in London, United Kingdom. Dr. Ogunwobi is currently a postdoctoral fellow in cancer biology at the University of Florida. In 2012 he was awarded an Advanced Post-Graduate Program in Clinical Investigation (APPCI) scholarship by the Clinical and Translational Science Institute (CTSI) of the University of Florida, through which he completed his Master of Science in Medical Sciences with a concentration in Clinical and Translational Science.