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Oxytocin Receptor and MicroRNA-198/c-MET Signaling as Mechanisms of Chemoresistance in Pancreatic Cancer

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OXYTOCIN RECEPTOR AND MICRORNA-198 SIGNALING AS MECHANISMS OF CHEMORESISTANCE IN PANCREATIC CANCER

by

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ABSTRACT

Oxytocin receptor and microRNA-198 signaling as mechanisms of chemoresistance in pancreatic cancer

by

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Advisor: Olorunseun O. Ogunwobi, MD, PhD

Despite some recent advances, the detailed molecular mechanisms of pancreatic cancer (PC) are still not clearly understood, and the prognosis remains very dismal for PC patients. Consequently, there is an urgent need for a better understanding of the detailed molecular mechanisms of PC and the development of novel prognostic biomarkers and more effective treatment options.

MicroRNAs (miRNAs) are small non-coding RNAs, about 19 to 22 nucleotides in length, that inhibit translation by typically binding to 3'UTR of the target mRNA. They have recently gained much attention as key regulators in tumor initiation, progression, and chemoresistance in a variety of cancers, including PC. MicroRNA-198 (miR-198) is significantly downregulated in a variety of cancers, including PC, acting as a tumor suppressor. However, its role in PC chemoresistance is not fully understood.

Hepatocyte growth factor receptor (c-MET), a well-known proto-oncogene, is overexpressed in PC and has been implicated in PC chemoresistance and is known to lead to cell proliferation via the extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling pathway. miR-198 directly regulates c-MET in hepatocellular carcinoma (HCC) and colorectal cancer (CRC).

There is an emerging link between oxytocin (OXT), the oxytocin receptor (OXTR), and cancer. However, the role of OXT or the OXTR in PC remains unknown. OXT binds to OXTR and

activates a series of downstream elements, which leads to the transient activation of ERK1/2, which promotes proliferation.

This study aimed to determine the role of miR-198 regulation of c-MET in PC chemoresistance and to investigate the role of OXT and OXTR in PC chemoresistance. We hypothesized that ERK1/2 signaling is a mediator of OXTR – dependent and miR-198/c-Met - dependent chemoresistance in PC. This hypothesis was tested using a combination of molecular and functional studies in human PC cell line models categorized in terms of their responsiveness to the current standard of care in PC, gemcitabine (Gemzar).

In this study, we have demonstrated that miR198 shows decreased expression in the human PC cell line that is more unresponsive to gemcitabine, PANC-1 than in the human PC cell line that is more responsive to gemcitabine, L3.6pl. We also observed that miR-198 regulates c-MET in the PANC-1 and L3.6pl cell lines, and specifically targets the 3'-UTR of c-MET in the PANC-1 cell line. We also found that miR-198 increases gemcitabine sensitivity in PANC-1, but not via the ERK1/2 signaling pathway.

It is also notable that OXT and OXTR are expressed in the human PC cell lines PANC-1 and L3.6pl. Inhibiting the OXTR increases gemcitabine sensitivity, but not via the ERK1/2 signaling pathway. Inhibition of OXTR also resulted in decreased ERK1/2, pERK1/2, and cyclin D1 protein expression as well as increased caspase 3 activity.

We also assessed the clinical relevance, in terms of overall survival and disease-free months, of OXT and OXTR genetic alterations in PC and HCC cases by analyzing information regarding these alterations from The Cancer Genome Atlas (TCGA). Overall, we found that PC and HCC patients with genetic alterations in OXT and OXTR have poorer outcomes.

The findings from this study indicate that miR-198 targets c-MET in PC and suggests that miR-198 plays a role in PC chemoresistance. Further, OXT and OXTR are important in PC and HCC progression and survival in a subset of patients. OXT and OXTR, therefore, could potentially have prognostic and therapeutic implications in a subset of PC and HCC patients.

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Figure 1 from Chapter 3 and Figures 5, 6, 7, and 8 from Chapter 4 in this dissertation have been published in *Hepatobiliary and Pancreatic Diseases International*. Figures 9,10, and 11 from Chapter 4 have been published in *SN Comprehensive Clinical Medicine*.

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LIST OF ABBREVIATIONS

3’-UTR	Three prime untranslated region
5-FU	Five-fluorouracil
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
c-MET	Hepatocyte growth factor receptor
CA19-9	Carbohydrate antigen 19-9
CEA	Carcinoembryonic antigen
Ct	Cycle threshold
Cyclin D1	Cyclin-dependent kinase inhibitor 1
DMEM	Dulbecco's Modified Eagle's Medium
ERK	Extracellular signal-regulated kinase 1 and 2
FBS	Fetal Bovine Serum
FOXM1	Forkhead box M1
FSTL1	Follistatin-related protein 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCC	Hepatocellular carcinoma
hENT-1	Human equilibrative transporter 1
HGF	Hepatocyte growth factor
L3.6pl	A human pancreatic cancer cell line
miR-198	MicroRNA-198
miR-370	MicroRNA-370

miRanda	A MicroRNA molecular target prediction algorithm
miRNAs	MicroRNAs
mRNA	Messenger Ribonucleic Acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
NC	Negative Control
OXT	Oxytocin
OXTR	Oxytocin receptor
OXTRi	Oxytocin receptor inhibitor
p-c-MET	Phosphorylated cellular Mesenchymal Epithelial Transition
PANC-1	A human pancreatic cancer cell line
PanIN	Pancreatic intraepithelial neoplasia
PC	Pancreatic cancer
PDAC	Pancreatic ductal adenocarcinoma
p-ERK1/2	Phosphorylated extracellular signal-regulated kinase 1 and 2
qPCR	Quantitative Polymerase Chain Reaction
SE	Standard error
TCGA	The Cancer Genome Atlas

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CHAPTER 1

INTRODUCTION

1.1 Pancreatic Cancer

According to the American Cancer Society 2020 Surveillance Research Report, pancreatic cancer (PC) is the 4th leading cause of cancer-related deaths in the United States.

Pancreatic ductal adenocarcinoma (PDAC) is the most common form of PC. PDAC has a median survival rate of only about 6 months. And while the 5-yr survival rate for PC has been rising slowly, and is now estimated to be 10%, the overall survival rate has not improved over 40 yrs as compared to other cancers. PC is expected to rise to the 2nd cause of cancer-related deaths in 2020.

PC remains one of the most devastating cancers with a poor prognosis [1]. Pancreatic ductal adenocarcinoma (PDAC) is the most common type of PC, and it is the fourth leading cause of cancer-related deaths in the United States [2, 3]. More than 70% of patients die within the first year of diagnosis with PC, and the 5-year survival rate is only 8% [4, 5] PC is commonly diagnosed at advanced, already metastatic stages where most patients will rely on chemotherapy, however, despite extensive research efforts in recent decades, PC remains resistant to almost all clinically available therapy regimens [6, 7].

PC remains one of the deadliest forms of cancer. It is the fourth leading cause of cancer-related deaths in the United States [2] and has the worst prognosis among all solid organ tumors. More than 70 percent of patients die within the first year of diagnosis with PC [4]. It is expected that PC will become the second leading cause of cancer-related deaths by 2030 [8]. Pancreatic ductal adenocarcinoma (PDAC) is the most common form of PC [6]; hence, for this project, we are focused on PDAC.

Consequently, the use of the term “pancreatic cancer” in this thesis proposal applies primarily to PDAC. Among all cancer types, PDAC is considered one of the most lethal tumors.

Although PDAC is responsible for only 3% of all new cancer cases, PDAC causes 7% of all cancer-related deaths [8].

PDAC originates in the epithelium of the pancreatic ducts and develops from a pre-malignant lesion termed pancreatic intraepithelial neoplasia (PanIN) [9]. The progression from dysplastic epithelium to invasive carcinoma parallels the progressive accumulation of genetic mutations, and almost all patients with fully established carcinoma carry one or more of four common genetic defects. K-RAS mutations are detected in ~30% of early neoplasms, and nearly 100% in advanced PDAC. [10] In 95% of tumors, the CDKN2A gene is inactivated, with a consequent loss of the p16 protein. In 50–75% of tumors, TP53 is abnormal, and SMAD4/DPC4 is downregulated in half the cases, resulting in an aberrant TGF-β signaling cascade [11].

The molecular mechanisms underlying PDAC oncogenesis are still obscure. The only environmental factor that has been agreed to confer a risk for PDAC is tobacco usage, with the threat to smokers 2.5–3.6 times that of non-smokers [12]. Increased risk of PDAC has also been reported in patients with chronic pancreatitis and diabetes, and some researchers attribute an increased risk to high-fat high-cholesterol diets. However, studies are inconclusive[13]. In 5–10% of patients, the disease has a familial pattern, either as part of a germ-line cancer-predisposing syndrome, or involving other genetic mutations clustering in families such as with BRCA2 [14].

At present, there are no specific symptoms or reliable early detection markers for the diagnosis of early-stage PDAC. The only tumor markers for PC that are currently being used in a clinical setting are carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA), both of which have profound limitations because of low sensitivity and specificity. Besides the inability of CA19-9 and CEA to discriminate malign and benign disease, they are non-specific tumor markers. In fact, in many diseases, the serum levels of these markers show variations [8].

As a result, PDAC typically will require invasive procedures to be diagnosed and is, in fact, a silent disease that, in most cases, would have been invasive and metastatic upon detection.

A tumor marker is a naturally occurring molecule that can identify the presence of cancer, assess prognosis, or monitor response to therapy. The lifetime risk of PC in the general population is low 1%. Because of this, a biomarker must have a high specificity (95%) to avoid unacceptable false-positive rates. The ability to identify PC early would likely improve the outcome of patients. Differentiating PC from diseases that can mimic it, such as chronic pancreatitis, is a problem for many new markers. CA19–9 remains the gold standard against which all new markers are compared.

Standard treatments for advanced stages of the disease include radiotherapy and chemotherapy regimens. Radiotherapy shows some utility in treating regionally confined cancers, but it is often too toxic for tissues surrounding the neoplasia [15]. Because in most PDAC cases, the disease is already metastatic at the time of detection, most patients will rely on chemotherapy. Widely used PDAC chemotherapeutic regimens include 5-fluorouracil (5-FU) and gemcitabine. Gemcitabine (2', 2'-difluorodeoxycytidine; dFdC) is the standard of care chemotherapeutic agent for PDAC patients. It is a nucleoside analog of cytidine and thus functions by inhibiting DNA synthesis. 5-FU is a nucleoside analog of thymine and uracil. It also blocks DNA synthesis by inhibition of thymidylate synthase (TS) activity and also induces DNA and RNA strand breaks and apoptosis by direct incorporation into DNA and RNA [16]. The current major problem with the utility of these chemotherapeutic agents is that up to 94% of PDAC patients are either unresponsive or develop resistance to them [17-20].

Several different cellular pathways, including alterations in DNA repair or apoptotic pathways, transcriptional factors, and nucleotide metabolism enzymes, have been linked to gemcitabine and 5-FU resistance and sensitivity [14, 16, 21, 22].

Human equilibrative transporter 1 (hENT-1) is a nucleoside transporter protein that mediates cellular entry of cytotoxic chemotherapies such as gemcitabine. Preclinical data have shown that sensitivity to nucleoside analogs correlates with the expression of hENT-1. Overexpression of hENT-1 enhances gemcitabine response in human PC, and cells lacking hENT-1 expression are highly resistant to gemcitabine. This protein has gained potential interest as a predictive or prognostic marker in PC. However, the prognostic value in resected PC is unclear [23].

In the case of 5-FU, it has been widely thought that TS is a main molecular mechanism governing 5-FU sensitivity, and targeting TS is a key strategy to reverse 5-FU resistance [24-26]; however, the causal relationship between TS levels and 5-FU sensitivity is still quite controversial [27].

Overall, the detailed molecular mechanisms governing resistance to gemcitabine and 5-FU treatments in PDAC are still unclear. As recent advancements have had little impact, new understanding and approaches are desperately needed. The mechanisms underlying chemoresistance need to be elucidated. There is also an urgent need for early detection biomarkers of PDAC, clinically useful biomarkers for monitoring response to chemotherapy, and novel therapeutic approaches.

While PC is a genetic disease, it should be noted that a single gene activation is not responsible for the conversion between cancer and non-cancer states [28]. In fact, to develop PC, multiple genetic alterations must be accumulated in a single cell. Such alterations include

overexpression of receptor-ligand systems, oncogene activation, and loss of tumor suppressor genes [28].

1.2 MicroRNAs in Cancer

MicroRNAs (miRNAs) are naturally occurring, small non-coding RNAs, commonly about 19-22 nucleotides in length, and are highly conserved across different species [29]. *In vivo*, miRNAs are biosynthesized from differential miRNA genes and matured from pri-miRNA and pre-miRNA to miRNA [29]. In the first step, RNA polymerase II synthesizes miRNA precursors (pri-miRNAs), which have comparatively longer sequences using miRNA genes as templates. Following this transcription, Drosha and Dicer cut the pri-miRNA to produce pre-miRNA duplex, which moves from the nucleus to the cytosol. In the cytosol, the miRNA duplex is dissociated, and the single-stranded miRNA becomes the mature miRNA. The mature miRNA is then incorporated into the complex known as RNA-induced silencing complex (RISC). In the majority of cases, the binding of miRNA to the 3'-UTR of target mRNAs through partial complementarity results in miRNA-induced silencing of protein expression of the target genes through translational repression or mRNA degradation [29].

Emerging studies have been focused on the biosynthesis, function, and molecular regulation of miRNAs in normal physiological and pathological conditions in chronic diseases, including different types of cancers [30-32]. Recent studies have shown miRNAs to play a role in cell growth, differentiation, and apoptosis through the regulation of specific gene expression and hence a role in tumor initiation, progression, and chemoresistance in several cancers including breast cancer, lung adenocarcinoma, colorectal cancer, PC [31, 32]. The deregulation of miRNAs is observed in virtually all tumors and shows tumor-specific profiles [8]. Such findings suggest

that miRNAs may serve as new promising diagnostic and prognostic biomarkers as well as therapeutic targets in various cancers.

In PC cells, several miRNAs act as oncogenic mediators, which inhibit the expression of tumor suppressors, resulting in the development and progression of PC. On the other hand, some miRNAs function as tumor suppressors, which down-regulate the expression of oncogenes and thereby inhibit cancer growth and aggressiveness [29]. Significantly increased levels of oncogenic miRNAs and decreased levels of tumor-suppressive miRNAs have been found in pancreatic pre-cancerous and cancerous cells, suggesting that the aberrant expressions of miRNAs together with the dysregulation of cellular signal transduction contribute to the molecular basis of PC development and progression. In PDAC, miRNAs have been found to regulate the K-Ras, PI3K/AKT, NF-kB, p53, and hedgehog pathways related to gemcitabine resistance [33]. Direct evidence, however, of attenuation of gemcitabine resistance by miRNA level perturbations is still scarce, sometimes contradicting, and cell-type-specific rather than generalizable to all cancer types [14].

Recent studies have shown that miRNAs can be used as diagnostic and prognostic biomarkers of various cancers [34-37]. The detection of miRNAs in samples acquired through minimally or noninvasive procedures, such as serum, plasma, urine, and saliva, can have a positive impact on the clinical management of cancer patients [8]. miRNAs enable disease screening in high-risk patients (as diagnostic biomarkers) and evaluation of several disease parameters (as prognostic biomarkers) [8].

Also, since each miRNA has hundreds of different conserved or non-conserved targets, the alterations in the level of a specific miRNA could cause significant alterations in the expression of many genes, and thereby promote aberrations in multiple cellular signal transduction pathways,

resulting in development and progression of PC. Therefore, targeting these miRNAs could provide an efficient approach for the prevention or treatment of PC through the regulation of multiple genes [38].

1.3 MicroRNA-198

MicroRNA-198 (miR-198) is located on exon 11 of the 3' UTR of Follistatin-related protein 1 (FSTL1). miR-198 was recently discovered to be significantly down-regulated in pancreatic [39], colorectal [40], liver [41], ovarian [42], prostate [43], and lung [44] cancers acting as a tumor suppressor. For example, using pancreatic adenocarcinoma cell lines, patient pancreatic adenocarcinoma samples, and mouse models of pancreatic adenocarcinoma, Marin-Muller et al. found that miR-198 is down-regulated in pancreatic adenocarcinoma [39]. Furthermore, they reported that re-expression of miR-198 in PC cells leads to reduced tumor growth, reduced tumor metastasis, and increased survival, thus showing that miR-198 has tumor suppressor activity in pancreatic adenocarcinoma.

Additionally, a recent study showed that miR-198 is significantly downregulated in hepatocellular carcinoma in comparison to normal liver tissue by more than 5-fold, thus suggesting that miR-198 may be a tumor suppressor [45]. Even further, Tan et al. showed that miR-198 diminishes hepatocyte growth factor (HGF) stimulated migration and invasion of hepatocellular carcinoma cell negatively by modulating c-MET expression, suggesting miR-198 could serve as a novel suppressor of hepatocellular carcinoma cell migration and invasion by negative regulating the HGF/c-MET pathway [40]. c-MET is a receptor tyrosine kinase that, after binding with its ligand, hepatocyte growth factor, activates a wide range of different cellular signaling pathways, including those involved in proliferation, such as the ERK1/2 signaling pathway, motility, migration and invasion. Although c-MET is important in the control of tissue homeostasis under

normal physiological conditions, it is also a well-known proto-oncogene as it has been found to be aberrantly activated in human cancers via mutation, amplification or protein overexpression. Targeting the c-MET pathway could serve as a possible treatment option. It is important to note that, although c-MET has been implicated in chemoresistance in PC [46], the potential role of miR-198 regulating c-MET in PC chemoresistance is unknown.

1.4 MicroRNA-370 (miR-370)

Interestingly, miR-198 and miR-370 have been shown to be members of a cluster of three miRNAs whose expression levels were identified to regulate unstable angina pectoris [47]. This finding suggests that it is possible that these two miRNAs typically function similarly in a given disease or pathological situation. Consequently, it is possible that these two miRNAs have similar roles in PC chemoresistance, perhaps, both acting as tumor suppressors.

The expression of miR-370 has been shown to be decreased in human gastric tumors [48]. In another study, downregulation of miR-370 expression was a frequent event in leukemia cell lines and primary leukemic cells obtained from patients with de novo acute myeloid leukemia [49]. Downregulation of miR-370 expression enhanced the proliferation of leukemic cells [49].

FOXM1 is a member of the forkhead transcription factor family and is required for cell cycle progression, apoptosis, angiogenesis, and DNA damage repair [50]. FOXM1 is overexpressed in the majority of cancers, including PC. This overexpression is implicated to have a role in tumorigenesis, cancer progression, and metastasis [51]. Additionally, the aberrant expression of FOXM1 has been linked to chemoresistance in breast and ovarian cancers [50].

There is reported involvement of the miR-370-FOXM1 signaling in tumors other than PC. For example, the expression of miR-370 decreased in human gastric tumors, and expression of FOXM1 was directly downregulated by miR-370 in gastric cancer cell lines [51]. While miR-370

has been reported to act as a tumor suppressor by regulating FOXM1 in gastric cancer and while FOXM1 has been reported to be involved in chemoresistance in breast and ovarian cancers, the role of miR-370 in PC or PC chemoresistance has not been reported.

1.5 Oxytocin in Cancer

Oxytocin (OXT) is a nine-amino acid peptide hormone synthesized mainly in the hypothalamus and secreted into the bloodstream [52]. OXT is well known for its importance in the female reproductive system, mainly during pregnancy and lactation in women[53]. Although originally thought to be limited in its role to the female reproductive system, further research revealed OXT to also play important roles in the male reproductive system [54-57]. In addition to physical function, it is now better understood that OXT also plays major roles in social behaviors, such as stress and trust, anxiety, social interaction and bonding, and parental care [58-61]. OXT has also been shown to play roles in neuropsychiatric disorders, which are linked to these social behaviors [62]. Additionally, emerging evidence has linked OXT to roles in cancer. The OXT peptide is synthesized as an inactive precursor protein from the OXT gene and gets activated upon binding to the OXTR, a G-protein coupled receptor [52]. Recent findings have shown that OXT promotes cell proliferation in breast, prostate, osteosarcoma, and lung cancers [63-71]. To promote cell proliferation, OXT binds to its receptor, which results in MAPK cascade activation, leading to transient ERK1/2 phosphorylation and cell proliferation [52, 72]. OXT is a possible candidate to be involved in PC chemoresistance as it can result in alterations in apoptotic pathways via MAPK cascade activation cell proliferation pathway[73]. OXT has been identified as a putative molecular target of miR-370 by the miRNA-molecular target prediction algorithm, miRanda. The putative sequence alignment is shown in Figure 1 below. OXT is a nine amino acid peptide

hormone synthesized mainly in the hypothalamus and secreted into the bloodstream. The OXT peptide is synthesized as an inactive precursor protein from the OXT gene. The OXTR is a G-protein coupled receptor. OXT is well known for its importance in the female reproductive system, particularly during pregnancy and lactation in women.

The role of OXT in cancer is a new area of study. Recent findings have shown that OXT promotes cell proliferation in breast, prostate, and lung cancers [52, 63, 64]. To promote cell proliferation, OXT binds to its receptor, which results in MAPK cascade activation, leading to transient ERK1/2 phosphorylation and cell proliferation, as shown in Figure 3. To date, the role of OXT in PC has not been reported. Moreover, the role of OXT in PC chemoresistance is unknown. Because one miRNA can have multiple molecular targets, some of which can be clustered in terms of their function in pathology, and studies have reported that FOXM1 (a molecular target of miR-370) is involved in chemoresistance in various cancers, it is likely that OXT (which is a putative molecular target of miR-370) may also be involved in chemoresistance in PC. Furthermore, OXT is a likely candidate to be involved in PC chemoresistance as it can possibly result in alterations in apoptotic pathways via MAPK cascade activation cell proliferation pathway.

In recent years, with the advancement of therapies, the overall survival of patients with metastatic PC slightly increased from 6 months to nearly 1 year. However, 5-year survival of all stages of PC is still only 7% with 2% for distant and 26% for localized disease [30], suggesting that the clinical outcome of patients with PC remains poor. Therefore, the discovery of new diagnostic and prognostic biomarkers in PC is particularly important for patient survival.

In this project, we seek to elucidate novel molecular mechanisms underlying PC chemoresistance. We hypothesize miR-198 and miR-370 are miRNAs, which may have a role in

PC chemoresistance and that miR-370's role in PC chemoresistance involves direct molecular targeting of OXT.

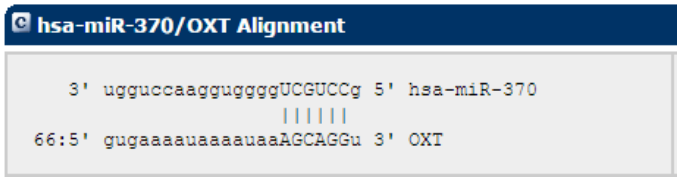


Figure 1.1: Shows the miR-370 and its alignment with the 3' UTR of its potential target OXT. Source: miRanda

1.6 Summary and Project Rationale

This study aimed to better understand the microRNA regulation of PDAC chemoresistance, with a specific focus on miR-198 and miR-370. Hepatocyte growth factor receptor (c-MET) is a well-known proto-oncogene is known to activate the ERK signaling pathway leading to cell proliferation. c-MET is overexpressed in PDAC and has been implicated in PDAC chemoresistance. Additionally, c-MET has been shown to be directly regulated by miR-198 in hepatocellular carcinoma. We, therefore, investigated whether the targeting of c-MET by miR-198 was regulating PDAC chemoresistance via the ERK signaling pathway. Additionally, after concluding that miR-370 does not target OXT in our panel of human PDAC cell lines, we focused our investigation on the roles of OXT and OXTR in PDAC chemoresistance. When OXT binds to its receptor, it is known to activate cell proliferation via ERK signaling. To the best of our knowledge, this was the first study that attempted to examine the roles of OXT and the OXTR PDAC and HCC progression and survival a well as in PDAC chemoresistance. It is likely that miR-198 and OXT could serve as novel, non-invasive, blood-based biomarkers in PDAC and HCC in the future. It is also likely that in the future, miR-198 could also serve as a therapeutic strategy

in PDAC chemoresistance. Even further, in the future, OXTR could also possibly have novel therapeutic value in a subset of PDAC and HCC patients.

CHAPTER 2

MATERIALS AND METHODS

2.1 Gene expression databases

The cBioPortal for Cancer Genomics (http://cbioportal.org), a Web resource for exploring, visualizing, and analyzing multidimensional cancer genomics data was used to retrieve information regarding OXT and OXTR alterations (mutations, putative copy-number alterations from GISTIC, mRNA expression Z-scores (RNA Seq V2 RSEM), protein expression Z-scores (RPPA) in PC. To visualize and analyze genomic alterations of OXT and OXTR in the TCGA data on PC, several options were selected in the web interface of cBioPortal. The cancer study "Pancreatic Adenocarcinoma (TCGA, Provisional)" and data type priority "Mutation," "CNA (DNA copy-number alterations)," "mRNA expression" and "Protein expression" were selected. For the gene set of interest, terms of "OXT" and "OXTR" were entered into the input box. Informed consent or statements of approval were not required for this study because the data was obtained from an open-access database.

2.2 Genomic alterations summary

The genomic alterations of OXT and OXTR in tumor samples are summarized in an OncoPrint the genomic alterations. On the table, rows represented genes and columns represented samples. Genomic alterations, including mutations, CNA (amplifications and homozygous deletions), and changes in gene expression, were summarized by glyphs and color-coding.

2.3 Survival analysis

From survival analysis, overall survival and disease-free survival differences were compared between samples with more than or equal to one alteration of query gene(s) and samples without alteration. This was done if the survival data were available.

2.4 Cell culture

Human pancreatic ductal adenocarcinoma cancer cell lines, PANC-1 purchased from the American Type Culture Collection (ATCC), USA, and L3.6pl obtained as a gift from Dr. Jose Trevino's laboratory from Department of Surgery, University of Florida (Gainesville, FL). The PANC-1 cell line is routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) with non-essential amino acids from Corning (Manassas, VA) and 10% fetal bovine serum (FBS). Both cell lines are maintained as adherent cultures at 37°C in a humidified atmosphere containing 5% CO_2.

2.5 Cell viability assay

Cell viability will be determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, as previously described [74]. Sample absorbance will be analyzed using a SpectraMax i3x Multi-Mode microplate reader at 490 nm. All experiments will be at least in triplicate.

2.6 Total RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from cell lines using the RNeasy Mini Kit from Qiagen, cat# 74104 (Germany). cDNA synthesis and quantitative real-time PCR (qRT-PCR) will be performed as previously described [75]. Briefly, extracted RNA was quantified with Nanodrop1000 spectrophotometer from NanoDrop (Madison, WI). 1 μg of RNA was reverse-transcribed into cDNA using QuantiTect Reverse Transcription kit from Qiagen, cat# 205311 (Germany). qRT-PCR was performed using SYBR Green to examine the mRNA expression levels of OXT and OXTR. Reactions were conducted in a 96-well spectrofluorometric thermal cycler ViiA™ 7 Real-Time PCR System from Life Technologies (Grand Island, NY). OXT and OXTR expressions were

normalized with GAPDH expression within each sample. Relative quantification of target gene expression was evaluated using the comparative cycle threshold (Ct) method. The following qPCR primers were used: forward human OXT: TTGAACAGAGCTCCACCGAC; reverse human OXT: TCTTCCGCGCAGCAGATATT; forward human OXTR: CCTTCATCGTGTGCTGGACG; reverse human OXTR: CTAGGAGCAGAGCACTTATG; forward human GAPDH: GAGTCAACGGATTTGGTCGT and reverse human GAPDH: TTGATTTTGGAGGGATCTCG. All primers were purchased from Sigma (St. Louis, MO).

2.7 Micro-RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

The expression of mature miR-198 and RNU6B_12 will be assessed by real-time PCR analysis. Total small RNA extraction will be accomplished by further processing using chloroform, isopropanol, and 75% ethanol and following the manufacturer's instructions. cDNA synthesis was performed using 0.6 ug of total RNA and miScript II RT kit (Qiagen, CA) following the 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). The target sequence for miR-198 is 5'GGUCCAGAGGGGAGAUAGGUUC, and for RNU6B_12 5'-CGCAAGGAUGACACGCAAAUUCGUGAAGCGUUCCAUAUUUUU in these assays.

2.8 Protein Extraction and Western Blotting

Cells will be grown in a monolayer in 6-well cell culture dishes to 70-80% confluency. Total protein will be extracted from the cells, and western blotting will be performed as previously described [76]. Primary antibodies used were against OXT: AB911 (Millipore, Burlington, Massachusetts), OXTR: ab2413 (Abcam plc, Cambridge, UK), and beta-actin: a5441 (Sigma-Aldrich, St. Louis, MO, USA), anti-ERK1/2 (#9102) and antiphosphorylated ERK1/2 (#9101) from Cell Signaling (Beverly, MA). Secondary antibodies used were either against mouse or rabbit (Sigma-Aldrich, St. Louis, MO, USA). Li-COR Odyssey CLx with infrared fluorescence, IRDye secondary antibodies, and imagers were also used to detect western blots without film or chemiluminescent substrates. Western blots were analyzed and quantified with Odyssey imager, Image Studio version 5.

2.9 miRNA and gemcitabine/5-FU Co-treatment Experiment

To determine the effects of miR-198 pretreatment on the gemcitabine potency, cells will be seeded in 100 mm Petri dishes at the initial density of $2x10^5$ cells and transfected with various concentrations of oligonucleotide control, oligonucleotide mimic or inhibitor of miR-198 for 24 h. Cells will then be collected and transferred to a 96 well plate ($3x10^3$ cells/well). After incubating for 24 h to allow cells to stabilize, gemcitabine will be added in a 7-point serial dilution series, 3 replicate plate columns per treatment. After 72 h of gemcitabine treatment, cell growth inhibition will be determined using MTT assay. qPCR analysis will be done to assess the expression levels of miR-198.

2.10 miRNA oligonucleotide transfection

The cells will be plated one day before transfection. Transfection will be done using Lipofectamine 2000 and Opti-MEM medium (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Transfection efficiency will be measured using a fluorescently labeled miR-198 oligo, and the amount of fluorescence will be determined by a Luminescence Spectrometer. We will study the effects of miR-198 on c-MET mRNA expression, c-MET, p-c-MET, ERK1/2, and p-ERK1/2 protein expression levels.

2.11 Statistical analysis

For survival analysis, Kaplan-Meier plots with a log-rank test were performed to compare the overall survival and the disease-free survival of pancreatic adenocarcinoma with at least one alteration or without alteration in the query gene(s). Samples with over-expression were identified by a threshold of $Z>2$ (mean expression over 2 SDs). The *a* level was set at 0.05. All the analyses mentioned above were performed in cBioPortal. All experimental data are expressed as the mean ± standard error (SE) from at least three separate experiments performed in triplicate unless otherwise noted. The differences between groups were analyzed using a double-sided Student's t-test when only two groups are present, and the null hypothesis was rejected at the 0.05 level unless otherwise specified. ANOVA was used when comparing more than two groups.

CHAPTER 3

MIR-198 SIGNALING AS A MECHANISM OF CHEMORESISTANCE IN PANCREATIC CANCER

3.1 Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that have recently gained much attention as key regulators in cancer progression as well as non-invasive blood-based biomarkers. miR-198 was recently discovered to be significantly down-regulated in hepatocellular carcinoma (HCC), CRC, and PC. Moreover, in HCC and CRC, miR-198 acts as a tumor suppressor by directly targeting the 3' UTR of c-MET. c-MET is a proto-oncogene that is overexpressed in human PC and is involved in growth and invasion of PC. Recent studies have suggested that chemoresistance in PC may be due to overexpression of c-MET. Also, there is currently one study that shows miR-198 to be involved in PC chemoresistance; however, the potential role of miR-198 regulation of c-MET in PC and/or CRC chemoresistance is unknown. A combination of molecular and functional studies in cell culture models was used to study the regulation of miR-198 of c-MET in PDAC chemoresistance. A number of different cellular pathways, including alterations in apoptotic pathways, have been linked to gemcitabine and 5-FU resistance and sensitivity. We, therefore, sought to investigate the downstream molecular target of c-MET involved in pathways involved in resistance to apoptosis, such as the MAPK pathway (ERK1/2). ERK1/2 protein expression has also been found to be overexpressed in PANC-1.

We studied the role of miR-198 targeting c-MET in PDAC chemoresistance. We used the human PDAC cell lines, PANC-1 and L3.6pl as models for studying chemoresistance as they are at the two extreme ends of responsiveness to gemcitabine and 5-FU, the common chemotherapeutic agents used to treat PDAC patients. PANC-1 is highly unresponsive, while L3.6pl is very responsive to gemcitabine and 5-FU. qPCR analysis was used to assess the expression of miR-198 and mRNA expression of c-MET in PANC-1 and L3.6pl. Protein expression of c-MET was obtained via western blot analysis. Transfections with the synthetic

oligonucleotide mimic and inhibitor of miR-198 followed by qPCR analyses were done to determine if miR-198 was targeting c-MET. Dual luciferase assay was also used to further validate whether miR-198 was targeting the 3'-UTR of c-MET. MTT assay was used to study the effect of the synthetic oligonucleotide miR-198 mimic on response to gemcitabine and 5-FU. To test our hypothesis that gemcitabine sensitivity was mediated via the ERK1/2 signaling pathway, we also probed for ERK1/2 and pERK1/2.

Together, these data suggest that miR1-98 does target the 3'-UTR of c-MET and is involved in PDAC chemoresistance but is not mediated via the ERK1/2 signaling pathway.

3.2 Results

3.2.1 – Effect of gemcitabine on cell viability in a panel of human PDAC cell lines.

We first assessed the responsiveness to gemcitabine in a panel of human PC cell lines (PANC-1, MIA PaCa-2, Capan-1, and L3.6pl). As is the case with nearly all PDAC cell lines, all four of these cell lines carry KRAS mutations.

Gemcitabine (2', 2'-difluorodeoxycytidine; dFdC) is the standard of care chemotherapeutic drug for treating PC patients. It is a nucleoside analog of cytidine and acts by inhibiting DNA synthesis [77]. Figure 3.1 shows the varying responses to gemcitabine in the panel of human PC cell lines. It is noted that PANC-1 is the most unresponsive cell line to gemcitabine, while L3.6pl is the most responsive cell in this panel. These two cell lines, therefore, served as models in our study to investigate potential mechanisms of chemoresistance in PDAC as they represent the two extremes of responsiveness to gemcitabine. It should be noted that response to various chemotherapeutic agents may vary from cell line to cell line due to variations in their gene expression [78].

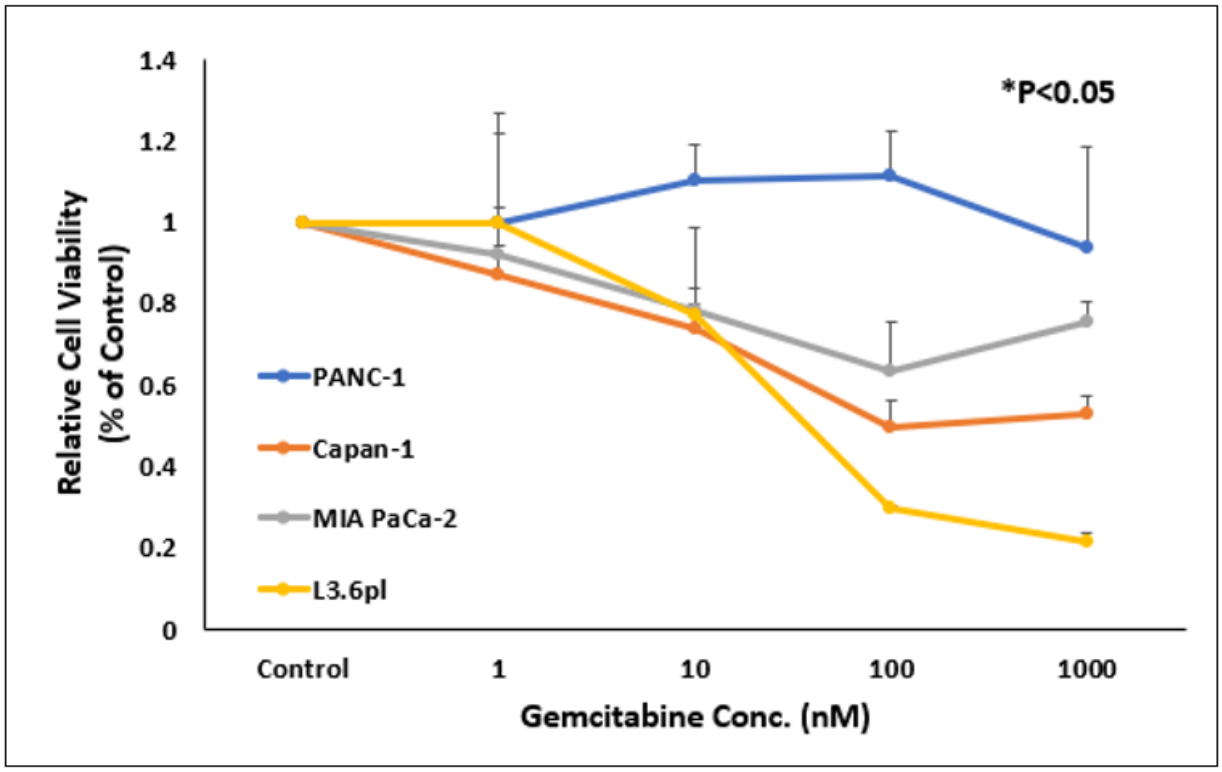


Figure 3.1: Effects of varying concentrations of gemcitabine on relative cell viability in a panel of human PDAC cell lines. Overall, PANC-1 is highly unresponsive, MIA PaCa-2 and Capan-1 moderately responsive, and L3.6pl highly responsive to gemcitabine. Student's paired *t* test or analysis of variance (ANOVA) was performed to compare cell proliferation of each cell line relative to PANC-1 across the varying concentrations.

3.2.2 – PANC-1 has lower miR-198 and higher c-MET mRNA expression than L3.6pl

Using several molecular target prediction algorithms tools (TargetScan, miRBase, and miRanda), c-MET was identified as one of the ten most frequently occurring directly molecular targets of miR-198. c-MET, the receptor for hepatocyte growth factor, is a proto-oncogene that is overexpressed in PDAC and is involved in growth and invasion of PDAC [38]. Some studies have also suggested that the overexpression of c-MET in PDAC may play a role in PDAC chemoresistance [46]. Other studies have shown that c-MET is directly targeted by miR-198 in

hepatocellular carcinoma [41]. However, whether miR-198 could be targeting c-MET in PDAC and playing a role in PC chemoresistance is still to be investigated.

We first performed RT-qPCR analysis to assess the expression levels of miR-198 in the PANC-1 and L3.6pl cell lines. We found that miR-198 expression was about 5-fold higher in L3.6pl as compared to PANC-1, as seen in Figure 3.2A. RT-qPCR analysis of c-MET mRNA expression showed that PANC-1 had a 5-fold higher expression level of c-MET than L3.6pl, as shown in Figure 3.2B. These findings suggest that miR-198 could be targeting c-MET.

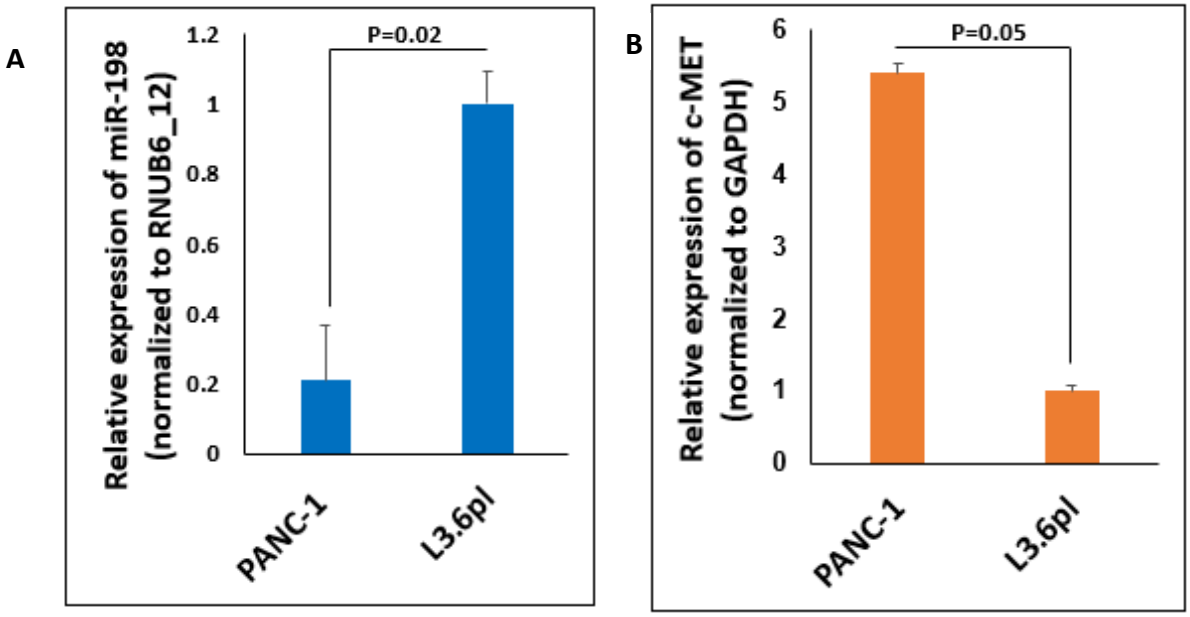


Figure 3.2: miR-198 expression is higher in L3.6pl cells than in PANC-1 cells and c-MET mRNA expression is lower in L3.6pl cells than in PANC-1 cells. miR-198 (A) and c-MET mRNA (B) expression in the human PDAC cell lines PANC-1 and L3.6pl via qPCR analysis. miR-198 expression was normalized against RNUB6_12, and c-MET expression was normalized against GAPDH. Data presented here are presented as mean ± the standard error of the mean (SEM).

3.2.3 – PANC-1 has higher c-MET protein expression than L3.6pl

Western blot analysis was also performed to assess the protein expression of c-MET in L3.6pl and PANC-1. As shown in Figure 3.3, we found that PANC-1 had a higher expression of c-MET at the protein level than L3.6pl. The differential expression of miR-198 and c-MET (at both the mRNA and protein levels) between these two cell lines, which are so varied in their response to the chemotherapeutic agent gemcitabine also suggests that miR-198 could be targeting c-MET and potentially plays a role in PDAC chemoresistance.

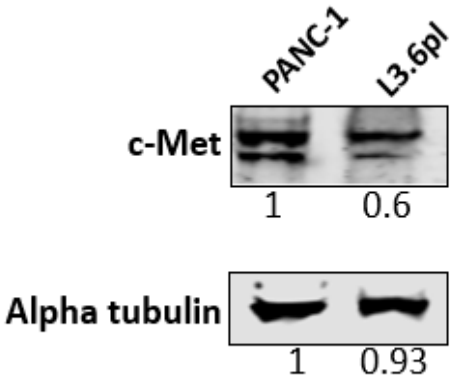


Figure 3.3: c-MET protein expression is higher in PANC-1 than in L3.6pl. Western blot analysis probing for c-MET in the human PDAC cell lines PANC-1 and L3.6pl. 20ug of whole-cell lysates were used. Numbers under the blot represent densiometric values normalized to the first band from the left in each row.

3.2.4 – miR-198 synthetic oligonucleotide mimic and inhibitor decrease and increase c-MET mRNA expression, respectively

To further investigate whether miR-198 was indeed targeting c-MET, we transfected PANC-1 cells (previously shown to have lower miR-198 expression than L3.6pl as seen in Figure 3.2A) with the synthetic oligonucleotide mimic of miR-198 and a negative control (NC) followed

by q-PCR analysis. We observed that at 10nM of the synthetic oligonucleotide mimic of miR-198 there was a decreased mRNA expression of c-MET. We also transfected L3.6pl cells (previously shown to have higher miR-198 expression than PANC-1 as seen in Figure 3.2A) with 1nM of the synthetic oligonucleotide inhibitor of miR-198 followed by q-PCR analysis and observed that there was an increase in c-MET mRNA expression. These findings further supported that miR-198 was targeting c-MET.

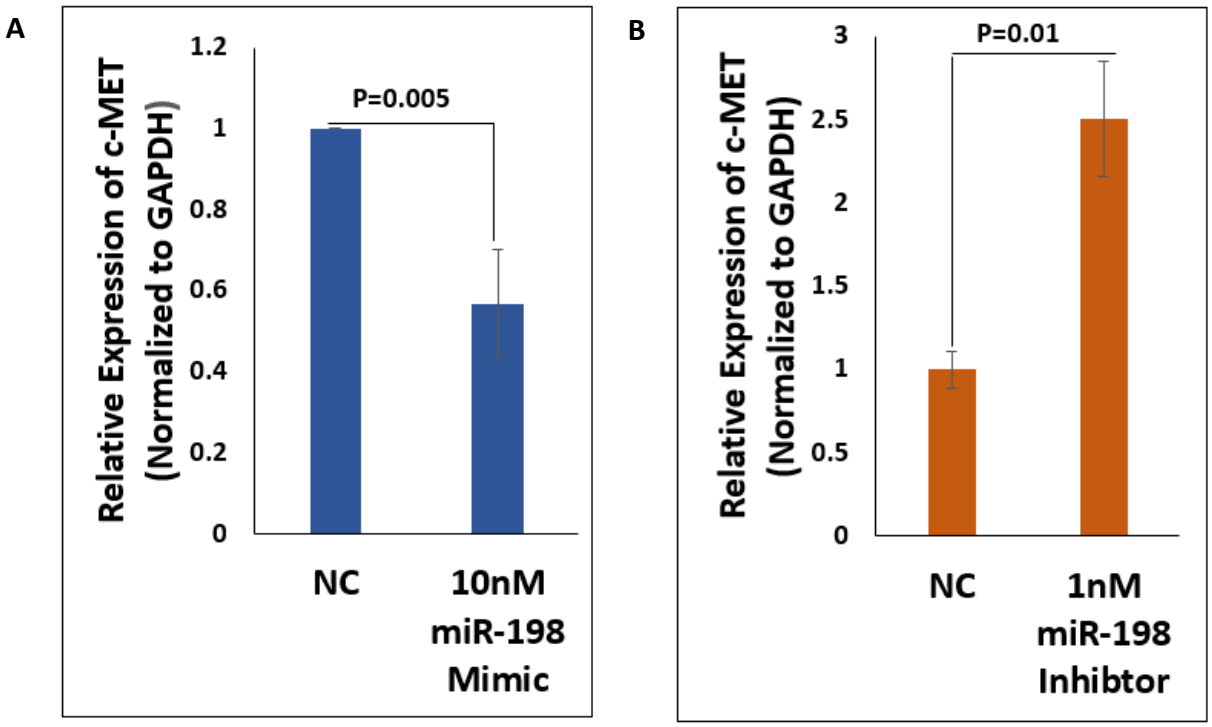


Figure 3.4: Transfection with the synthetic oligonucleotide mimic and inhibitor of mir-198 decreases and increases c-MET mRNA expression, respectively. c-MET mRNA expression after PANC-1 cells were transfected for 48hrs with the synthetic oligonucleotide mimic of miR-198 (A) and c-MET mRNA expression after L3.6pl cells were transfected with the synthetic oligonucleotide inhibitor of miR-198 (B) via qPCR analysis. c-MET mRNA expression was normalized against GAPDH. Data presented here are presented as mean ± the standard error of the mean (SEM).

3.2.5 – Dual luciferase assay confirms that miR-198 targets the 3'-UTR of c-MET in PANC-1

Previous studies have shown that miR-198 acts as a tumor suppressor by directly targeting the 3'-UTR of c-MET in HCC and CRC. To further confirm that miR-198 targets the 3'-UTR of c-MET in PANC-1, we performed dual luciferase assay where firefly luciferase was used to report the interaction between miR-198 and c-MET and Renilla luciferase was used to normalize expression between replicates. Our data, as shown in Figure 3.5, indicates that miR-198 does target the 3'-UTR of c-MET in PANC-1. We also performed dual luciferase assay to determine whether miR-198 was targeting the 3'-UTR in the L3.6pl cell line. However, our data indicated that it does not. A possible explanation might be that miR-198 targeting of the 3'-UTR of c-MET differs from one PDAC cell line to the other.

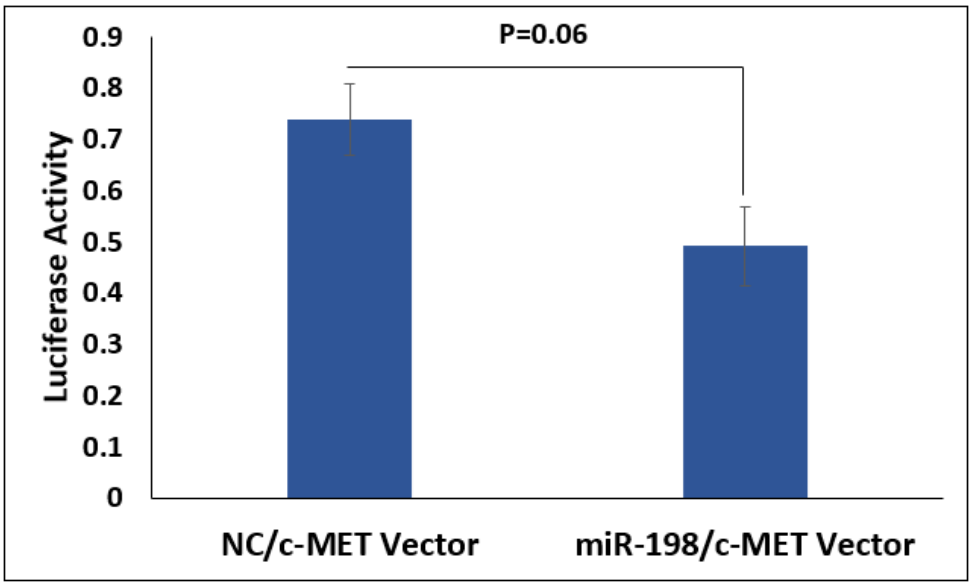


Figure 3.5: Dual-luciferase assay confirms that miR-198 targets the 3'-UTR of c-MET in PANC-1. Luc-Pair™ Duo-Luciferase Assay was used to determine if miR-198 binds to the 3'-UTR of c-MET. PANC-1 cells were co-transfected with GeneCopoeia pEZX-MT06 miRNA reporter empty vector or c-MET 3'UTR-containing plasmid with a 50nM non-targeting negative control or miR-198 50nM mimic for 24 hours. FLuc and RLuc activity were measured. Activity is normalized to the negative control luciferase activity set to 1.0. Data presented here are presented as mean ± the standard error of the mean (SEM).

3.2.6 – miR-198 synthetic oligonucleotide mimic increases PANC-1 gemcitabine sensitivity

To assess the effect of miR-198 on the response to gemcitabine in PANC-1 cells, PANC-1 cells were pre-treated with 10nM of the synthetic oligonucleotide mimic of miR-198 for 48hrs followed by treatment with varying concentrations of gemcitabine (1nM – 1000nM) for 72hrs. MTT assay was then performed to assess the cell viability of the cells. The negative controls were cells that were not transfected as well as cells that were transfected with a scrambled oligonucleotide sequence (NC). We found that cells that were transfected with the synthetic oligonucleotide mimic of miR-198 showed increased responsiveness to gemcitabine as compared to the negative controls.

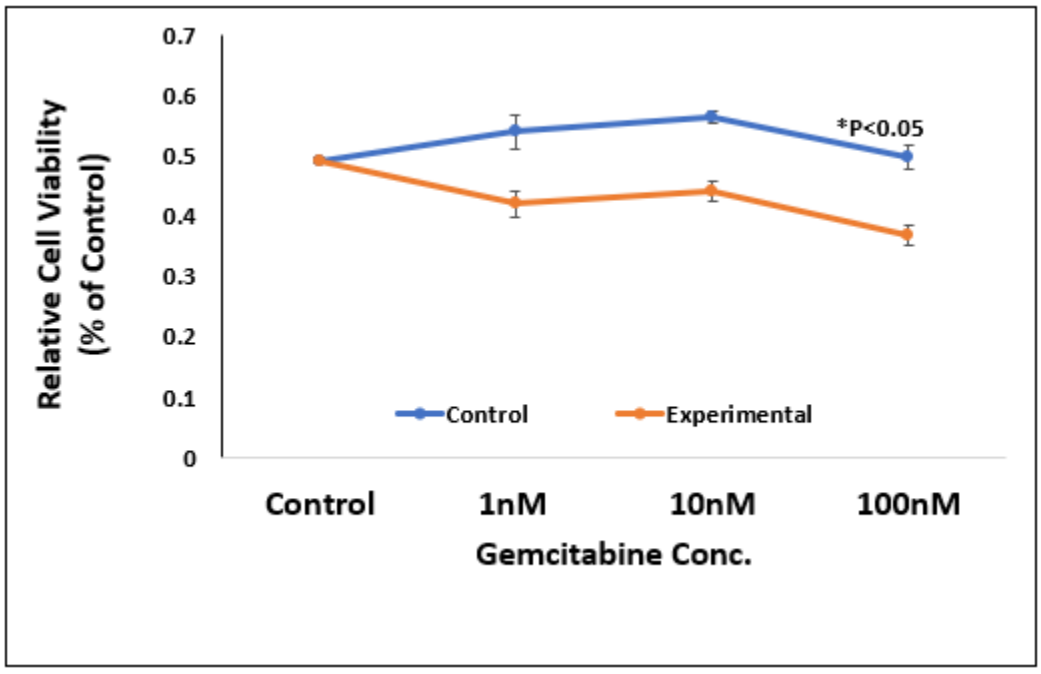


Figure 3.6: miR-198 synthetic oligonucleotide mimic increases PANC-1 gemcitabine sensitivity. MTT assay was performed to assess the cell viability under control (cells not pretreated with the synthetic oligonucleotide mimic of miR-198) and experimental (cells pre-treated with the synthetic oligonucleotide mimic of miR-198 for 48hrs) conditions. Data presented here are presented as mean ± the standard error of the mean (SEM).

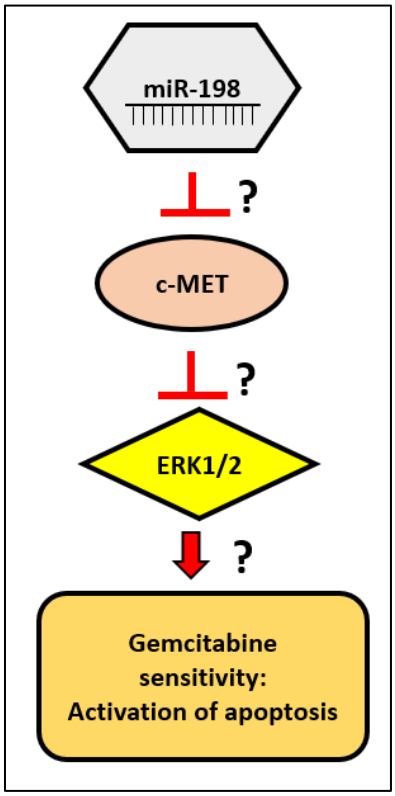
3.2.7 – miR-198/c-MET-dependent gemcitabine sensitivity does not occur via ERK1/2 signaling

The schematic in Figure 3.7 is a representation of the proposed mechanism of action of miR-198 targeting c-MET and playing a role in PDAC chemoresistance. c-MET is known to activate several cell proliferation pathways, including the MAPK pathway. As shown in the schematic in Figure 3.7, miR-198 is proposed to bind to c-MET and inhibit its translation, which in turn inhibits the MAPK pathway, specifically the ERK1/2 signaling pathway resulting in gemcitabine sensitivity by activating apoptosis.

To assess whether miR-198 is targeting c-MET, which then affects the downstream MAPK pathway, we investigated the protein expression via western blot analysis of ERK1/2 and p-ERK1/2, c-MET, and p-c-MET in PANC-1 and L3.6pl when treated with the miR-198 oligonucleotide mimic or inhibitor, respectively.

We expected to find in PANC-1 cells treated with the miR-198 oligonucleotide mimic, the ERK1/2, and p-ERK1/2 protein expression would decrease as compared to PANC-1 cell treated with the negative control. L3.6pl cells treated with the miR-198 inhibitor were expected to show an increase in ERK1/2 and p-ERK1/2 protein expression as compared to L3.6pl cells treated with the NC. However, as shown in Figure 3.8, we did not observe a change in p-ERK1/2 protein expression. It is interesting to note that a change in ERK1/2 expression was observed as shown in Figure 3.8, however. A possible explanation as to why a similar change in pERK1/2 expression was not observed could be due to a time-point issue. The specific experimental conditions here were 48hrs pre-treated with 1nM of the miR-198 inhibitor followed by gemcitabine treatments at 72hrs. It is likely that at different time-points it might be possible to observe a change in pERK1/2 expression, if it is indeed relevant to miR-198's activity.

The ERK1/2 and p-ERK1/2 protein expression data in PANC-1 cells treated with the miR-198 oligonucleotide mimic is not shown here as we were unable to obtain clear western blot images. The western blot data for c-MET and p-c-MET protein expression in PANC-1 and L3.6pl when treated with the miR-198 oligonucleotide mimic or inhibitor, respectively is not shown in this dissertation as the images were not very clear which we attributed to technically difficulties with the particular antibodies.



<u>Figure 3.7</u>: Schematic representing the proposed mechanism of action of miR-198 targeting c-MET and playing a role in PDAC chemoresistance. miR-198 is proposed to bind to the 3'-UTR of c-MET, thus inactivating it, which in turn does not lead to an activation of the MAPK pathway, thus resulting in cells being more sensitive to gemcitabine via the activation of apoptosis pathway.

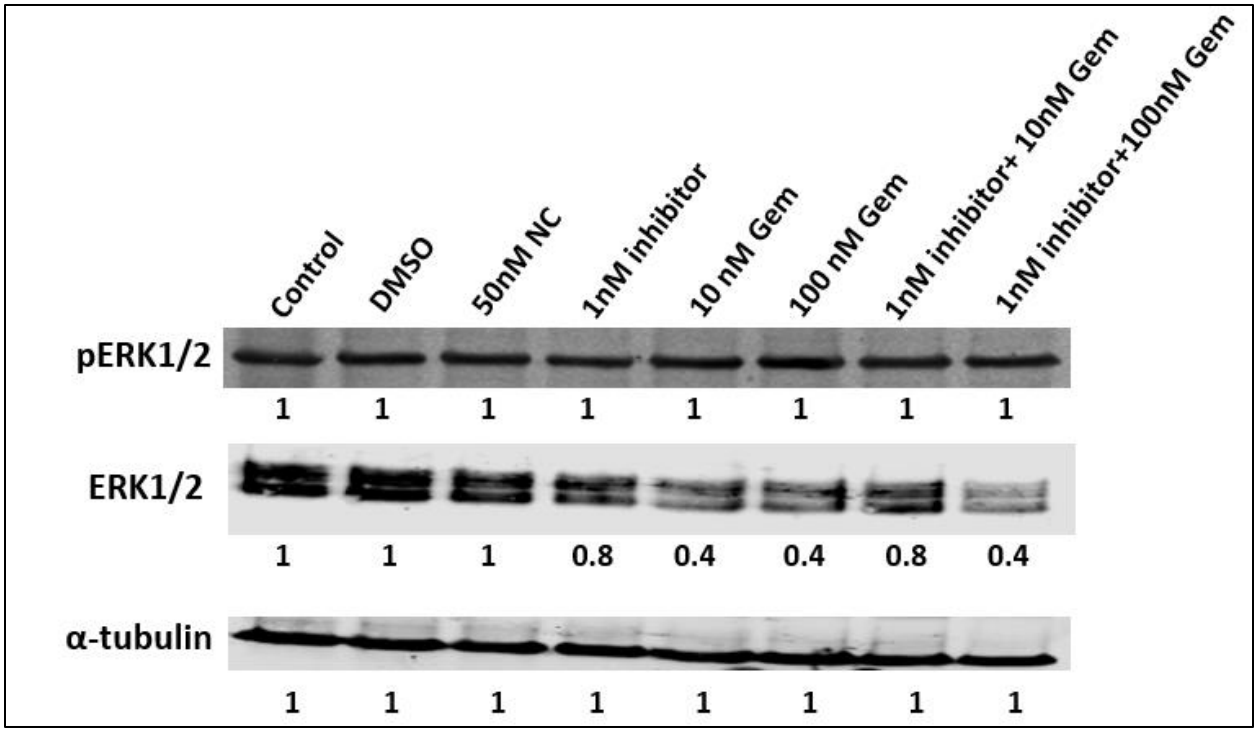


Figure 3.8: Inhibition of miR-198 does not affect ERK1/2 signaling in L3.6pl cells. Western blot analysis probing for ERK1/2 and p-ERK1/2 in the human PDAC cell line L3.6pl. 20ug of whole cell lysates were used. Numbers under the blot represent densiometric values normalized to the first band from the left in each row.

3.3 Discussion

The differential expression in miR-198, c-MET mRNA, and c-MET protein expression observed between PANC-1 (highly unresponsive to gemcitabine) and L3.6pl (highly responsive to gemcitabine) suggests that miR-198 could be involved in PDAC chemoresistance. Additional studies are needed to further elucidate this mechanism.

In addition to studying the effect of the synthetic oligonucleotide mimic of miR-198 on response to gemcitabine, we also looked at its effect on response to 5-FU in PANC-1. We did not observe a significant decrease in cell viability in cells pre-treated with 10nM of the synthetic oligonucleotide mimic of miR-198 followed by 5-FU (1nM – 1000nM) treatment for 72hrs. This

data (not shown) suggest that unlike gemcitabine, the mechanisms involving 5-FU sensitivity are not regulated by miR-198.

L3.6pl cells were also pre-treated with the synthetic oligonucleotide inhibitor of miR-198 followed by gemcitabine and 5-FU treatments. We expected to observe that inhibition of miR-198 would result in L3.6pl cells becoming less responsive to gemcitabine and 5-FU. However, we did not observe a significant difference between cells pre-treated with the synthetic oligonucleotide inhibitor of miR-198 and cells that were not before they were exposed to either gemcitabine or 5-FU. It is likely that miR-198 plays different roles in varying human PDAC cell lines. It would be worthy to investigate whether miR-198 regulates gemcitabine and 5-FU sensitivity in Capan-1 and MIAPaCa-2. These two cell lines have been shown to be moderately responsive to gemcitabine (Figure 3.1) and 5-FU (data not shown). Even though these two cell lines are not as responsive as L3.6pl to gemcitabine and 5-FU, being moderately responsive, they could serve as alternative models for studying PDAC chemoresistance, representing cell lines more responsive than PANC-1 to gemcitabine and 5-FU.

Previous studies have reported that activated ERK1/2 (pERK1/2) is overexpressed in PDAC. In the human PDAC cell line, BxPC-3 other studies have shown that knockdown of pERK1/2 leads to gemcitabine sensitivity. However, these mechanisms are still not very well understood. Although our data shows that miR-198 increases responsiveness to gemcitabine but not via the ERK1/2 signaling pathway, it is likely that it could be acting via one of the other cell proliferation pathways activated by c-MET such as STAT, PKC, FAK, and mTOR mediated pathways. This aspect could be further investigated in the future to reveal a better understanding. Thus, these are pathways which could be investigated in the future.

One of the initial steps which we took at the start of this thesis project was to identify the putative direct molecular targets of miR-198 using several microRNA target prediction algorithms. In addition to c-MET, we also found that other putative direct molecular targets of miR-198 included neurotrophic tyrosine kinase receptor type 3 (NTRK-3), forkhead box protein M1 (FOXM-1), and JUN. Using q-PCR analysis we investigated the mRNA expression of NTRK-3 and FOXM-1 in PANC-1 and L3.6pl and found them both to be differentially expressed between these two cell lines. PANC-1 showed a 3-fold higher mRNA expression of both NTRK-3 and FOXM-1 than L3.6pl. NTRK-3 is a membrane-bound kinase which phosphorylates itself and members of the MAPK pathway and regulates cell survival. Mutations in this gene have been associated with medulloblastomas, secretory breast carcinomas, pancreatic cancer and other cancers [79]. FOXM-1 is a key transcription factor that is widely expressed during the cell cycle and plays roles in cell proliferation, self-renewal, and tumorigenesis. In most human cancers, FOXM1 has been shown to be overexpressed and associated with poorer patient outcomes [80]. While NTRK-3 and FOXM-1 have been shown to play roles in PDAC chemoresistance, their potential regulation by miR-198 in PDAC chemoresistance have not been reported to date but would be interesting to investigate further.

CHAPTER 4

OXYTOCIN AND THE OXYTOCIN RECEPTOR IN CHEMORESISTANCE IN PANCREATIC CANCER

4.1 Introduction

Pancreatic and liver cancers are among the leading causes of cancer-related deaths. Pancreatic ductal adenocarcinoma (PDAC) and hepatocellular carcinoma (HCC) are the most common forms of pancreatic and liver cancers, respectively. Despite some advances, the detailed molecular mechanisms of PDAC and HCC are still not clearly understood, and the prognosis remains poor for these patients. Chemoresistance is a major problem for both PDAC and HCC patients as these diseases are commonly diagnosed at advanced, already metastatic stages where most patients will rely on chemotherapy, but most patients are resistant to almost all clinically available therapy regimens [6, 7]. Consequently, there is an urgent need for better understanding the detailed molecular mechanisms of these cancers and the development of novel prognostic biomarkers and more effective treatment options.

OXT is a nine-amino acid peptide hormone synthesized mainly in the hypothalamus and secreted into the bloodstream [52]. OXT is well known for its importance in the female reproductive system, mainly during pregnancy and lactation in women[53]. Emerging evidence has linked OXT to roles in cancer. The OXT peptide is synthesized as an inactive precursor protein from the OXT gene and gets activated upon bonding to the OXTR, a G-protein coupled receptor [52]. Recent findings have shown that OXT promotes cell proliferation in breast, prostate, osteosarcoma, and lung cancers [63-71]. To promote cell proliferation, OXT binds to its receptor, which results in MAPK cascade activation, leading to transient ERK1/2 phosphorylation and cell proliferation [52, 72]. OXT is a possible candidate to be involved in PC chemoresistance, as it can possibly result in alterations in apoptotic pathways via MAPK cascade activation[73].

The OXTR is known to be expressed in several human tissues, including the pancreas and liver, where it believed to play roles in regulating glycogen levels. However, the role of OXT and OXTR in PDAC and HCC has not been previously reported.

Here, we studied the role of OXT and OXTR in PDAC chemoresistance by investigating the mRNA (via qPCR analysis) and protein expression (via western blot analysis) of the OXT and OXTR in the human PDAC cell lines, PANC-1 and L3.6pl. PANC-1 is highly unresponsive while L3.6pl is very responsive to the chemotherapeutic agents commonly used to treat PDAC patients, gemcitabine, and 5-FU. Being at the two extreme ends of responsiveness to these chemotherapeutic agents, PANC-1 and L3.6pl served as our models for studying chemoresistance. Using MTT assay, we assessed the effect of inhibiting the OXTR on response to gemcitabine and 5-FU. We also tested our hypothesis that the OXTR inhibition was working via the ERK1/2 signaling pathway and plays a role in response to gemcitabine by probing for ERK1/2, pERK1/2, cyclin D1, and caspase 3 activity via western blotting.

We also assessed the clinical relevance, in terms of overall survival and disease-free months, of OXT and OXTR genetic alterations by retrieving and analyzed information regarding OXT and OXTR genetic alterations for 185 individual PDAC and 360 individual HCC patient data from The Cancer Genome Atlas (TCGA) provisional datasets via cBioPortal accessed in April 2018. The data we obtained herein suggest a role for OXT and OXTR in PDAC and HCC overall survival and disease-free months as well as PDAC chemoresistance.

4.2 Results

4.2.1 – miR-370 as a putative target of OXT

miR-370 has been identified as a miRNA which targets OXT via its 3' UTR by the miRNA molecular target prediction algorithm, miRanda. Figure 1 shows the potential sequence alignment between miR-370 and OXT. We were interested in knowing whether miR-370 is involved in PDAC chemoresistance via direct molecular targeting of OXT. Previous studies have shown that miR-370 acts as tumor suppressor in various cancers, including PC by directly targeting FOXM1.

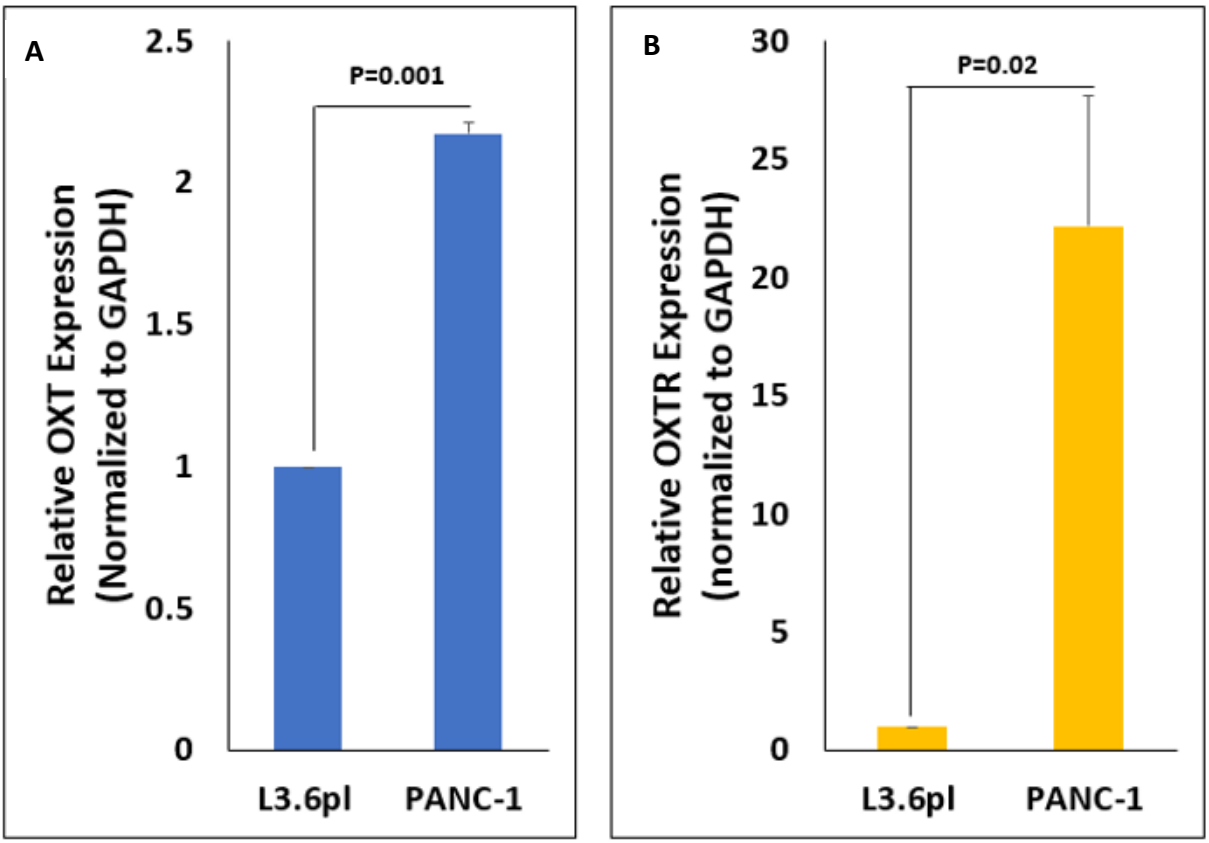
For this reason, we expected to find that L3.6pl (the highly responsive cell line) would have a higher expression level of miR-370 and lower expression level of FOXM1 as compared to PANC-1. However, we did not observe this. In fact, we found that PANC-1 had a 6-fold higher miR-370 expression level as compared to L3.6pl as well as about a 2-fold higher FOXM1 and OXT mRNA expression level (data not shown). Such expression patterns were not consistent with our hypothesis and did not suggest that miR-370 was directly targeting OXT. We, therefore, decided to no longer focus on the miRNA regulation of OXT, but instead to focus on the mechanisms downstream of the OXTR in this thesis work.

4.2.2 – OXT and the OXTR mRNA and protein expression are observed in PDAC cell lines

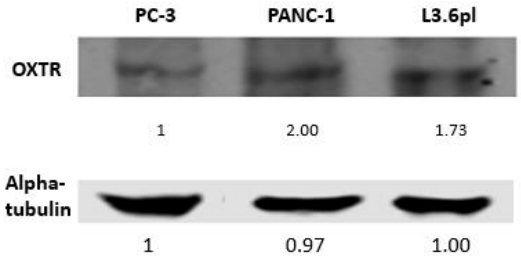
To study the possible role of OXT and OXTR in PC chemoresistance, we first assessed whether OXT and OXTR are expressed in human PDAC cell lines. qPCR analysis revealed for the first time that OXT and OXTR are expressed at the mRNA level in the two human PDAC cell lines, PANC-1 and L3.6pl, which are highly unresponsive and responsive to gemcitabine, respectively. We found that OXT mRNA expression is 1-fold higher in PANC-1 as compared to L3.6pl (Figure 4.1A). The analysis of *OXTR* mRNA expression is nearly 10-fold higher in PANC-

1 as compared to L3.6pl, as shown in Figure 4.1B. This differential mRNA expression observed between the cell lines studied that are varying in their responsiveness to gemcitabine suggest that OXT and OXTR gene expression changes may be involved in PDAC chemoresistance.

Western blot analysis was done to assess the protein expression levels of OXTR in PANC-1 and L3.6pl. Figure 4.2 shows that OXTR protein expression is higher in PANC-1 as compared to L3.6pl. Due to difficulties with the OXT antibody, we did not include OXT protein expression data here. It is interesting to note that while there is also a differential OXTR protein expression between PANC-1 and L3.6pl observed, this differential expression is not as large as the differential expression observed at the OXTR mRNA level between the two cell lines. This may suggest that it is possible that there are important post-transcriptional modifications involved here which should be explored in subsequent studies.



<u>Figure 4.1</u>: OXT and OXTR mRNA expression is lower in L3.6pl than in PANC-1. OXT (A) and OXTR (B) expression in the human PDAC cell lines PANC-1 and L3.6pl via qPCR analysis. OXT and OXTR mRNA expression were normalized against GAPDH. Data presented here are presented as mean ± the standard error of the mean (SEM).



<u>Figure 4.2</u>: OXTR protein expression is higher in PANC-1 as compared to L3.6pl. OXTR protein expression using western blotting analysis. PC-3 is a human prostate cancer cell line that served as a positive control. 20ug of whole-cell lysates were used. Numbers under the blot represent densiometric values normalized to the first band from the left in each row.

4.2.3 – Inhibiting the OXTR increases sensitivity to gemcitabine in PANC-1

To assess the effect of inhibiting the OXTR on response to gemcitabine, PANC-1 cells were pre-treated with 5uM of a synthetic OXTR inhibitor called L-371,267 for 24hrs followed by gemcitabine treatment at concentrations ranging from 10nM to 1000nM for 72hrs. It was observed via MTT assay that inhibiting the OXTR resulted in increased sensitivity to gemcitabine in PANC-1, as shown in Figure 4.3 suggesting that the OXTR may play a role in PDAC chemoresistance.

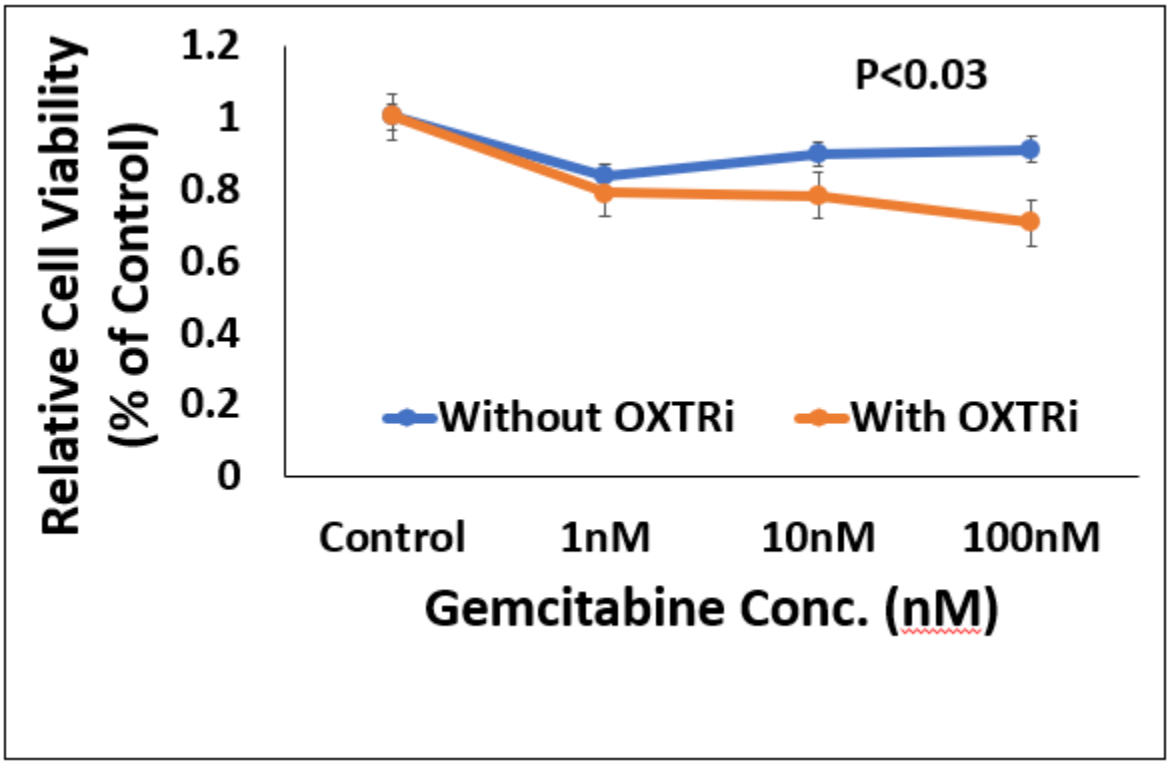


Figure 4.3: Inhibiting the OXTR increases sensitivity to gemcitabine in PANC-1. MTT assay was performed to assess the cell viability under the conditions where PANC-1 cells not treated (control) or treated with 5uM of the OXTR inhibitor, L-371,267 for 24hrs followed by gemcitabine treatment at varying concentrations for 72hrs. In the presence of the OXTR inhibitor, PANC-1 cells become more responsive to gemcitabine at concentrations ranging from 10nM – 1000nM. where cells were not pretreated with the OXTR inhibitor. Data presented here are presented as mean ± the standard error of the mean (SEM).

4.2.4 – Inhibiting the OXTR decreases ERK1/2, pERK1/2, and cyclin D1 protein expression in PANC-1

Previous studies have shown that the extracellular signal-regulated protein kinases (ERK) 1 and 2 activities contribute to gemcitabine resistance in PC cells [81]. The ERK1/2 signaling pathway is involved in the control of diverse cellular processes such as proliferation, survival, differentiation, and motility. This pathway is often upregulated in human tumors and represents an attractive target for the development of anticancer drugs. Phospho-ERK1/2 is activated upon OXT binding to the OXTR, leading to cell proliferation (Figure 4.4). Whether OXT contributes to PDAC chemoresistance via this pathway remains unknown. We, therefore, examined protein expression of ERK1/2 and p-ERK1/2 in PANC-1 and L3.6pl in absence and presence of the OXTR inhibitor, L-371,267 (5uM). A decreased expression in ERK1/2 was observed under the conditions when the OXTR was inhibited for 48hrs and when the OXTR was inhibited for 48hrs followed by 100nM gemcitabine treatment for 72hrs. A decrease in pERK1/2 expression was also observed under the condition when the OXTR was inhibited for 48hrs in PANC-1. These data suggest that the OXTR works via ERK1/2 signaling (Figure 4.4) in PANC-1 which has not been previously reported. No change in pERK1/2 expression was observed when the OXTR was inhibited for 48hrs, followed by 100nM gemcitabine treatment for 72hrs, however. Changes in pERK1/2 expression were also not observed under the conditions when PANC-1 cells were treated with 100nM gemcitabine or the OXTRi for 48hrs followed by 10nM gemcitabine. It could be possible that there might have been a time-point issue here. The specific experimental conditions here were either treatment with 100nM gemcitabine for 72hrs or inhibition of OXTR for 48hrs followed by gemcitabine treatment for 72hrs. Perhaps, at different time-points different pERK1/2 expression could be detected.

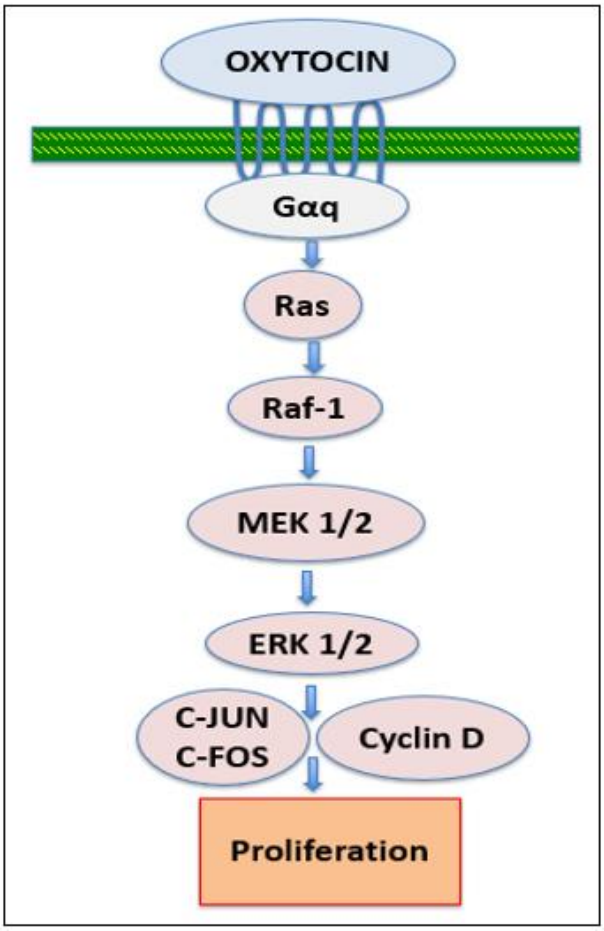


Figure 4.4: OXT related cell proliferation signaling pathway. By coupling of OXT to its receptor subunit, Gαq/11, Ras is activated, which then activates the MAPK pathway (Raf-1, MEK1/2, ERK1/2) leading to cell proliferation. Modified from: Lerman B, Harricharran T, Ogunwobi OO. OXT and cancer: An emerging link. *World J Clin Oncol.* 2018; 9(5):74-82.

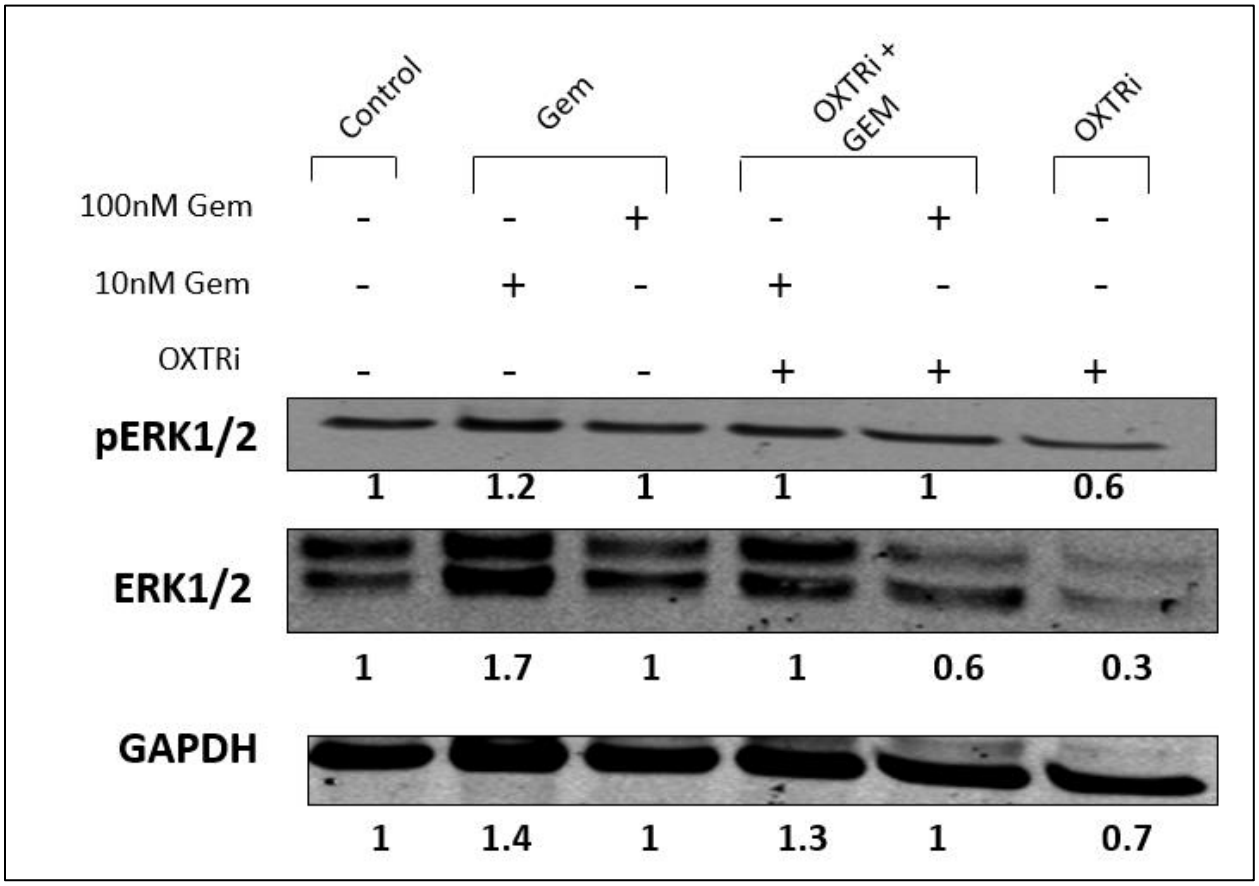


Figure 4.5: Inhibition of the OXTR decreases ERK1/2 and p-ERK1/2 protein expression. pERK1/2 and ERK1/2 protein expression using western blotting analysis. From left to right, PANC-1 cells: untreated (control), treated with 10nM gemcitabine for 72hrs, treated with 100nM gemcitabine, pre-treated with 5uM L-371,267 (an OXTR inhibitor) for 48hrs followed by treatment of 10nM gemcitabine for 72hrs, treated with 5uM L-371,267 for 48hrs followed by treatment with 100nM gemcitabine for 72hrs, and treated with 5uM L-371,267 for 48hrs. 20ug of whole-cell lysates were used. Numbers under the blot represent densiometric values normalized to the first band from the left in each row.

Cyclin-dependent kinase inhibitor 1 (Cyclin D1), an important cell cycle regulator of the G1-S transition [82] is downstream of ERK in the OXT-mediated cell proliferation pathways [72], as shown in Figure 4.4. We therefore also probed for the protein expression of cyclin D1 under the same treatment conditions that we probed for ERK1/2 and p-ERK1/2 expression. We observed that there was a decrease in cyclin D1 protein expression under the condition when the

OXTR was inhibited in PANC-1 cells, followed by gemcitabine treatment at a 100nM for 72hrs. We also observed a decrease in cyclin D1 protein expression under the condition when the OXTR was inhibited in PANC-1 cells. These data suggest that the OXTR could be acting via the proposed oxytocin-mediated cell proliferation pathway shown in Figure 4.4. It is also possible that this pathway is involved in the observed increase in gemcitabine sensitivity as shown in Figure 4.3.

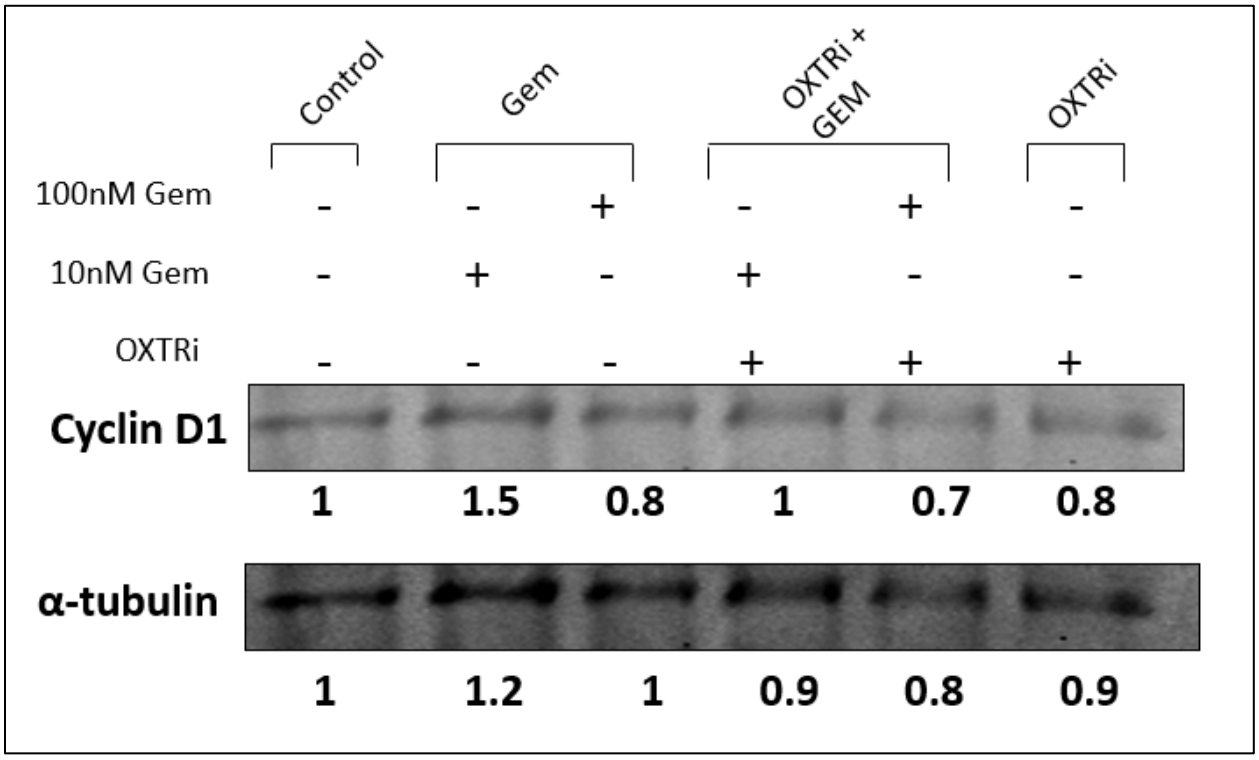


Figure 4.6: Inhibition of the OXTR decreases cyclin D1 protein expression. Cyclin D1 protein expression using western blotting analysis. From left to right, PANC-1 cells: untreated (control), treated with 10nM gemcitabine for 72hrs, treated with 100nM gemcitabine, pre-treated with 5uM L-371,267 (an OXTR inhibitor) for 48hrs followed by treatment of 10nM gemcitabine for 72hrs, treated with 5uM L-371,267 for 48hrs followed by treatment with 100nM gemcitabine for 72hrs, and treated with 5uM L-371,267 for 48hrs. 20ug of whole-cell lysates were used. Numbers under the blot represent densiometric values normalized to the first band from the left in each row.

4.2.5 – Inhibiting the OXTR increases apoptosis in PANC-1 cells

A number of factors have been shown to be correlated with gemcitabine resistance, including gemcitabine transport metabolism defects and alterations in the apoptotic pathways [78]. Caspase-3 is a cysteine–aspartic acid protease and is a well-known executioner caspase at the end of the intrinsic apoptotic cascade [83]. We therefore probed for pro- and cleaved- caspase 3 under the same treatment conditions that we probed for ERK1/2, p-ERK1/2, and cyclin D1. An increase in cleaved caspase 3 was observed under the condition when the OXTR was inhibited in PANC-1 cells. These data suggest that the OXTR plays a role in PC and could be a plausible therapeutic target.

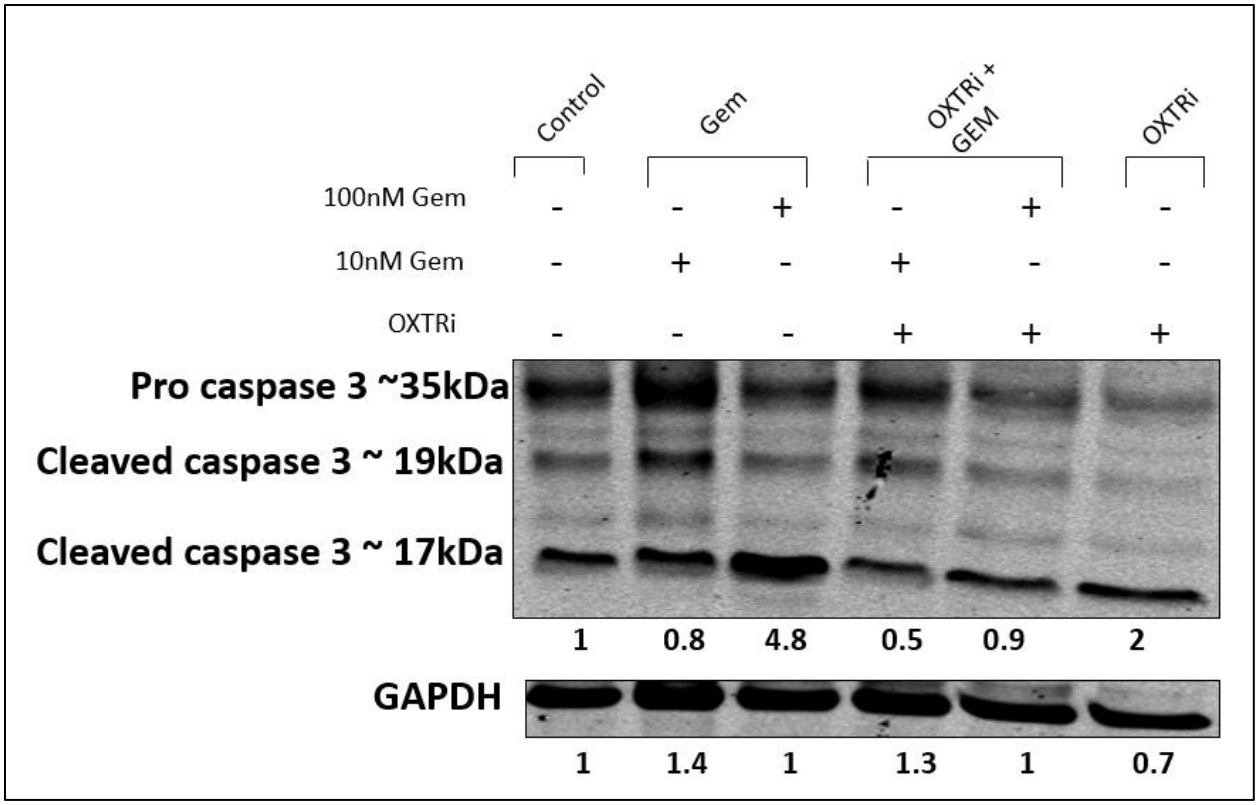


Figure 4.7: Inhibition of the OXTR increases apoptosis. Pro- and cleaved caspase 3 protein expression using western blotting analysis. From left to right, PANC-1 cells: untreated (control), treated with 10nM gemcitabine for 72hrs, treated with 100nM gemcitabine, pre-treated with 5uM L-371,267 (an OXTR inhibitor) for 48hrs followed by treatment of 10nM gemcitabine for 72hrs, treated with 5uM L-371,267 for 48hrs followed by treatment with 100nM gemcitabine for 72hrs, and treated with 5uM L-371,267 for 48hrs. 20ug of whole-cell lysates were used. Numbers under the blot represent densiometric values normalized to the first band from the left in each row.

4. 2. 6– Poorer survival outcomes with OXT and OXTR genetic alteration in PDAC patients

PC is a genetic disease, but the transition between cancer and non-cancer is not regulated by a simple switch activated by a single gene [28]. In fact, multiple genetic alterations, whose number is increasing, must accumulate in a single cell, including overexpression of receptor-ligand systems, oncogene activation, and loss of tumor suppressor genes, to develop PDAC.

Some *in vitro* and *in vivo* studies revealed that OXT is responsible for bivariate biological functions involved in cancer [52]. Preliminary studies have shown that most physiological

situations related to OXT secretion have some linkage with cancer outcome, either tumor regression or progression in different kinds of cancer [52]. To our knowledge, no previous studies have tried to explore whether OXT and OXTR genetic alterations were associated with clinical features in PDAC [84].

The Cancer Genome Atlas (TCGA) Data Portal contains information on DNA, RNA, proteins, and survival status in various cancers. We assessed the genetic alterations of OXT and OXTR in PC in the TCGA data sets using the cBioPortal for Cancer Genomics online platform. Additionally, we investigated whether these changes were associated with clinical outcomes.

We found that 5% (9 out of 185) of the sequenced PDAC cases/patients have genetic alterations or changes in gene expression in the OXT and *OXTR* genes, respectively (Figure 4.8). The OXT gene expression changes were all by an upregulation in OXT mRNA (Figure 4.8. The *OXTR* genetic alterations_and gene expression changes, (as shown in Figure 4.9), were either missense mutation (2 cases), amplification (1 case), or mRNA upregulation (6 cases).

We also obtained overall survival Kaplan-Meier estimates whenever present. This analysis shows that the median months survival was 18.66 for PC cases with gene expression changes in the OXT gene and 19.94 for cases without such gene expression changes (Figure 4.10). The median months survival was 15.11 for PC cases with genetic alterations and gene expression changes in the *OXTR* gene and 20.17 for cases without such genetic alterations or gene expression changes (Figure 4.11).

Disease/progression-free Kaplan-Meier estimates were also obtained whenever available. These data indicate that the median months disease-free was 14.75 for PC cases with changes in gene expression and 17.05 for cases without such changes in gene expression in the OXT gene. These findings show that OXT and OXTR genetic alterations and changes in gene expression could have a significant clinical impact in a subset of PDAC patients (Figure 4.12).

Figure 4.8: OXT genetic alterations in pancreatic cancer. Altered in 9 (5%) of 185 sequenced cases/patients (185 total). Tumor Samples with mRNA data (RNA Seq V2) included.

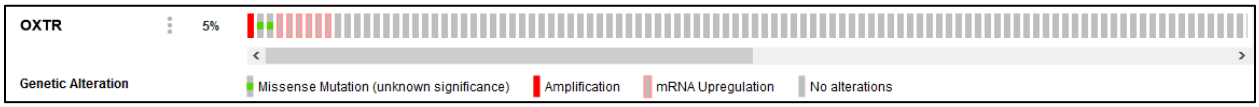
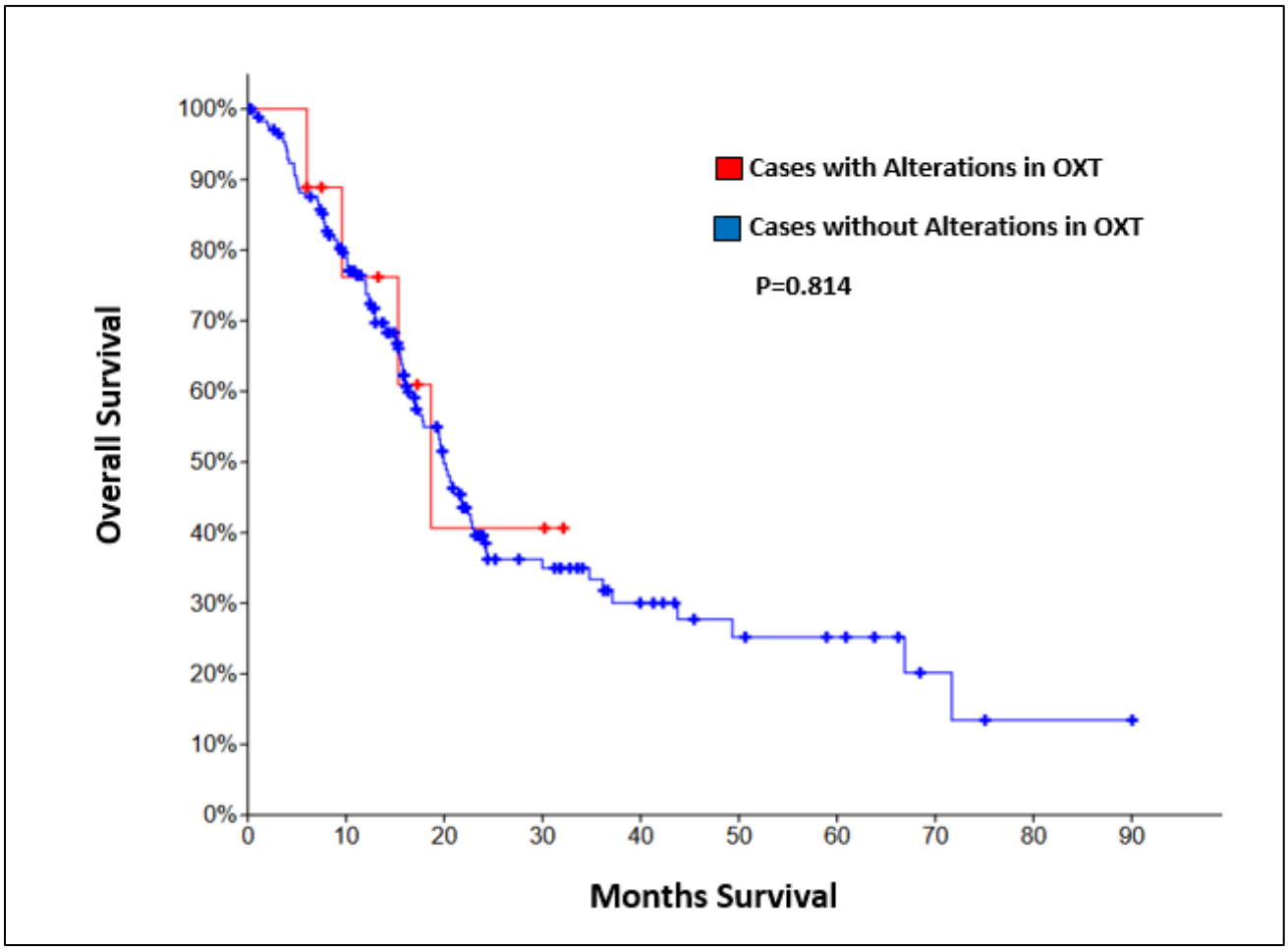


Figure 4.9: OXTR genetic alterations in pancreatic cancer. Altered in 9 (5%) of 185 sequenced cases/patients (185 total). Tumor Samples with mRNA data (RNA Seq V2) included.



<u>Figure 4.10</u>: Overall Survival Kaplan-Meier Estimate in pancreatic cancer. The median months survival was 18.66 for cases with alterations (mutations, putative copy-number alterations from GISTIC, mRNA expression Z-scores (RNA Seq V2 RSEM), protein expression Z-scores (RPPA) and 19.94 for cases without alterations in the OXT gene.

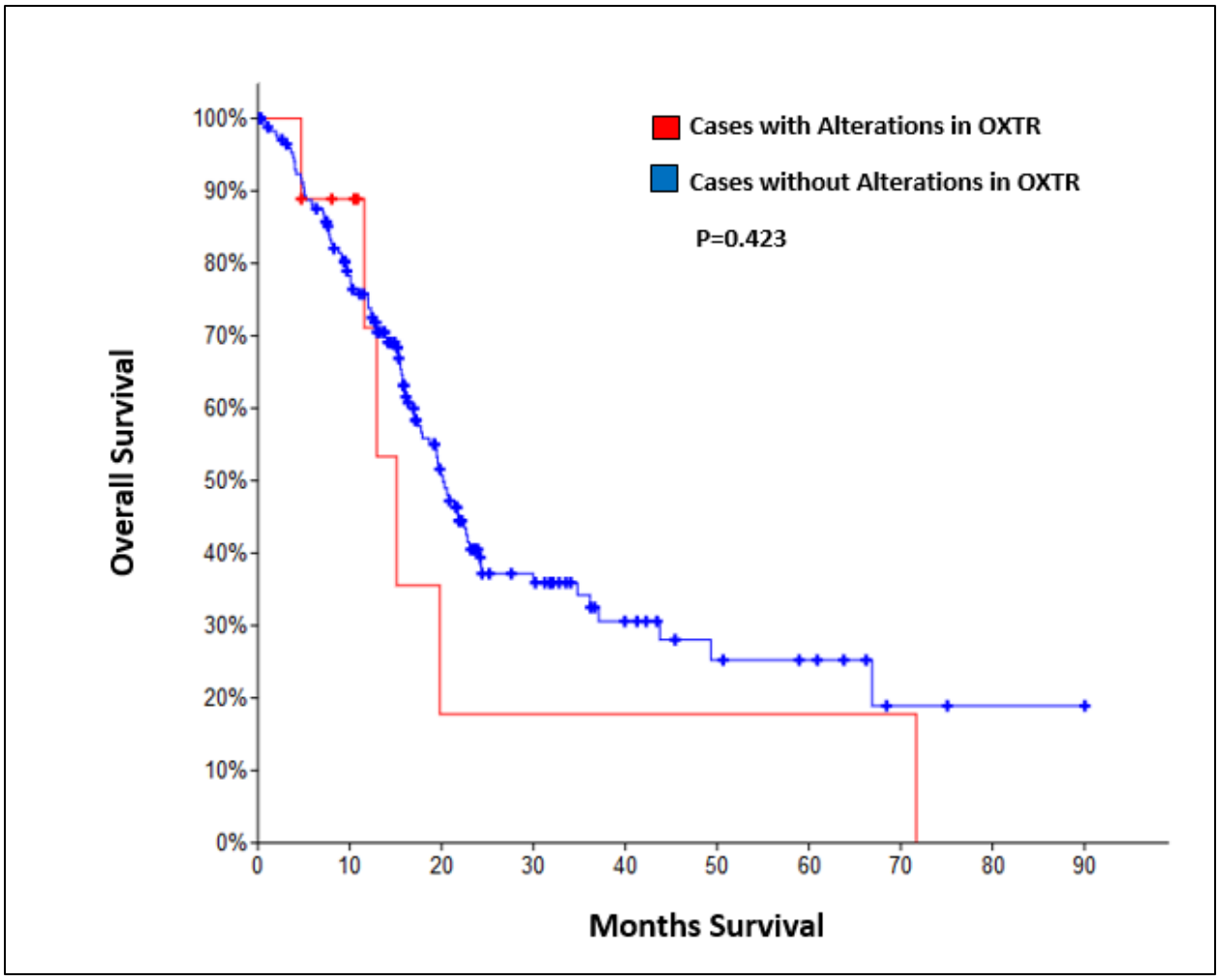
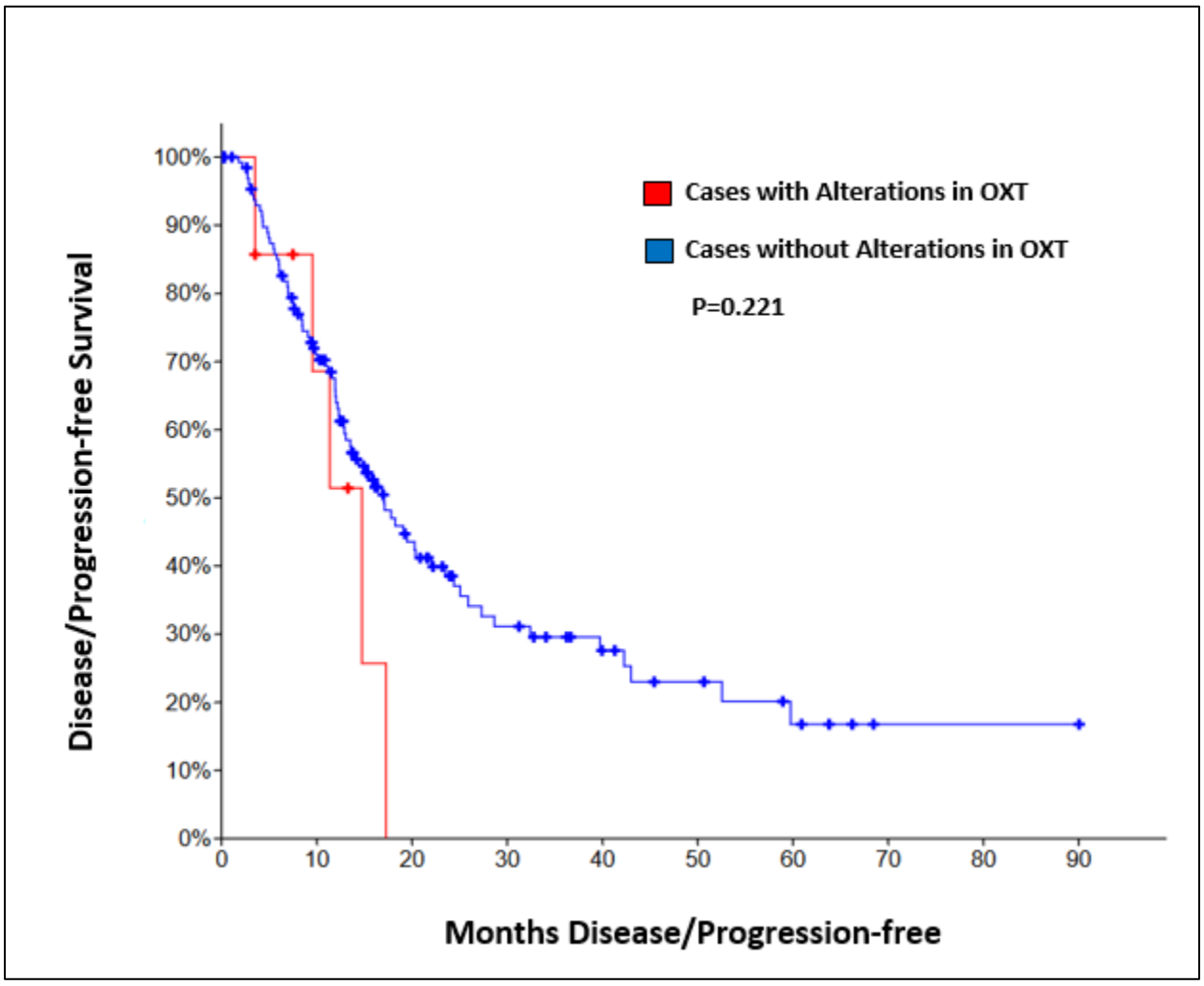


Figure 4.11: Overall Survival Kaplan-Meier Estimate in pancreatic cancer. The median months survival was 15.11 for cases with alterations (mutations, putative copy-number alterations from GISTIC, mRNA expression Z-scores (RNA Seq V2 RSEM), protein expression Z-scores (RPPA) and 20.17 for cases without alterations in the OXTR gene.



<u>Figure 4.12</u>: Disease/Progression-free Kaplan-Meier Estimate in pancreatic cancer. The median months disease-free was 14.75 for cases with alterations (mutations, putative copy-number alterations from GISTIC, mRNA expression Z-scores (RNA Seq V2 RSEM), protein expression Z-scores (RPPA) and 17.05 for cases without alterations in the OXT gene.

4. 2. 7 – Poorer survival outcomes with OXTR genetic alteration in HCC patients

While it is known that OXTR is expressed in liver tissues where it likely plays a role in regulating glycogen level [6], a role for OXT and OXTR in HCC has not been reported to date.

We, therefore, took a similar approach as we did for the TCGA PDAC cases and investigated whether OXT and OXTR genetic alterations were associated with clinical features in HCC.

We found that 1.7% (6 out of 360) and 3.0% (11 out of 360) of HCC patients have genetic alterations in the OXT and OXTR genes, respectively, in their HCC tissues. The OXT genetic alterations were amplification (1 case), and mRNA upregulation (5 cases) and the OXTR genetic alterations were either missense mutation (1 case), amplification (2 cases), or mRNA upregulation (9 cases) as shown in Figures 4.13 and 4.14.

The median months survival was 33.02 for HCC cases with genetic alterations in the OXTR gene and 60.84 for cases without such alterations (Figure 4.15). Overall survival data were not available for the OXT gene. The median months disease-free was 8.64 for cases with alterations and 21.55 for cases without alterations in the OXTR gene, as shown in Figure 4.16.

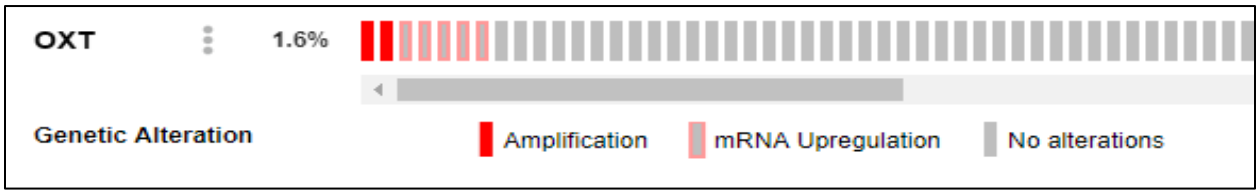


Figure 4.13: OXT genetic alterations in hepatocellular carcinoma (HCC). Altered in 6 (1.6%) of 360 sequenced cases/patients (360 total). Tumor Samples with mRNA data (RNA Seq V2) included.

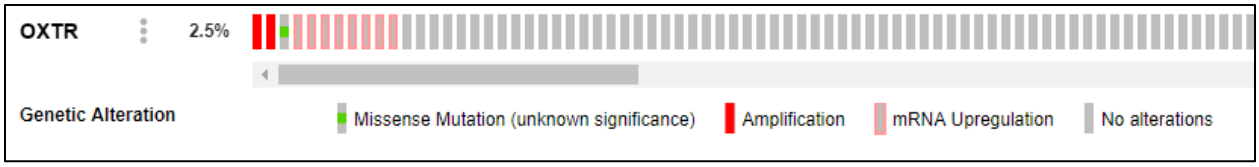


Figure 4.14: OXTR genetic alterations in hepatocellular carcinoma (HCC). Altered in 11 (2.5%) of 360 sequenced cases/patients (360 total). Tumor Samples with mRNA data (RNA Seq V2) included.

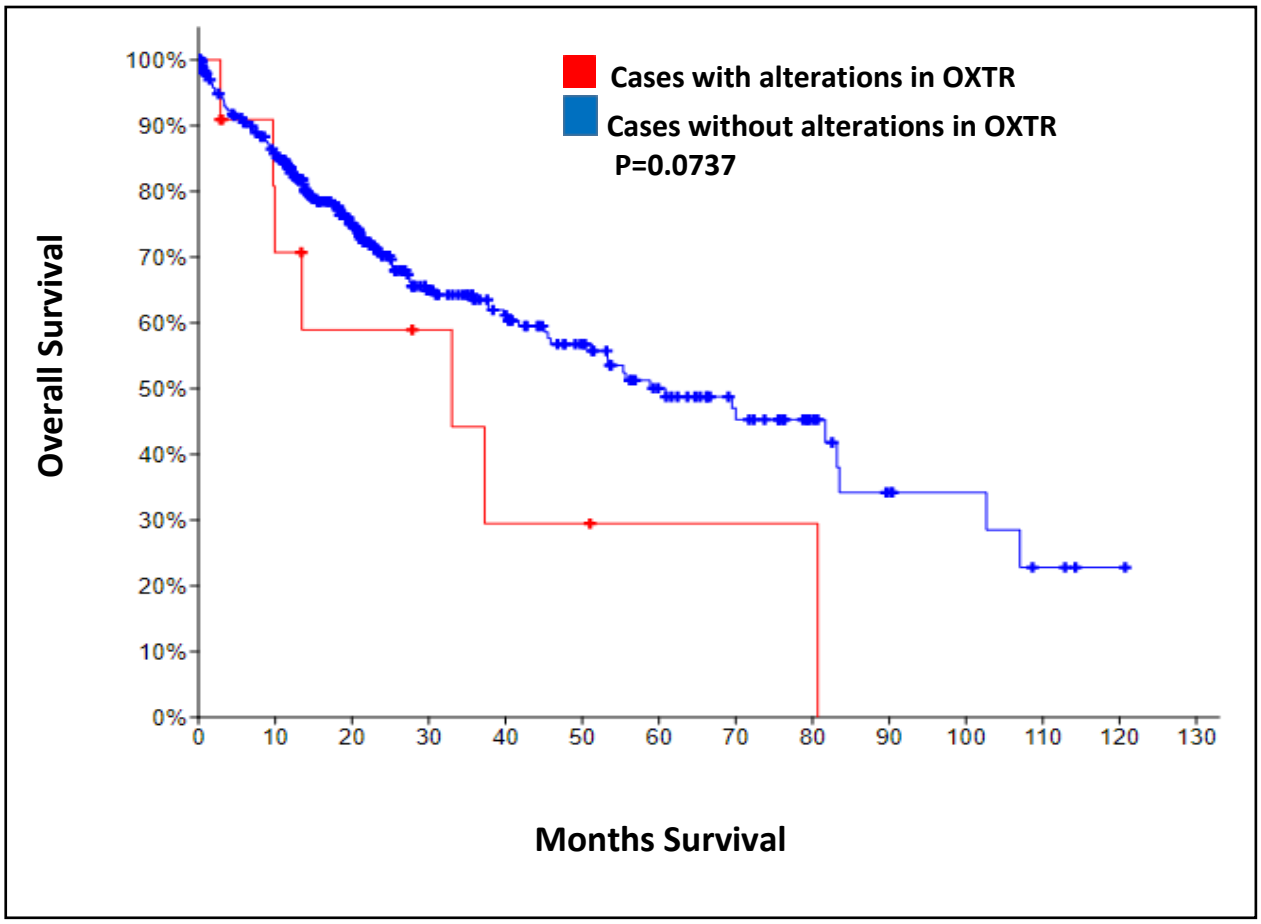


Figure 4.15: Overall survival Kaplan-Meier estimate in hepatocellular carcinoma. The median months survival was 33.02 for cases with alterations (mutations, copy-number alterations, mRNA expression, protein expression, and 60.84 for cases without alterations in the OXTR gene.

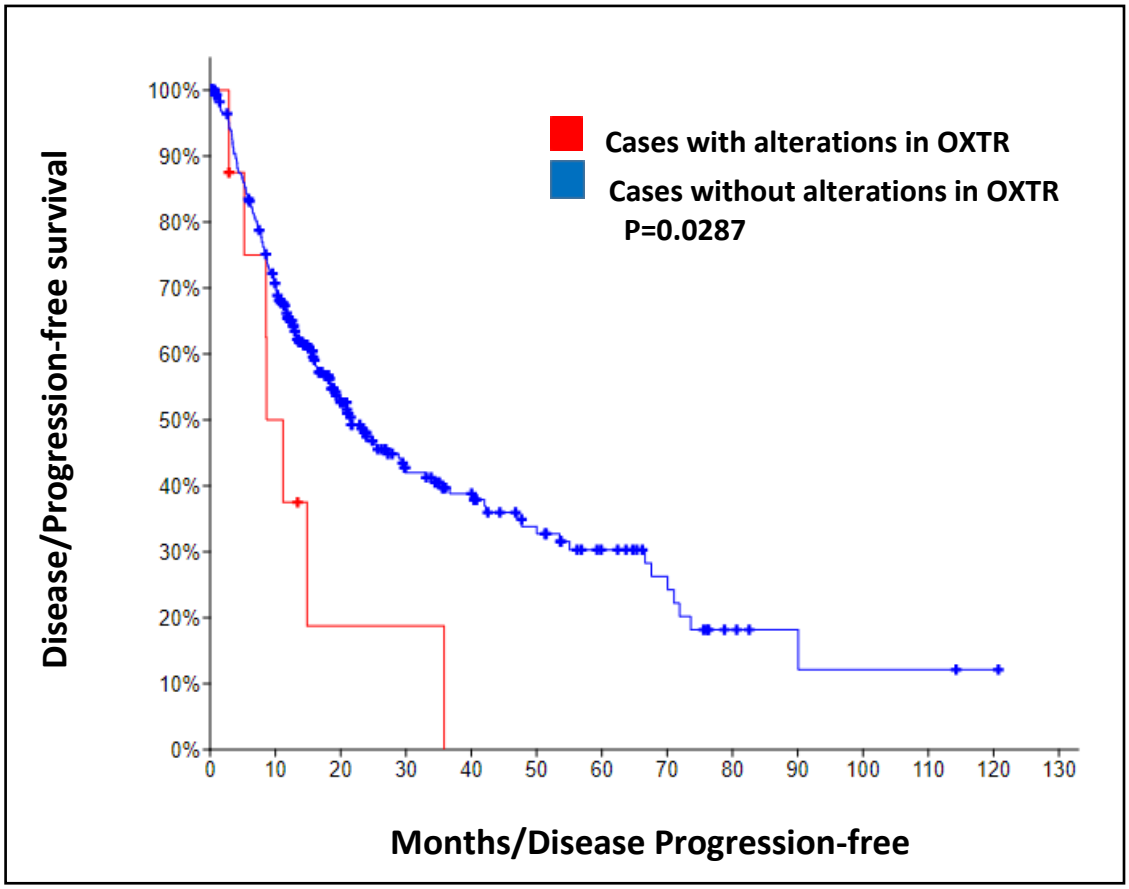


Figure 4.16: Progression-free Kaplan-Meier estimate in hepatocellular carcinoma. The median months disease-free was 8.64 for cases with alterations (mutations, copy-number alterations, mRNA expression, protein expression, and 21.55 for cases without alterations in the OXTR gene.

4.3 Discussion

To the best of our knowledge, this is the first study that investigated the role of OXT and OXTR in PDAC chemoresistance. Moreover, this is the first study that investigated the clinical significance in terms of overall survival and disease-free progression months of OXT and OXTR in PDAC and HCC and reported that patients with genetic alterations and changes in gene expression in OXT and OXTR genes have poorer survival outcomes and months disease-free.

Our data presented herein is very interesting since we have reported that OXT and OXTR are expressed in human PDAC cell lines for the first time**.** We also looked at the mRNA expression in PANC-1 and L3.6pl, which are two human PDAC cell lines with varying responsiveness to the chemotherapeutic agents, gemcitabine (Figure 3.1), and 5-FU. Our analysis showed a differential mRNA expression between the cell lines based on their responsiveness to the chemotherapeutic agent gemcitabine, suggesting that OXT and OXTR may play roles in PDAC chemoresistance, which could be further elucidated with subsequent experiments.

HCC is among the leading causes of cancer-related deaths with a very poor prognosis. Consequently, there is an urgent need for better understanding the molecular mechanisms, novel prognostic biomarkers, and more effective treatment options. There is an emerging link between OXT, OXTR, and cancer. However, the role of OXT or the OXTR in HCC remains unknown.

Amico and colleagues had shown the presence of OXT in human pancreatic extracts, which suggested the possibility of local synthesis of OXT, especially since OXT is known to be an endocrine mediator of insulin and glucagon release [8]. There is also the possibility of OXT from the pancreas having a local effect on the liver, due to the proximity of these two organs. Importantly, OXT is a systemic hormone that could expectedly have widespread effects on all body organs, including the liver. For this reason, we took a similar approach as we did in

investigating the clinical significance of OXT and OXTR genetic alterations in PDAC, and conducted a similar investigation using TCGA HCC patient data.

Notably, HCC cases with alterations in OXT had progression-free median months survival of 55.06 versus 20.99 for HCC cases without OXT gene alterations. However, it is important to note that only 6 out of 360 (1.7%) HCC patients in this entire cohort had OXT gene alterations. Future studies are needed to further determine the clinical implications of this finding. The current data, nevertheless, suggests that OXT likely plays a different role from OXTR in HCC. It is critical that future studies clarify what these disparate roles and their underlying molecular mechanisms are.

HCC cases with genetic alterations in the OXTR gene showed significantly lower median months survival as well as progression-free survival as compared to cases without OXTR gene alterations. These findings indicate the need for future experiments to elucidate the detailed molecular mechanisms of OXT and OXTR signaling in HCC. Genetic alterations in several genes encoding proteins have been implicated in HCC pathogenesis. These include P53, P16, P73, APC, PTEN, IGF-2, BRCA2, SMAD2, SOCS, beta-catenin, retinoblastoma, c-myc, and cyclin D1 [7]. Additionally, several signaling pathways are known to be involved in HCC development, including MAPK pathway activation via the Ras protein leading to ERK1/2 activation and, ultimately, proliferation [7]. Such players and signaling pathways could be investigated in the future.

The OXTR has three subunits, the Gαi, Gαs, and the Gαq subunits. The Gαq subunit's activation can lead to cell proliferation by the MAPK pathway activation via the Ras protein leading to ERK1/2 activation. Alternatively, this subunit of the OXTR can lead to cell proliferation by the MEK5 pathway activation via ERK5 activation, C-JUN, and C-FOS activation [72]. Our

data suggests that the OXTR inhibitor on its own acts via the ERK signaling pathway. However, when PANC-1 cells were pre-treated with the OXTR inhibitor followed by gemcitabine treatment, we did not observe an activation of the ERK signaling pathway. We did, however, observe a decrease of cyclin D1 for both of these conditions. We also observed an increase in cleaved caspase 3 for both of these conditions, indicating that there was increased apoptosis. We would like to propose that while the OXTRi might be acting via the Ras-mediated ERK1/2 signaling pathway, in the presence of gemcitabine, the OXTRi potentially acts via the ERK5/C-JUN/C-FOS signaling pathway. Further investigation is needed to further elucidate this new hypothesis.

The interesting data presented in this work suggest that OXT and OXTR could play key roles in the detailed molecular mechanisms in PDAC, PDAC chemoresistance and possibly other GI-related cancers as OXT and the OXTR have been shown to be expressed at the mRNA level throughout the GI tract [85]; however, only a few studies have investigated the potential physiological and/or pathophysiological roles of OXT and OXTR expression in the human GI tract.

It is interesting to note that Ras mutations are involved in both PC and HCC development The OXTRi is known to act via the Ras-mediated ERK1/2 signaling pathway leading to cell proliferation. The OXTR is therefore a promising therapeutic target in both PC and HCC.

One limitation of this study is that mRNA and protein expression in normal PC conditions were not available here for analysis. Nevertheless, these findings suggest that OXT and OTXR antagonists could be promising therapeutic agents for the treatment of PC in the future, and OXT could potentially be a non-invasive blood-based biomarker in PC, for example, in terms of monitoring or predicting response to chemotherapy.

CHAPTER 5

CONCLUSIONS, SIGNIFICANCE, AND FUTURE DIRECTIONS

Thus far, we have shown that miR198 is significantly lower in the more unresponsive PDAC cell line PANC-1 than in the more responsive PDAC cell line L3.6pl. Our qPCR data also suggest that miR-198 regulates c-MET in the PANC-1 and the L3.6pl cell lines. Dual-luciferase assay confirmed that miR-198 targets the 3'-UTR of c-MET in the PANC-1 cell line. We have also shown that miR-198 increases gemcitabine sensitivity in PANC-1. A subsequent experiment would be to investigate whether this observed gemcitabine sensitivity is directly linked to miR-198's regulation of c-MET. We attempted to make this investigation via western blot analysis looking at the expression of c-MET, p-c-MET, and the proposed downstream targets ERK1/2 and pERK1/2 protein expression in cells that were either pre-treated with the synthetic oligonucleotide miR-198 mimic (in the PANC-1 cells) or inhibitor (in the L3.6pl cells) followed by gemcitabine or 5-FU treatments as compared to cells not pre-treated with the synthetic oligonucleotide mimic or inhibitor. However, we were unable to obtain clear western blot images, which we assumed to be due to technical difficulties with the c-MET and p-c-MET antibodies at that time.

During our investigation of the role of OXT and OXTR in PDAC chemoresistance, we found that OXT and OXTR are expressed in human PDAC cell lines and that there is a differential expression observed between the more unresponsive PDAC cell line PANC-1 as compared to the more responsive PDAC cell line L3.6pl in terms of mRNA expression of OXT and OXTR and protein expression of OXTR. Inhibition of the OXTR increases gemcitabine sensitivity, decreases ERK1/2 and pERK1/2, and cyclin D1 protein expression while it increases caspase 3 activity. In our retrospective clinical analyses using TCGA datasets, we found that PDAC and HCC patients with genetic alterations in OXT and OXTR have poorer outcomes.

These interesting data suggest that OXT and the OXTR may be important in PDAC and HCC progression and survival as well as in PDAC chemoresistance. Based on the very promising

HCC clinical data we presented here, the investigation of the detailed molecular mechanisms of OXT and OXTR in HCC is warranted. OXT and OXTR, therefore, could potentially have prognostic and therapeutic implications in a subset of PDAC and HCC patients. For example, OXT, like miR-198, may be a promising prognostic blood-based biomarker in PDAC and HCC. It is also possible that miR-198 and OXTR antagonists could serve as therapeutic agents in the future and be beneficial to a subset of PDAC and HCC patients.

One limitation of some of our experimental analyses was the absence of miRNA, mRNA, and protein expression data from normal PC cells to serve as controls during our analyses. In future work, such relevant data should be included.

Some other future works could investigate the potential role of OXT and OXTR in other GI-related cancers. The rationale here is that OXT and the OXTR have been shown to be expressed at the mRNA level throughout the GI tract [85], however, only few studies have investigated the potential physiological and pathophysiological roles of OXT and OXTR expression in the human GI tract.

miR-198 has not only been shown to act as a tumor suppressor in PDAC but also in HCC and colorectal cancer (CRC). c-MET has been shown to be directly regulated by miR-198 in hepatocellular carcinoma. Chemoresistance is a major problem in not only PDAC but also in HCC and CRC. It would be interesting to investigate in future studies if miR-198 regulation of c-MET is involved in HCC and CRC chemoresistance.

During this thesis work, an attempt was made to investigate the expression of miR-198 amongst PDAC patients with varying responses to chemotherapy. However, this aspect of this thesis work was not completed due to the lack of comprehensive patient data publicly available in TCGA datasets or the difficulty in obtaining PDAC patient samples (urine, plasma, and tissues).

Such a study would have the potential of identifying miR-198 as a novel, non-invasive biomarker for predicting and monitoring response to chemotherapy in PDAC.

Although our data shows that miR-198 increases responsiveness to gemcitabine but not via the ERK1/2 signaling pathway, it is likely that it could be acting via one of the other cell proliferation pathways activated by c-MET such as STAT, PKC, FAK, or mTOR mediated pathways. This aspect could be further investigated in the future.

Other top validated direct molecular targets of miR-198 which we found in miRanda included NTRK-3, FOXM-1, and JUN. Interestingly, all of these targets have been shown to play important roles in PC and CRC chemoresistance, but their regulation by miR-198 and this regulation having roles in PDAC or CRC chemoresistance have not been reported to date. Hence further interesting future studies may include investigating if the miR-198 regulation of NTRK-3, FOXM-1, and JUN plays a role in PDAC and CRC chemoresistance.

Although we did not find miR-370 to be targeting OXT in our PDAC cell lines, it would be interesting to study whether miR-370 could be targeting OXT to regulate the role of OXT and OXTR in other cancer types.

Emerging studies are pointing to a critical role of the nervous system in carcinogenesis [86, 87]. For example, many neuropeptides are aberrantly expressed in cancer cells. One recently discovered example is OXT. OXT is produced by hypothalamic neurons and has multiple roles in the central nervous system. Cortisol has also been linked to some functions of OXT [140]. Some studies have shown that higher OXT levels and increased social support (a known prognostic player in cancer) are associated with diminished effects of stress. In a study by Mankorious and colleagues, it was shown that there is a cross-talk network between OXT and cortisol at the

molecular level, where the carcinogenic effect of cortisol was reversed by OXT via autophagy in human ovarian cancer cells in vitro [140].

Additionally, serum levels of cortisol have been shown to be higher in HCC patients than in healthy individuals [134]. Studies by Wu and colleagues have shown that exposing HCC cell cultures to cortisol represses p53 expression by upregulating expression of the p53 suppressor Bcl2L12. It would be interesting to determine whether there could be an interaction between OXT and cortisol, which could be involved in a potential neural regulation of HCC as well as other gastrointestinal cancers.

Studies have shown FOXM1, a transcription factor, to upregulate the expression of c-MET and to play critical roles in the activation of HGF/c-Met signaling. There is a positive feedback loop where the activation of HGF/c-MET signaling leads to increased expression and transcriptional activity of FOXM1 via downstream signaling mechanisms of ERK1/2, AKT, and STAT3, mTOR, FAK which affect cell survival, motility, and proliferation. It would be interesting to study the possible regulation of miR-198 of c-MET and FOXM-1 as well.

CHAPTER 6

BIBLIOGRAPHY

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