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Anti-Cancer Effects Of Oil Palm Phenolics On Pancreatic Cancer - Histological Evidence

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ANTI-CANCER EFFECTS OF OIL PALM PHENOLICS ON PANCREATIC CANCER – HISTOLOGICAL EVIDENCE

by

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_____

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DEDICATION

I would like to solely dedicate my Masters Thesis to my advisor Dr. Smiti Gupta who has been a wonderful guide and supported me throughout my course of study. The moral support and confidence that she has instilled in me is indescribable. I am very thankful to her for allowing me to be a part of her lab and her research.

I would also like to dedicate this project to my parents, without whom my entire course of study itself would be impossible. I thank them very much for supporting my education guiding me in the right direction. I am very thankful to them for all the values that they have inculcated in me for the person that I am today. I also dedicate this to my husband for his complete support and patience during my entire course of study.

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CHAPTER 1

INTRODUCTION

The American Cancer Society estimates based of the National Cancer Institute, CDC and the North American Association of Central Cancer Registries suggest that a total of 1,660,290 new cancer cases and 580,350 cancer deaths are projected to occur in the United States in 2013. During the years 2005-2009, cancer incidence rates declined by 0.6% in men and remained stable in women and overall cancer deaths declined by 20% since 1991 to 2009. Death rates for lung, colorectal, breast and prostrate have declined in the past years **[1].** In spite of the reduced death rates, some demographic groups like African-Americans who have a higher cancer incidence and death rates, compared to any other race, have not benefitted equally especially with colorectal and breast cancer. Suggestions have been given for improvement of this limitation by applying more knowledge about cancer control. **[2]**

Cancer

Cancer is a class of diseases that are characterized by out-of-control cell growth. This proliferation of cells eventually forms lumps or tissue masses called tumors at specified organs, which can grow, interfere, produce hormones that alter and interfere with other regular functions of the body. They exist as benign (those that remain in the originated area and demonstrate limited growth) and malignant tumors (those that manage to move through the circulatory system destroying healthy tissues and eventually divide and grow developing their own functions like producing hormones, making their own blood vessels known as angiogenisis). When this tumor spreads to the other regions of the body, destroying healthy tissues, the process is termed as metastasis. The resulting condition is very challenging to treat. Most of the deaths resulting from

cancer are due to metastatic tumors and only 10% of deaths are caused by the primary tumors **[30].**

Cancer begins to develop when the process of programmed cell death (PCD) or apoptosis seizes. While the normal cells undergo apoptosis, cancer cells do not, but instead continue to proliferate and divide which leads to a mass of abnormal cells that grow out of control **[3].** There are many factors that lead to the initiation of cancer. It can be initiated with a mutation in one or more genes which leads to accumulation of defects in a number of genes, leading to malignancy. Other factors may include loss of normal signals to stop proliferation and differentiation, cell division, absence of apoptosis, ability to invade tissues and organs and angiogenesis **[4].**

Cancer is initiated primarily by three factors – Genes, diet and environment. All the genes in our body are highly polymorphic and contribute to metabolism, cell cycle, protein repair, transcription factor genes. High penetrant genes are inherited genes which directly contribute to the development of cancer. However, the existence of these genes is very rare. Low penetrance or susceptibility genes are those which are not sufficient to cause the disease but can cause damage when exposed to environment like diet and smoking.

There are several environmental factors that contribute to mutations in the body, but the most common one is oxidative stress. **[5]** Oxidative stress can directly affect the functioning of the cell cycle and can cause cells to proliferate. Cancer can be caused when a person is born with a mutation or by getting exposed to the environment which affect the normal biological functioning and leads to the formation of reactive oxygenated metabolites and reactive oxygen species which results in DNA damage. Some of the DNA is repaired but some others go on to

being fixed mutations which result in abnormal gene expressions. As time progresses, these mutated genes become more permanent and therefore will initiate cancer and tumor formation**[3]**.

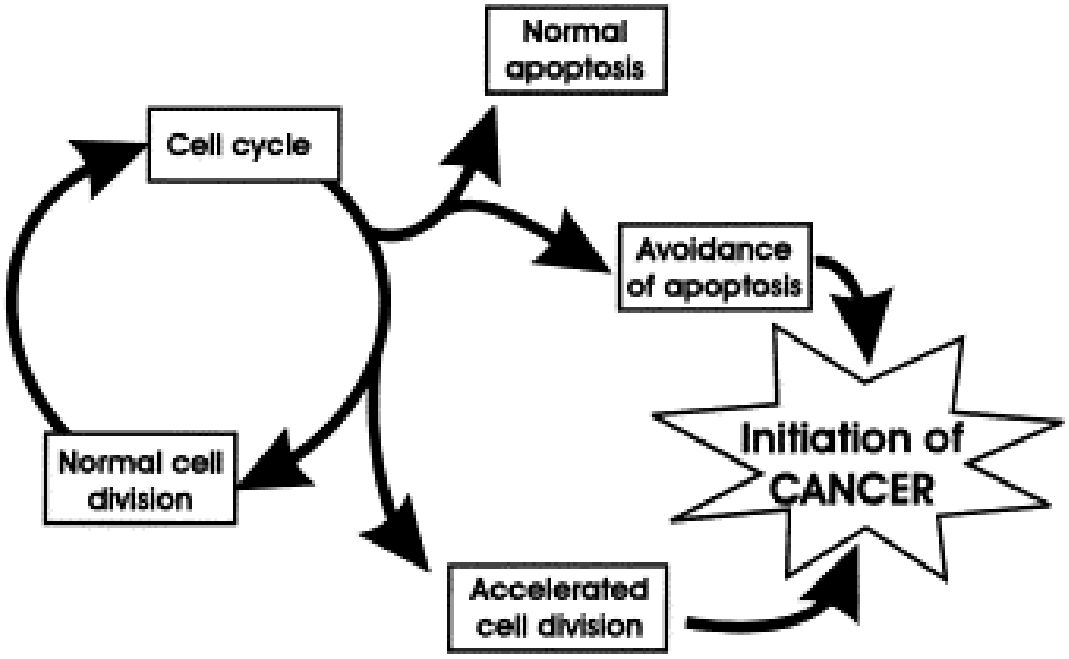


Fig 1: Process of initiation of cancer [3]

Cancer, signaling pathways and transcription factors:

Cancer can be a genetic predisposition that can be inherited from family with genetic mutations that can develop into cancer later in life or can be developed during the natural course of aging during the course of life. As we age, there are a lot of possible chances for cancer causing mutations to develop in the DNA, which is a major risk factor for cancer. There are two types of primary genes that control cell division and control cancer – proto-oncogenes, that activate the cell cycle and facilitate the proliferation of cells and tumor suppressor genes which slow down the growth of cells. A high proportion of oncogenes and tumor suppressor genes encode transcription factors **[6].**

There are currently 17 known signal transducting pathways, at least 2 stress response pathways and several transcription factors that are responsible for the up or down- regulation of specific genes. All the environmental factors produce signals that affect these transduction/stress response pathways, leading to cell response. DNA methylation, genetic instability and loss of communication between the cells result in tumor progression **[3].**

A transcription factor is a molecule participating alone or as a part of a complex, whilst binding to the enhancer response element or promoter, with the outcome being the up or down-regulation of the gene. Some examples are TCF1, ERα, ERβ, AHR, NFκB, RB1, E2F, TBP, TP53 and all the DNA repair transcription factors **[7]**. A complex of more than 20 proteins, regarded as transcription factors, participate in the initiation of transcription in the promotion region of most genes.Polymorphisims resulting from this are most likely to cause cancer and since all the genes in the human genome are highly variable on a gene-by gene basis, transcription factor genes might be no more or no less responsible for cancer than any other type of genes**[8]**.

Many of the transducting pathways are activated during early embryogenesis and fetogenisis when the balance is comprehended between the cell division and apoptosis mechanism in the developing organism. As every pathway has a receptor, the signal is received by the reception mechanism, and the cytosolic protens including the kinases and phosphatases convey the signals. The transcription factors therefore, downstream which up-regulate or down-regulate the expression of specific genes (**Fig.2) [3].** All primary and modifier genes participate in one or more of these pathways. One of the few examples is the APC tumor suppressor which participates though WNT β-catenin transduction pathway. Another example is the estrogen and retinoic acid receptors and the AH dioxin binding receptor through specific nuclear-translocation

pathways. Several exogenous and endogenous signals interact with our cells every day and can cause mutations. In addition to this, environmental factors may act as activators or inhibitors, co-activators or repressors or agonists or antagonists leading to disturbances in cell response to these signals. During the process of initiation and progression of cancer, some genes involved in the biological processes may be disturbed and altered contributing to malignancy **[3].**

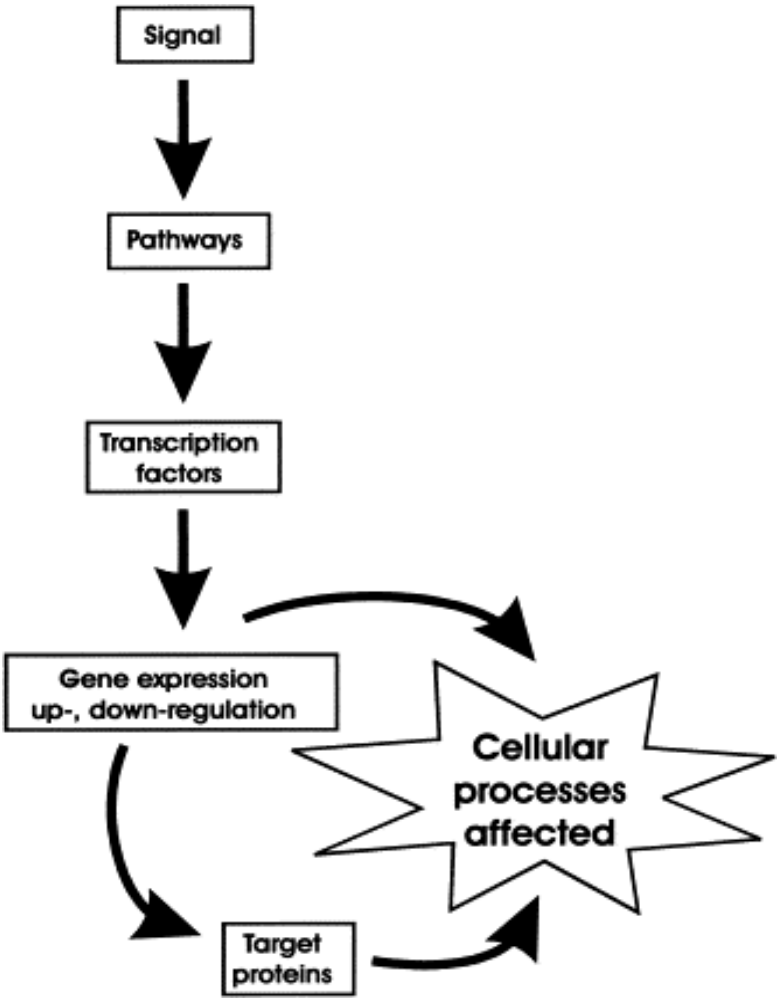


Fig 2: Transcription factors affect cellular processes [3]

Some of the pathways affecting gene expressions are Sonic Hedgehog (SHH), TGFβ receptor (Ser/Thr kinase), NOTCH/DELTA (*HER* genes) pathway, IL-1/TOLL receptor (NFκB)

pathway, p53 pathway, etc. A majority of these pathways, fall up on transcription factors that eventually direct gene expression patterns and result in formation of tumors and metastasis. Since the expressions of these transcription factors are tightly regulated, they interfere in logicalpoints of therapeutic cancer development and progression **[3].**

Three major transcription factors play an important role in human cancer and are important targets in drug discovery for cancer therapy – NF-κB and AP-1 families of transcription factors, STAT family members and Steroid receptors. These transcription factors, when inhibited, are used to validate them as drug targets and in therapeutic applications **[3].** In the overall study, changes in expression of various genes involved in the carcinogenic pathway such as MMP9, CCND1, BCL2 and COX-2 were investigated.

Cancer and diet:

Studies clearly suggest that dietary factors relate to one-third of the variation in cancer risk, combined with physical activity and weight, while smoking and other environmental factors contributed to the other third. It is clearly evident, that lifestyle factors are great determinants of cancer, however 75-80% of cancer cases under 65 years of age are preventable **[9]**. Carcinogens present in the environment, eg., Some foods, tobacco, asbestos, radiation, gamma rays, X-rays, etc, are directly responsible for damaging DNA which promote cancer by producing free radicals that interfere with the regular functions of the body **[30].**

Diet serves as a source of carcinogen exposure. Norat et al **[10]** was able to relate a positive correlation between colorectal cancer and intakes of red and processed meat, high intake of fish, which included parameters like anthropometric measurements and physical

activity, smoking status, dietary fiber and folate, and alcohol consumption. An increased risk of pancreatic cancer was observed with different methods of food preparation involving high salted foods, smoked meat, dehydrated and fried food and refined sugar. It has also been reported that consumption of grilled or barbequed red meat increased pancreatic risk **[11]**.

Antioxidants or bioactive components that are present in fruits and vegetables, like Vitamin E, β-carotene, lycopene, and selenium to name a few, have been found to reduce the risk of lung, prostate, stomach, or total cancers, as well as oral pre-cancers, in epidemiologic studies. It has been shown that the level of consumption of these antioxidants have shown a mighty reduction in risk of cancer in all vulnerable subjects like smokers and the elderly. Some of these nutrients have also shown to reduce the growth of tumors in mice. Antioxidants have the ability to scavenge free radicals and also have other molecular consequences which include inhibiting the activation of carcinogens and limiting the damage that are caused to DNA and membranes **[12].**

Although, there is much evidence and studies done on this ever-debating topic, researchers are still trying to explore the link between diet and cancer as there is still not enough data to develop guidelines regarding specific foods and cancer risk. The goal of the overall study was to investigate the effects of a water soluble extract from oil palm, rich in phenolic compounds (OPP) on the progression of pancreatic cancer in a mouse model.

II. Pancreatic Cancer

Pancreas:

The pancreas is an elongated organ located at the back of the abdomen. The right side of the organ is called the head and is the widest part while the left side extends upwards and ends near the spleen. The pancreas consists of exocrine and endocrine glands.

The function of the exocrine gland is to help break down all the vital nutrients like carbohydrates into starch and sugars by carbohydrases, fats into fatty acids and glycerol by lipases, and proteins into small peptides and amino acids by proteases in the duodenum. When these digestive enzymes enter the duodenum, they get activated and secrete bicarbonate to neutralize stomach acids in the duodenum. The endocrine gland produces hormones namely insulin, glucagon and somatostatin which regulate the glucose levels in the blood while somatostatin limits the production or release of these hormones.

Pancreatic cancer:

Pancreatic cancer is a disease caused due to DNA mutations which can be inherited or acquired as a person ages. Although the actual cause is not known, there are several environmental factors that have been associated with pancreatic cancer, the major one being smoking. The risk of pancreatic cancer has proven to be three times more in smokers and tobacco users than in non-smokers. There is limited evidence on the intake of alcohol, coffee and pancreatic cancer association. Studies have shown that people with a severe case of diabetes, cirrhosis, high fat and high cholesterol diet are linked to an increased incidence **[13]**.

Deaths arising from pancreatic adenocarcinomas or pancreatic cancer are ranked fourth in the United States. It is more common in older people but only less than 20% of the pancreatic

tumors are curable in all. The overall five-year survival rate in pancreatic cancer is less than 5% **[13]**.

Pancreatic cancer is caused when there is an outbreak of abnormal growth of cells within the organ. More than 95% of pancreatic cancer is categorized as exocrine tumors and within this, the majority type of exocrine cancer are adenocarcinomas. The common symptoms are venous thromboembolism, pain, malignany bilary obstruction, malignant gastric outlet obstruction, pancreatic exocrine insufficiency which causes abdominal pain, indigestion, weight loss and anorexia and depression. Treatment of exocrine tumors is based upon what stage the cancer is at **[14].**

Acinar cell carcinoma, a rare form of pancreatic cancer of the exocrine gland, causes excessive production of lipase enzyme which digests fat. The most common histological subtype is ductal adenocarcinomas that originate on the epithelial cells of the exocrine ducts. Invasive ductal adenocarcinma, Adeno squamous carcinoma , giant cell tumor, intra-ductal papillary mucinous neoplasm, acinar cell neoplasms, epithelial cell neoplasms, mouse Pancreatic Intraepithelial Neoplasia (mPanIN) are some types of pancreatic cancers and tumors. Malignant pancreatic tumors are heterogeneous with respect to their underlying cellular and histological phenotype. Since pancreatic cancer is usually diagnosed and detected only towards the final stages, it becomes very crucial for early detection and treatment for treating pancreatic cancer in the initial stages, so as to control or retard the progression of pancreatic cancer to later stages.

Stages of Pancreatic cancer:

The American Joint Committee on Cancer (AJCC) developed a standardized way which explains the severity of cancer. This method is used to stage pancreatic cancer based on the size

of the tumor (T), lymph nodes (N) and metastasis (M). Once this information is determined, the cancer is categorized as Stage 0, IA, IB, IIA, IIB, III and IV based on the TNM categorization.

At Stage 0, tumor is confined to the primary lining of the pancreatic duct cells and has not invaded deep into the tissues or outside of the pancreas. Such tumors are referred to as pancreatic carcinoma in situ or pancreatic intraepithelial neoplasia III (mPanINIII). At stage I, which is divided into IA and IB based on the size of the tumors (less than 2cm in IA, greater than 2cm in IB), the tumor is not spread to the lymph nodes. At stage IIA, the tumor is growing outside the pancreas but has not spread to the nearby or distant blood vessels or lymph node sites. The tumors at Stage IIB are growing outside the pancreas but do not metastasize to other sites. Stage III tumors grow outside the pancreas, near blood vessels, but do not spread to distant sites.The final stage of pancreatic cancer, Stage IV, has its tumors spread and metastasized to distant sites such as organs, tissues, blood vessels, etc.

Studies suggest that accumulation of gene mutations result in pancreatic cancer. The cancer originates in the ductal epithelium of the exocrine gland which evolves from pre-malignant lesions to a highly invasive cancer called pancreatic intra-epithelial neoplasia (mPanIN). mPanIN is best characterized in the histological analysis of pancreatic cancer. This categorization of mPanIN lesions progresses from mPanIN-1A, mPanIN-1B to more severe mPanIN-2 and mPanIN-3 which eventually leads to invasive adenocarcinomas. Simultaneously, there is a large accumulation of mutations which involve the activation of KRAS2, inactivation of tumor suppressor gene CDKN2A and TP53 also including inactivation of SMAD4/DPC4 gene**[13].** These genes are accompanied by transcriptomic alterations that facilitate cell cycle deregulation, cell survival, invasion and metastases. In this study, we used the mPanIN grading system to look at the differences in OPP fed mice compared to control mice with cancer.

Current therapeutic options:

Although pancreatic cancer represents only 3% of cancer diagnosis in the U.S, it is the fourth leading cause of cancer deaths. Despite the newer anti-neoplastic combinations, the survival rate for all stages of pancreatic cancer is 6% **[15]**.

Since pancreatic cancer is associated with very poor prognosis and surgery is considered as the only radical therapy after drug therapy. Newly diagnosed pancreatic cancer cases have developed distant metastasis to about 85% and only 5-25% of pancreatic head cancer and less than 10% of pancreatic body cancer can be treated with surgical excision. Although this may be excised, the recurrence rate post operation is high. This explains why radiotherapy has become the predominant treatment option for advanced pancreatic cancer **[16]**. There has also been magnetic resonance spectroscopy (MRS) research in malignant diseases which has provided data on the metabolism and biochemistry of tumors, its effects on nutrients, hormones and growth factors**[17].** Thus, there is a need for better therapeutic or dietary regimens to improve patient survival or progress.

A common drug, Gemcitabine is being used extensively for the standard care and treatment of locally advanced and metastatic pancreatic cancer. The effects of the drug are however moderate which results in the survival range of 8 months to 1 year. Numerous trials are being investigated based on administering Gemcitabine alone and in combination with secondary agents such as fluropyramidine, multitarget anti-folate or topoisomerase inhibitors. This combination therapy with Gemcitabine and other agents has proven better survival rates than administering Gemcitabine alone in mouse models**[18].** In this study, we investigated the effect

of OPP alone and in combination of the current drug, Gemcitabine, on progression of pancreatic cancer in mouse model.

III. Oil Palm Phenolics:

Composition:

Oil palm (*Elaeis guineensis*) belongs to the family of *Arecaceae* and is a high oil tropical plant that has effective antioxidative components. The oil palm fruit contains lipid soluble phytochemicals like carotenoids, tocopherols and tocotrienols. The extraction of water soluble materials from the oil palm gives another class of phytochemicals including phenolics and organic acids called Oil Palm Phenolics (OPP).

OPP contains a high range of antioxidants and has conferred positive outcomes on degenerative diseases in various animal models without any evidence of toxicity **[19], [20].** A recovery procedure for oil palm phenolics that get discarded into the waste stream during milling of oil palm has been developed to extract the phenolic content. This isolation of the bioactive components has proven to show positive correlation in health and wellness **[19].**

Ravigadevi Sambanthamurthi used several methods like HPLC, GC-MS and NMR for analyzing the composition of OPP and reported that it contains caffeic acid, protocatechic acid and p-hydrobenzoic acid.The phenolic constituent caffeoylshikimic acid also existed in three isomeric forms, 3- caffeoylshikimic, 4- caffeoylshikimic and 5- caffeoylshikimic as major components. The isomers are also known as dactyfilic acid, isodactylifiric acid, neonactylifric acid respectively. Caffeoylshikimic accounted for more than half of the total phenolic content **[19].**

Properties:

OPP has proven to exhibit significant biological properties against LDL oxidation in vitro, by up-regulation four lipid catabolism genes and down-regulating five cholesterol biosynthesis genes proving that it played a role in reducing cardiovascular disease in mice. OPP also up-regulated eighteen blood coagulation genes in the spleens of mice **[21], [22]**. It is also reported that gene expression changes caused by OPP in mice fed a low fat normal diet which indicated to have novel health promoting properties including hepatoprotective, antidyslipidemic, anti-thrombotic and caloric restriction effects. Effects of OPP on human subjects are yet to be shown and trials are being run currently for human intervention. It can act as a potential anti-ooxidative agent because of its low toxicity **[22]**.

IV. Hypothesis:

The overall goal of this research is to see whether OPP has a beneficial anti-cancerous effect on pancreatic cancer in a triple transgenic mouse model and is expected that OPP holds a chemo-preventive agent against pancreatic cancer. Our hypothesis was that OPP would function as a chemo-therapeutic agent against pancreatic cancer in the mouse model. The changes in regression of pancreatic cancer will be observed in the histological slides from the pancreas. In the overall study, the change in tumor progression or regression was monitored by MRI. This was confirmed by gene expression and histology. The histological changes due to OPP intake is presented here.

To study this hypothesis, the following aims have been developed:

Specific Aim I: To investigate the in-vivo effects of OPP on pancreatic cancer in a transgenic mouse model. The overall goal of the project was to observe for changes in anthropometric, diet and fluid intake. Pancreatic cancer progression in whole animals was

monitored by MRI and ultrasound techniques. However, that data will be presented elsewhere. The focus of this work is to investigate the histological changes produced due to cancer and the effect of OPP on it.

Specific aim II: To investigate histological changes produced in the pancreas of OPP fed transgenic mouse by Hematoxylin and Eosin staining and immuno-histochemistry.

The goal of this project was to validate the histological changes in the pancreatic tissue of the transgenic mice using the PanIN grading system. These lesions are confirmed by performing immuno-histochemisty using specific anti-bodies for S100P and Smad4/DPC4 genes.

CHAPTER 2

METHOD

Specific Aim I: To investigate the in-vivo effects of OPP on pancreatic cancer in a transgenic mouse model.

1. Animals

A total of 42 male mice (control = 10; KPC (LSL.$Kras^{G12D}$/+; $p53^{R172H}$/+; PdxCretg/+) = 32) aged 6-8 weeks old, were obtained from Van Andel Institute,MI and divided into various study groups**.** The weights of the mice ranged between 20-30 grams and were housed individually in solid bedding cages at Scott Hall, Wayne State University. They were maintained at a standard temperature of 70 °F with a relative humidity of 45-48% and with a 12-h light/12-h dark cycle. The mice were allowed to acclimatize for one week prior to the six week long study. The protocol followed was approved by the Animal Investigation Committee of Wayne State University.

The mouse model for this study was developed by genetically modifying $Kras^{G12D}$, p53R172H and the PdxCretg genes. $Kras^{G12D}$ is specific to developing pancreatic progenitor cells by crossing an activated Kras allele to the transgenic strains that express Cre recombinase , PdxCretg. Once the mice are generated with conditional mutations to PdxCre-expressing compound mutant animals (KPC), they develop murine mPanIN with 100% penetrance and also develop Pancreatic Ductile Adenocarcinoma at a very early stage **[23]**. This model was selected because many aspects in these KPC mice exhibit similarities in many human diseases, including histopathology, neoplastic tissues, occurance of metastasis, activation of biochemical pathways and genomic instabilities.

2. Experimental diet:

Diet for the mice was purchased from Dyets Inc., (Bethlehem, PA) for the entire study **(Table 1).** The diet which was provided for the control group was a purified isocaloric AIN-93G diet and for the treatment mice received a modified diet that was combined with 5% OPP.

Regular diet				**5% OPP diet**		
Ingredient	**Kcal/gm**	**Grams/kg**	**Kcal/kg**	**Kcal/gm**	**Gram/kg**	**Kcal/kg**
Casein	3.58	200	716	3.58	200	716
L-Cystine	4	3	12	4	3	12
Sucrose	4	100	400	4	100	400
Cornstarch	3.6	397.486	1430.9496	3.6	347.486	1250.9496
Dyetrose		132	501.6	3.8	132	501.6
Soybean oil	9	70	630	9	70	630
t-Butylhydroquinone	0	0.014	0	0	0.014	0
Cellulose	0	50	0	0	50	0
Mineral Mix	0.88	35	30.8	0.88	35	30.8
Vitamin Mix	3.87	10	38.7	3.87	10	38.7
Choline Bitartrate	0	2.5	0	0	2.5	0
OPP	-	-	-	0	50	0

Table 1: Composition of the Regular and Experimental Diets

3. Experimental groups and protocol:

After a week of acclimatization, the animals were divided into their respective control (n=18) and treatment groups (n=24).

3a. Control groups –CC, CP and KC

The control groups CC, CP were regular mice and mice from the KC group were KPC mutated transgenic mice that were all given a regular purified diet **(Table 2).** The three control groups were administered with saline injections at 200 µl per animal once every week from Week 1- Week 5.

3b. Treatment groups– KP, KG and KPG

The treatment groups were KPC mutated transgenic mice that were provided with different combination diets. The KP group was given the 5% OPP diet with no drug administration, KG was given a regular diet administered with Gemcitabine while KPG was given the OPP diet along with the administration of Gemcitabine **(Table 2).** The KP mouse was given placebo injections of saline (0.85% NaCl), while the KG and KPG mice were given chemotherapy injections with 100mg/5ml Gemcitabine stock (5ul/g body weight) once a week from Week 1 to Week 5.

4. Anthropometric:

The body weight, diet and water consumption of all the groups was measured two times every week from Week 1 to Week 6. Ultrasound (Week 1,5) and MRI (Week 3,6) was conducted at the beginning and the end of the study. Urine of the mice was collected, centrifuged and stored in -80C once every week during Weeks 2, 4 and 6. At the end of the study (Week 6), the animals were sacrificed by anesthetizing them with 5µl/g body weight on a combination of 80mg/ml Ketamine and 20mg/ml Xylazine. Blood and tissues were harvested

after sacrifice, and the vital organs required for the experimental study (pancreas, liver, heart, lungs, fore-stomach and testes) were stored in liquid nitrogen(-196C) for future analyses. For histological study, the tissues were collected in 10% neutral buffered formalin tubes. They were then transferred into 70% ethanol tubes after 48 hours of collection and shipped in 50% ethanol to Michigan State University for H&E staining.

MICE (n=42)			
GROUPS	**CANCER**	**DIET**	**DRUG**
CC (n=5)	No	Regular	No
CP (n=5)	No	OPP	No
KC (n=8)	Yes	Regular	No
KP (n=8)	Yes	OPP	No
KG (n=8)	Yes	Regular	Gemcitabine
KPG (n=8)	Yes	OPP	Gemcitabine

Table 2: Group divisions of the control and experimental mice

CC: Control mice on control diet; KC – KRAS mutated mice on control diet; KP- KRAS mutated mice on OPP diet; KG – KRAS mutated mice on regular diet and administered with Gemcitabine; KPG – KRAS mutated mice on OPP diet and administered with Gemcitabine.

Specific aim II: To investigate histological changes produced in the pancreas of OPP fed transgenic mouse by Hematoxylin and Eosin staining and immunohistochemistry.

1. Hematoxylin & Eosin staining:

Hematoxylin & Eosin staining is a staining method used to recognize various types of tissues and morphologic changes in cancer diagnosis. Hematoxylin is a deep blue-purple stain which stains nucleic acids and Eosin is a pink stain that stains non-specific proteins. H&E stain is particularly used for cancer diagnosis because it has the ability to fix itself on cytoplasmic, nuclear and extracellular matrix features making cancer studies more efficient by disclosing structural information and some functional implications. **[24]**

For this study, tissue samples were processed and vacuum in-filtrated with paraffin on Thermo Fisher Excelsior tissue processor by previously fixing it with 10% neutral buffered formalin and later embedding with the ThermoFisher HistoCentreIII embedding station. As soon as the blocks were cooled, the paraffin was removed and placed on a Reichert Jung 2030 rotary microtome and faced to expose the tissue sample. The cooled blocks were then sectioned at 4-5 microns and dried at 56 degree C slide incubator to ensure adherence to the slides. The time for this process did not exceed 24 hours, as it would destroy antigenic components. The slides were removed from the incubator and then stained for H&E.

2. H&E Staining protocol:

H&E staining was performed at the Histopathology laboratory, Michigan State University. First, Xylene was run on the slides for 5 minutes and the process was repeated twice. Next, absolute alcohol and 95% ethanol was run two times at 2 minutes each. Tap water rinse was done two times for two minutes, Gill 2 Hematoxylin (ThermoFisher – Pittsburg,PA) for 1.5

minutes and last in 1%aqueous glacial acetic acid and running tap water for two minutes to enrich the nuclear detail differentiation. After completing the tap water run, the slides were placed in 95% ethanol , 1% alcoholic Eosin-Phloxine B, 95% ethanol, four changes of 100% ethanol, four changes of xylene all for two minutes each. The slides were then cover-slipped with synthetic mounting media for permanent adhesion and visualization with light microscopy.

Microscopic examination of the slides were done using the Nikkon Eclipse 80i at the Nutrition and Food Science Department, Wayne State University. The camera used was a Nikkon DS- U2/L2 and pictures were taken at 2x, 10x, 20x and 40x oil magnification. The slides stained for histology were sectioned under the microscope in order to be able to observe the architechural and the cytological changes in the pancreatic tissues. Once this was done, PanIN grading was performed based on the criteria for the grading system.

3. **Immuno-histochemical labeling using SMAD4 (DPC4) and S100p:**

Immuno-histochemistry (IHC) is a combination of anatomical, immunological and biochemical technique used to assess binding of antigen and DNA/RNA expression with the help of antibodies within cells in biological tissues IHC is used to determine a benign or a malignant cancer, the stage, the grade of the tumor and even the origin of a metastasis to find the site of primary tumor. It is also used to determine the efficiency of a drug by observing the up-regulation or down-regulation of the disease targets **[25].**

S-100 protein (S-100p) is a 10.4kDa acidic, calcium binding protein which is present in the cytoplasm of most cells **[26].** It has been reported that S-100P regulates proliferation, migration and survival of pancreatic cancer cells and also increase their invasive nature. Over-expression of S-100p led to disruption of the intermediate filaments of the exocrine pancreas.

Further studies were done and reported that the metastatic capability of S-100P decreased when S-100p was silenced **[27]**.

Smad4/DPC4 is a tumor suppressor gene of pancreatic cancer which shows loss of heterozygosity at the 18q chromosome. These proteins are crucial to the TGFβ signaling pathway which negatively regulates growth of the epithelial cells in the pancreatic tissue. The signals of TGFβ are detected by TGFβRI and TGFβRII receptors which have serine/threonine kinase activity. When TGFβ binds to TGFβRII, the receptor gets phosphorylated and activates TGFβRI which in turn phosphorylates Smad2 and Smad 3. A complex is formed during this phosphorylation with Smad4 which translocated to the nucleus. This interacts with the DNA binding proteins by regulating the transcription of the target genes and allowing cell proliferation **[28].**

3a. Monoclonal Rabbit anti – SMAD4:

Specimens were fixed in 10% neutral formalin and later processed by embedding in paraffin. They were sectioned using a rotary microtome at 4-5 microns. The slides on which the sections were placed were coated with 2% 3-Aminopropyltriethoxysilane and dried at 56 degree C overnight. After deparafinizing them with Xylene, they were hydrated through varying grades of ethyl alcohol and distilled water. The slides were then placed in 7.4 pH of Tris Buffered Saline for five minutes for correcting the pH. Epitome retrival using heat induction was performed using citrate plus pH 6.0 buffer in a rice steamer for 30 minutes and then a 10 minute incubation at 25 degree C (Scytek). 3% hydrogen peroxide/ methanol bath was used to block endogenous peroxidase. A distilled water rinse was performed which was then followed by avidin-biotin complex that was used to stain at room temperature using the DAKO autostainer. Tris Buffered Saline and Tween were used to rinse after every step. After blocking all the non-

specific protein with normal Goat serum (vector labs, Burlingame,CA) for 30 minutes, the sections were incubated with avidin/biotin blocking system for 15 minutes each (Avidin – Vector labs, CA; Biotin – St.Louis, MO). Primary antibody slides were incubated fpr 30 minutes with 1:100 diluted Rabbit Monoclonal antibody SMAD4. Biotinylated Goat anti Rabbit IgG was prepared at 11.0 µg/ml in Normal Antibody Diluent incubated for 30 minutes. Next, incubation with Vectastain Elite ABS reagent was done for 30 minutes. Following this, incubation with Vector Nova Red peroxidase chromogen for 15 minutes was done along with counterstaining with Gill 2 Hematoxylin (Thermo Fisher, Kalamazoo, MI) for 30 seconds. Lastly, differentiation, dehydration and mounting with synthetic media were done.

3b. Polyclonal Rabbit anti- S100P:

Immuno-histochemical staining of S100P antibody was done by following the same steps as in the Monoclonal Rabbit anti-SMAD4 protocol, except that the diluents used for incubating the primary antibody slides was incubated at 60 minutes with Rabbit Polyclonal Anti S100P diluted in Normal Antibody Diluent at 1:200. The remaining steps were followed where the reaction development utilized the Vector Nova Red peroxidase chromogen incubation of 15 minutes followed by counterstain in Gill 2 Hematoxylin for 30 seconds with differentiation, dehydration and mounting with synthetic mounting media.

4. Statistical Analysis:

All data derived from the experiments are presented as mean ± SD. Significant differences between the experimental groups were determined using one way ANOVA test on the EZ-ANOVA software. $P<0.05$ was used to indicate a statistically significant difference.

CHAPTER 3

RESULTS

Specific Aim I: To investigate the in-vivo effects of OPP on pancreatic cancer in a transgenic mouse model.

1. **Food and water consumption:**

   Overall food and water intake data is presented in **Fig 3** and **Fig 4**. Both the control and treatment groups did not show any significant differences ($p>0/05$) in the average consumption of food and water during Week1 or Week 5. The water intake in the control groups CC and CP and the combination group KPG was significantly higher during week 1 as compared to Week 5 ($p<0.05$).

2. **Body weight:**

   No significant differences in body weights were detected among the experimental groups KC, KP, KG and KPG in weeks 1 and 5. ($p>0.05$) **(Fig 5 and Fig 6).**

3. **Survival data:**

   The control group CC which was given a regular purified diet and the CP group which was given the modified OPP diet showed a 100% survival rate. Among the transgenic KPC mice, the KC control mice showed an 87% survival rate, the KP and the KPG group showed a survival rate of 87% and the KG group a 71% survival at the time of sacrifice.

Group	Total	Survived	Died	%Survival Rate
CC	5	5	-	100%
CP	5	5	-	100%
KC	8	7	1	87%
KG	8	6	2	75%
KP	8	6	1	87%
KPG	8	7	1	87%

Table 3: Survival data of control groups (CC, CP) showing a 100% and transgenic KPC group KC showing an 87% survival. The KG group showed lowest survival rate at 75%.

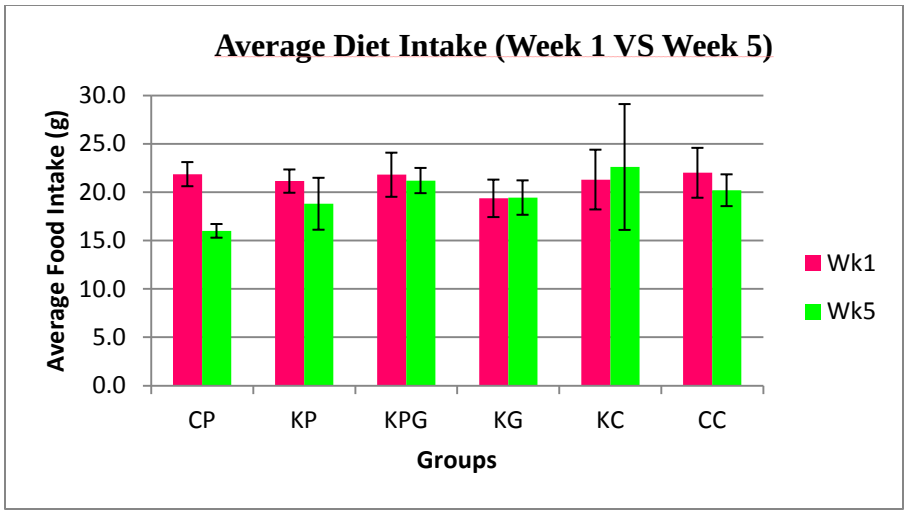


Figure 3: Food intake for all groups during Week 1 and Week 5 were not significantly different between different groups. Values are expressed as mean ± SD ($p>0.05$).

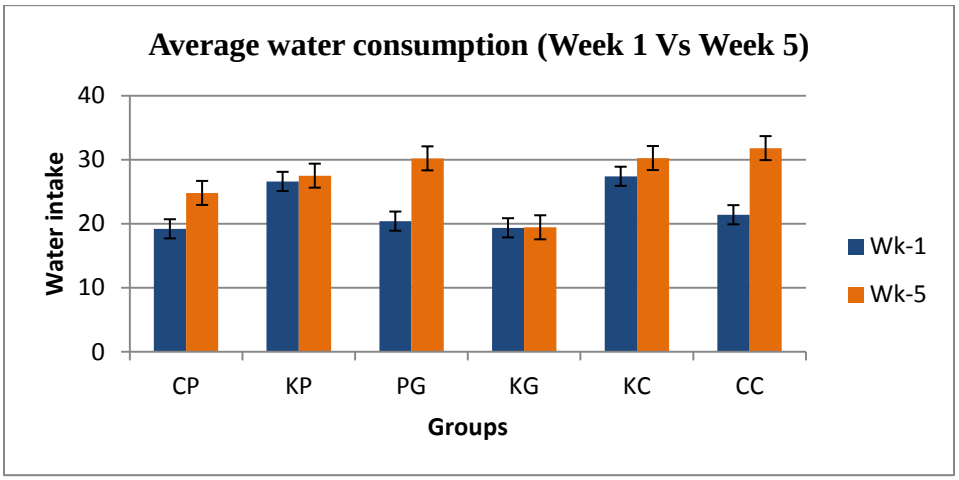


Figure 4: Water intake for all groups during Week 1 and Week 5 were significantly different between different groups CC, CP and KPG during week 1 and week 5 ($p<0.05$). Other groups showed no significant differences ($p>0/05$). Values are expressed as mean ± SD.

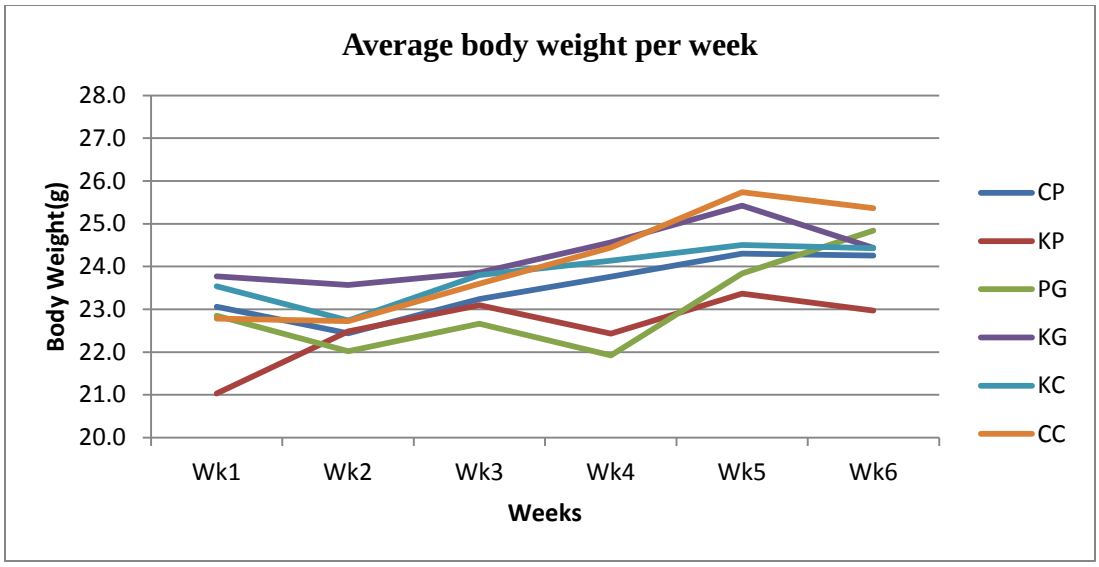


Figure 5: Average body weights of all groups from Week 1 to Week 6

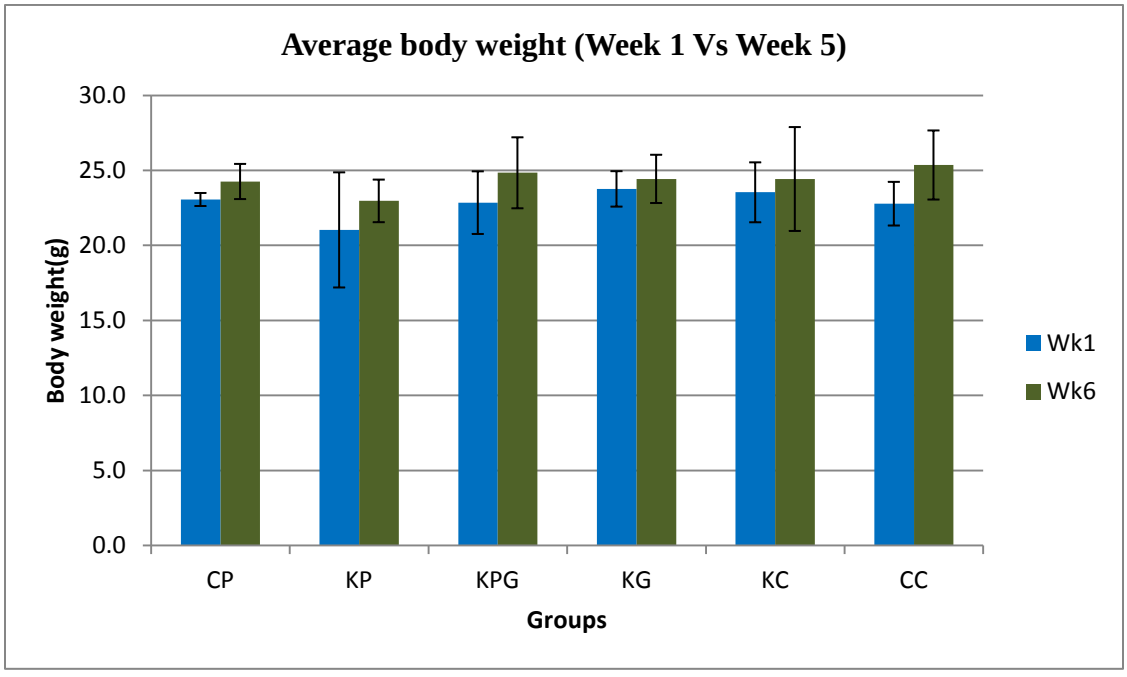


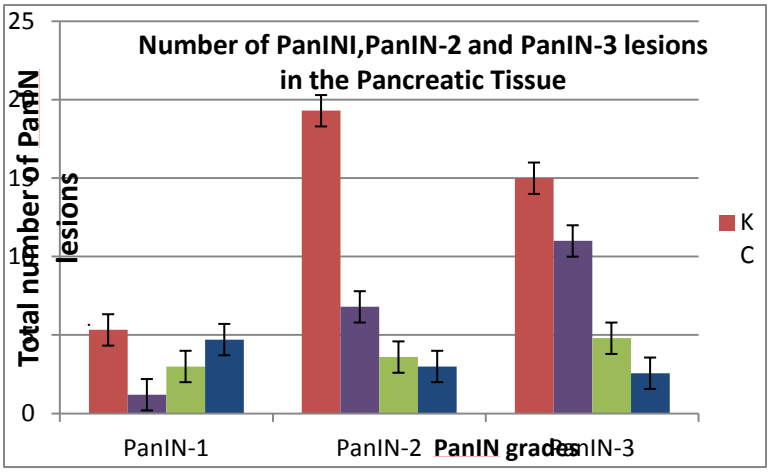
Figure 6: Body weights for all groups during Week 1 and Week 5 were not significantly different between different groups. Values are expressed as mean ± SD ($p>0.05$).

Specific aim II: To investigate histological changes produced in the liver and pancreas of OPP fed transgenic mouse by Hematoxylin and Eosin staining and immunohistochemistry.

All the slides were supervised and confirmed by Dr. Doina David, School of Medicine, Wayne State University. Each slide was studied with careful examination in every compartment of the tissue section and documented.

After careful validation of the histological slides, it was noticed that the control group KC and the treatment groups KP, KG and KPG groups showed that the acinar ducts of the exocrine region were affected and had multiple areas of adenocarcinomas in the KC group, malignant neoplasms, mitosis and desmoplasia in the KP, KG and also some in the KPG group**.** There were significant differences observed in the histological analysis between the control groups and treatment groups as hypothesized. The CP control group showed normal pancreatic tissue as compared to the CC group thereby confirming no signs of toxicity from the 5% OPP administered. The KC group showed a significantly higher number of PanIN-I, PanIN-II and PanIN-III lesions when compared to the treatment groups as hypothesized. Among the treatment groups, the KP group showed a significantly lower and higher number of PanIN-I and PanIN-III, respectively, when compared to the KG and the KPG groups. However, when the total PanIN counts were done by totaling the PanIN-I, PanIN-II and PanIN-III lesions, it was observed that the KC showed a significant increase in the overall number of PanINs when compared to the treatment groups ($p<0.05$) **(**Fig 7a). Among the treatment groups, the OPP diet given to the KP group did help in a significant reduction in the total number of PanINs as compared to the KP group. But when the KP was compared to the KG group which was administered Gemcitabine drug, it was seen that there was a significant reduction of total PanINs in the KG group as compared to the KG group. However, when OPP was given in combination with Gemcitabine in

the KPG group, there was a significant reduction in the total mPanIN lesions ($p<0.05$) (Fig 7b). The H&E staining showed evident all the mutation changes in the cancer tissue which was confirmed by performing immunohistochemistry by using specific SMAD4/DPC4 and S100P antibodies.



7a. Number of total mPanIN lesions in control (KC) and treatment (KP,KPG,KG) groups. KC groups showing significant reduction in PanIN-I,II and III lesions and KP showing significantly low number of PanIN-I lesions. Values are expressed as mean ± SD ($p<0.05$).

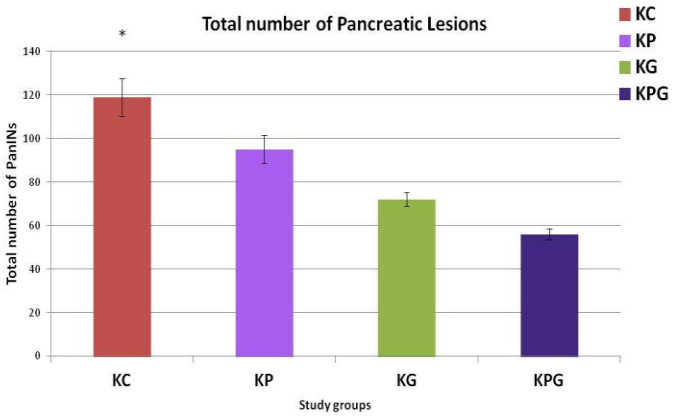
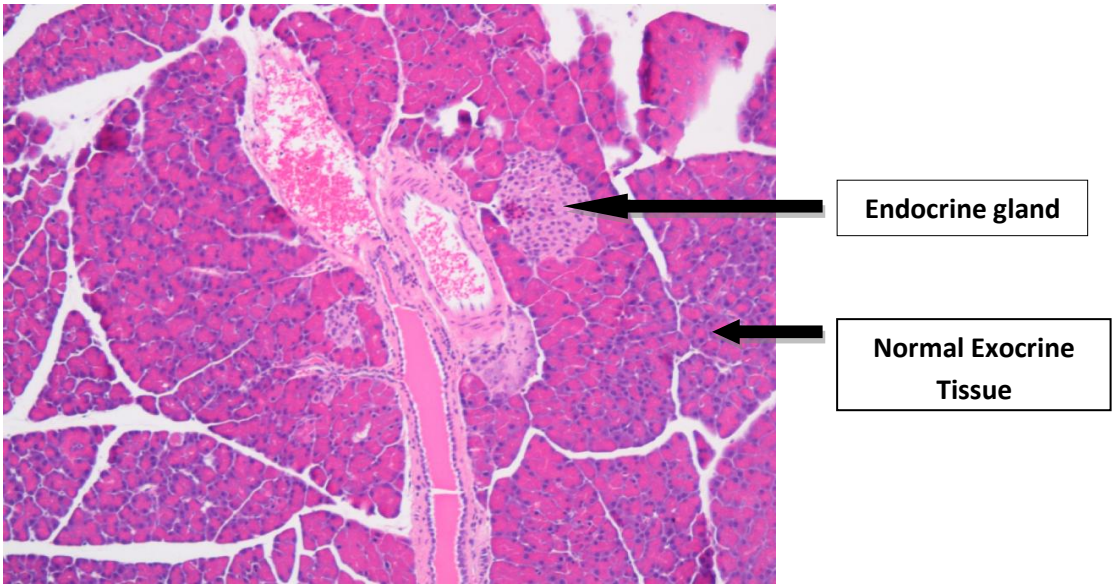


Figure 7b: Total number of mPanIN lesions in the KC and treatment groups. KPG showing significantly low number of total PanIN lesions. Values are expressed as mean ± SD ($p<0.05$).

8a)



8b)

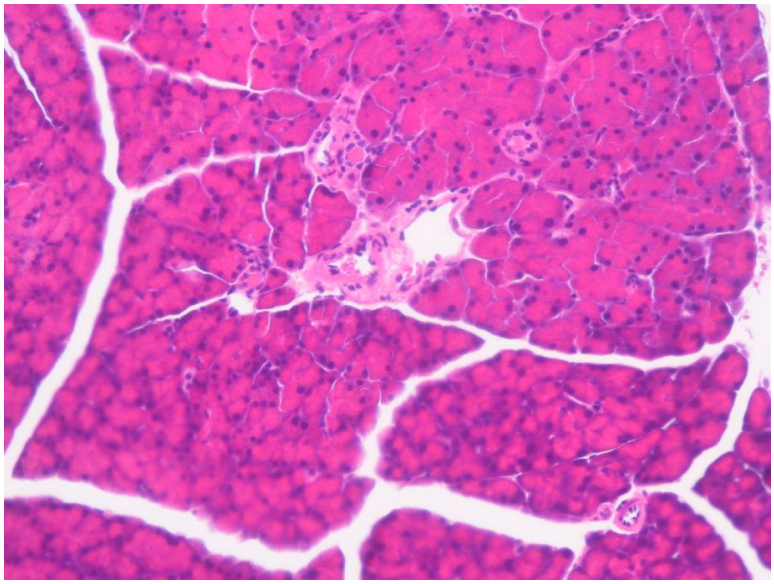
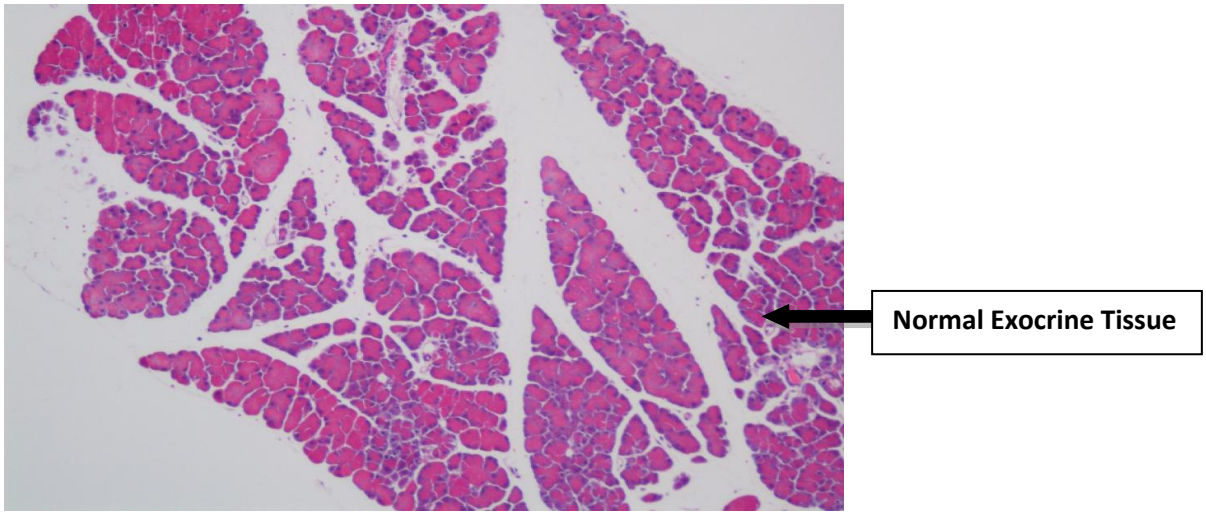


Figure 8: Microscopic regions of a control mouse on a regular diet (CC) 8a. H&E staining at 10X magnification focusing the normal exocrine and endocrine region of the pancreatic tissue; 8b. H&E staining at 20X magnification focusing the endocrine gland in a normal pancreatic tissue.

9a)



9b)

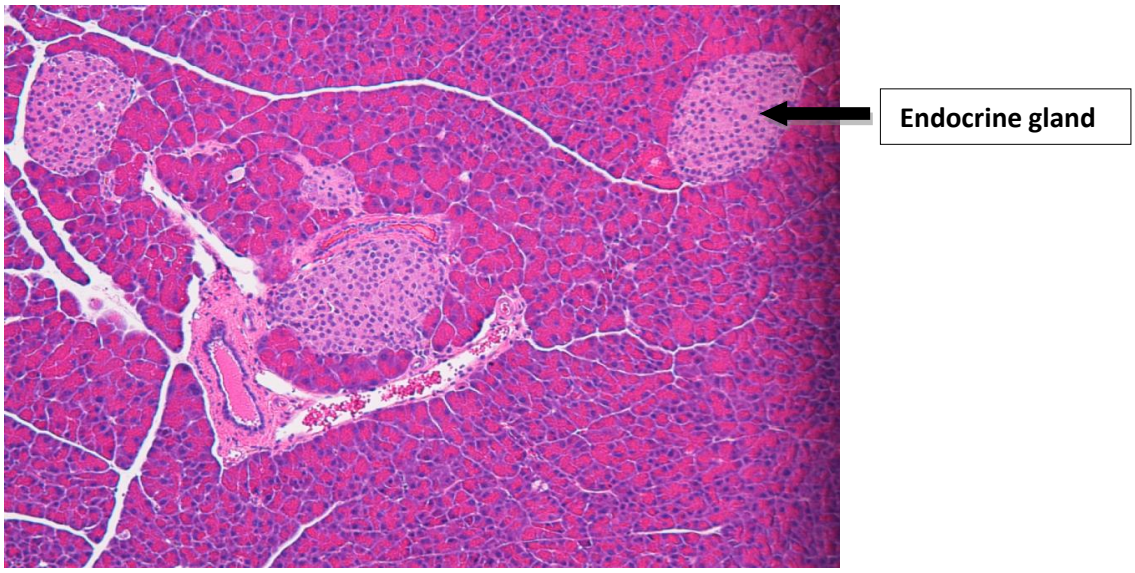
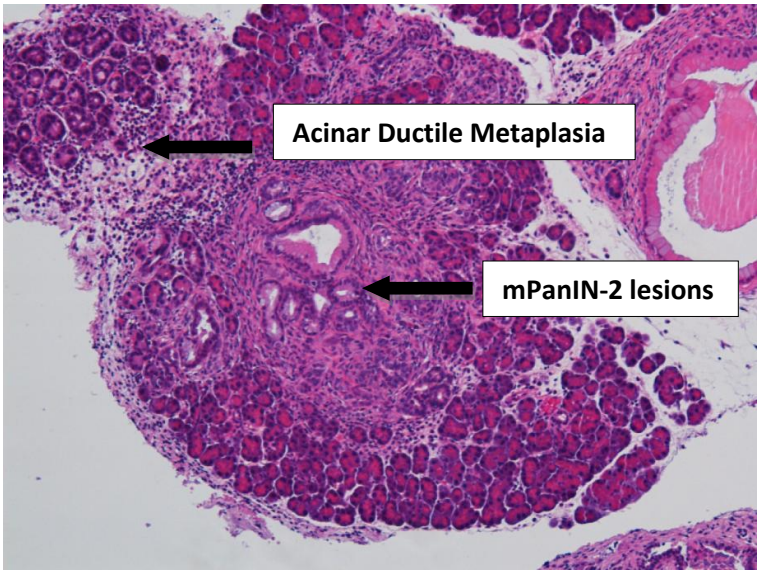


Figure 9: Microscopic region of a control mouse on a 5% OPP diet (CP) - 9a. H&E staining at 10X magnification focusing normal exocrine region of the pancreatic tissue. 9b. H&E staining at 20X magnification focusing the endocrine gland in a normal pancreatic tissue showing no signs of toxicity from the OPP administered.

10a)



10b)

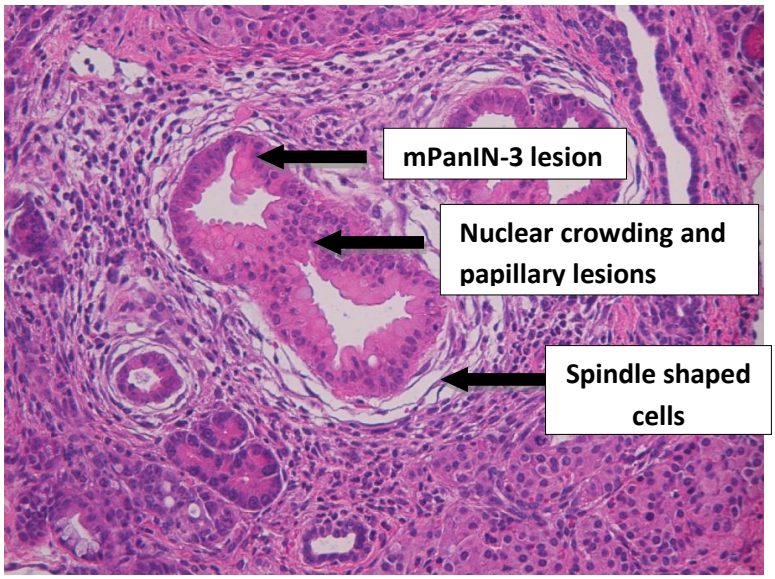
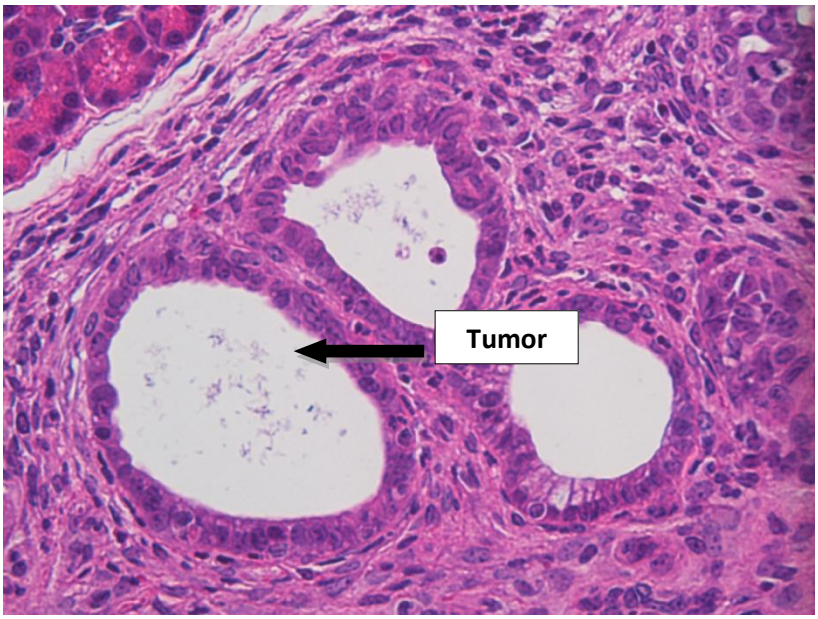


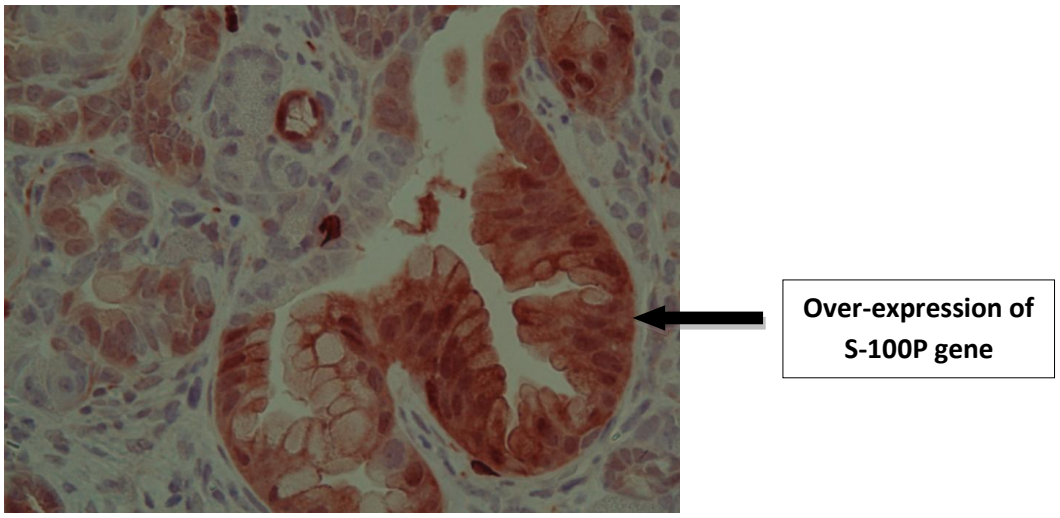
Figure 10: Microscopic region of a transgenic KPC mouse on a regular diet (KC) - 10a. H&E staining at 10X magnification focusing the emergence of ductile metaplasia and mPanIN-2 lesions in an affected exocrine portion of the pancreas; 10b. 20X magnification focusing PanIN3 with noticeable nuclear crowding, papillary lesions, budding; emerging adenocarcinoma with spindle shaped cells.

10c)

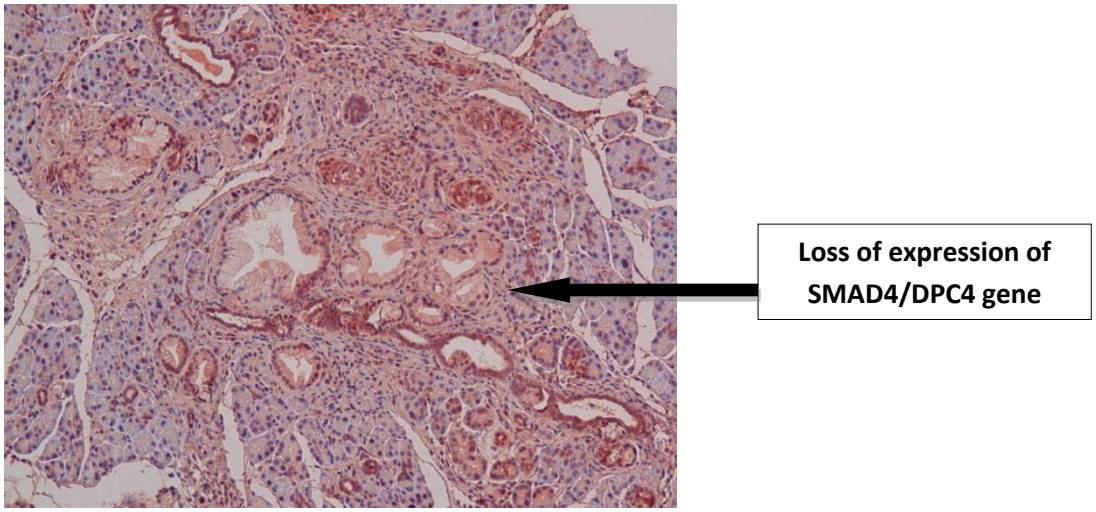


10c) 20X magnification focusing on cystic neoplasm in the exocrine region of the pancreas.

10 d)



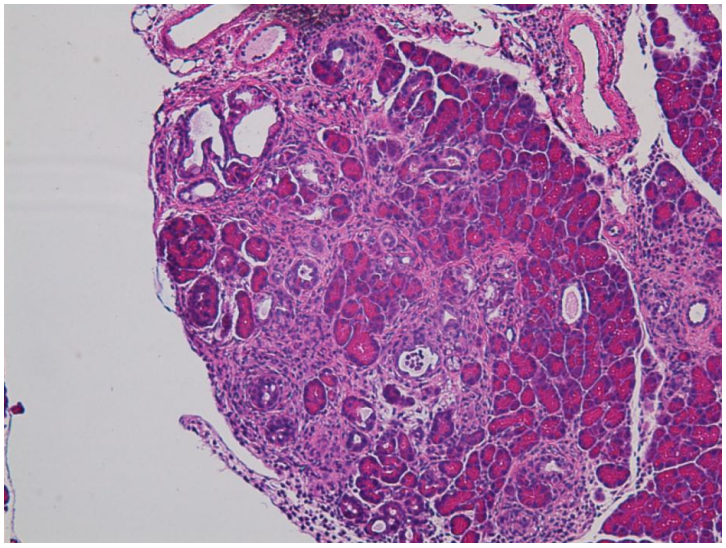
10 e)



10d. IHC – 20X magnification confirming the over-expression of S100P protein due to excessive stain adhesion

10e. IHC - 20X magnification confirming loss of expression of SMAD4/DPC4 due to less stain adhesion.

11a)



11b)

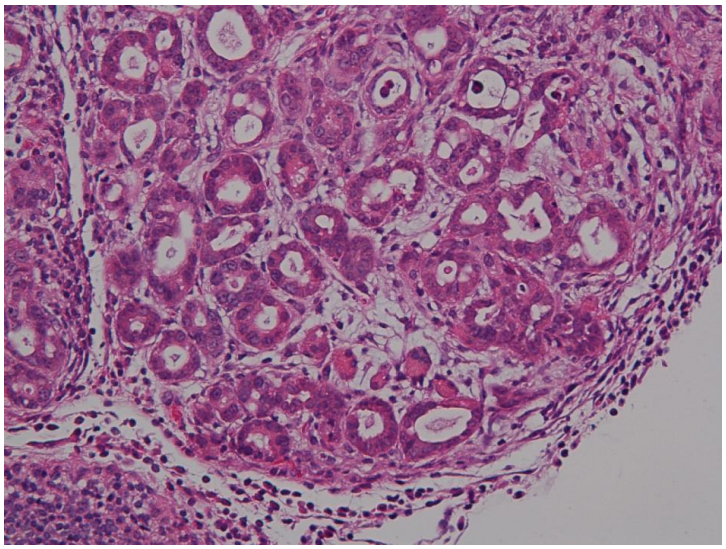
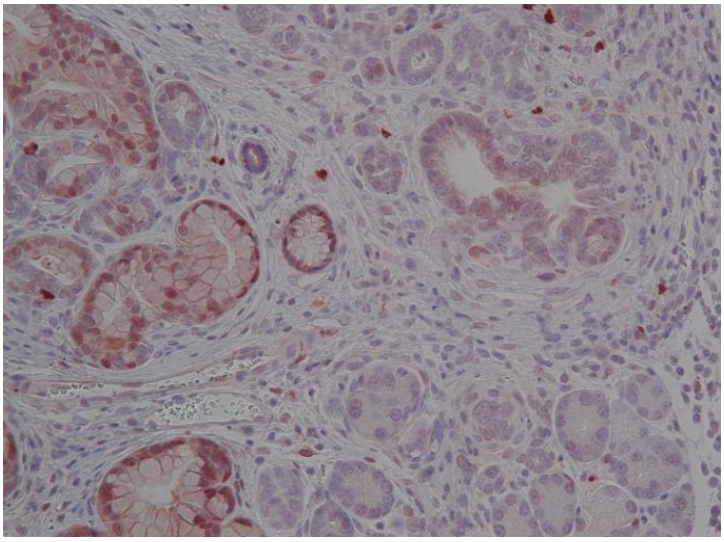
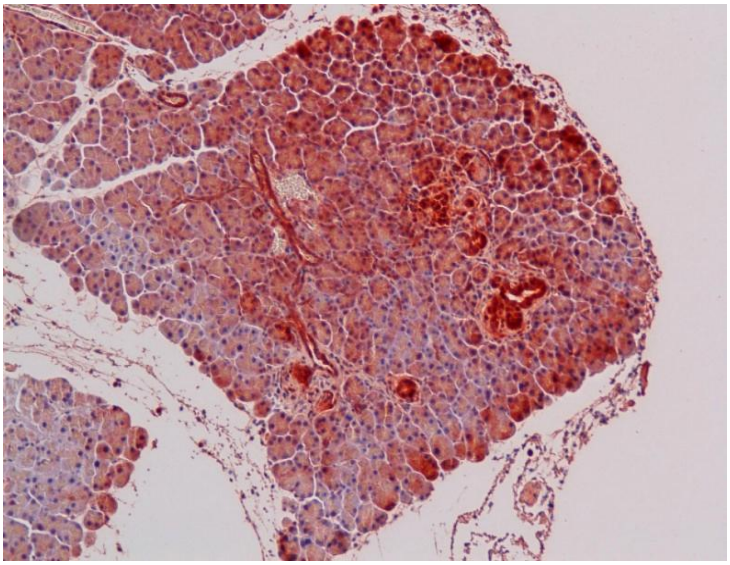


Figure 11: Microscopic regions of a transgenic KPC mouse given an OPP diet (KP) - 11a. H&E staining at 10X magnification focusing affected exocrine area with Acinar ductile metaplasia and some mPanIN lesions; 11b. 20X magnification focusing colony of ductile metaplasia that may progess to PanIN-I lesions.

11c)



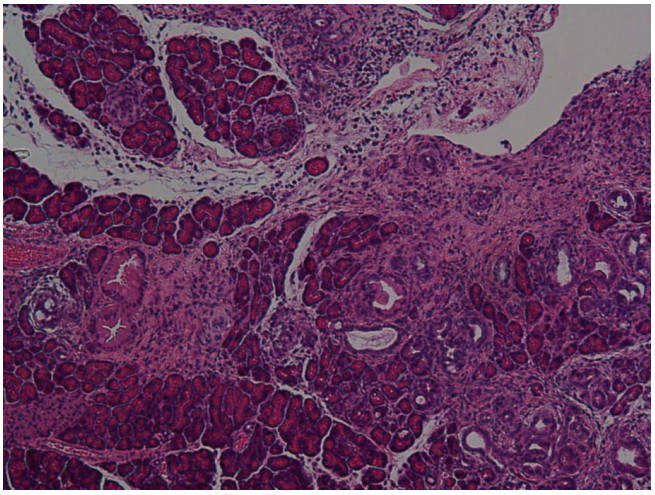
11d)



11c. IHC – 20X magnification confirming the over-expression of S100P due to less stain adhesion

11d. IHC- 20X magnification showing loss of expression of SMAD4/DPC4.

12a)



12b)

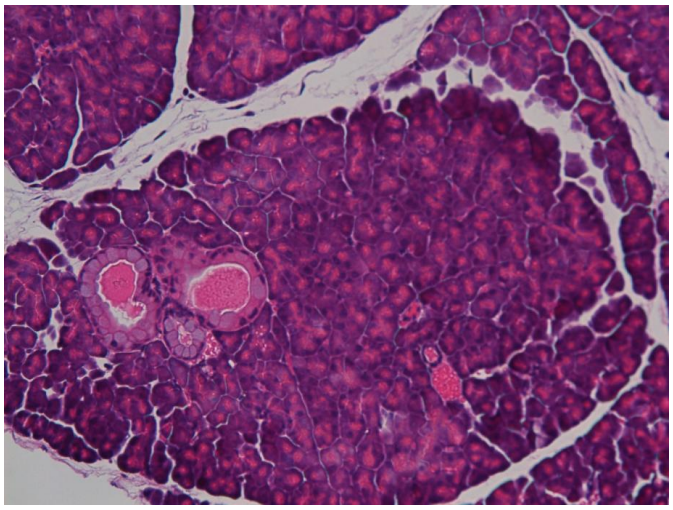
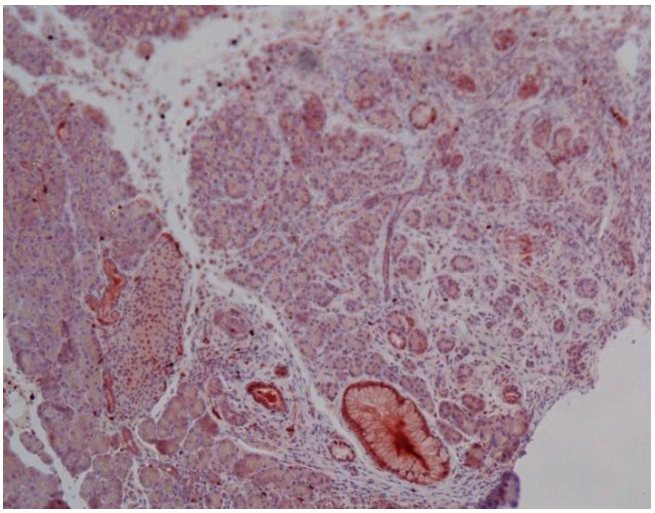
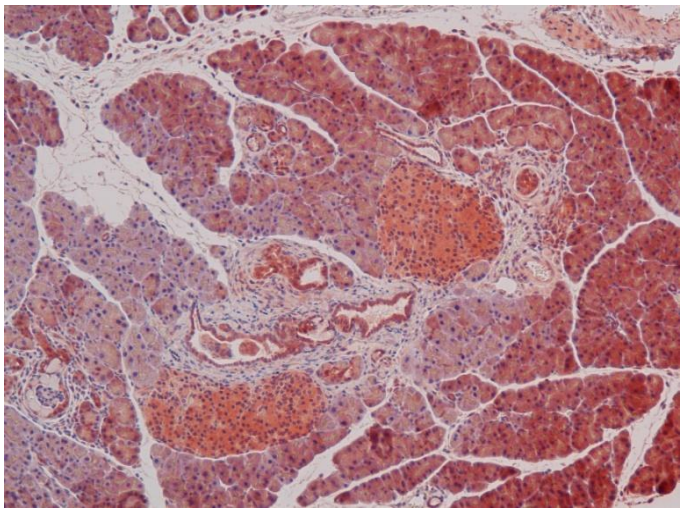


Figure 12: Microscopic regions of a transgenic KPC mouse on a regular diet and administered with Gemcitabine(KG) - 12a. H&E staining at 10X magnification focusing affected areas of the exocrine gland with traces of acinar ductile metaplasia and mPanIN lesions; 12b. 20X magnification focusing on PanIN1 lesions with basally located flat nuclei and supra-nuclear mucin on the exocrine area.

12c)



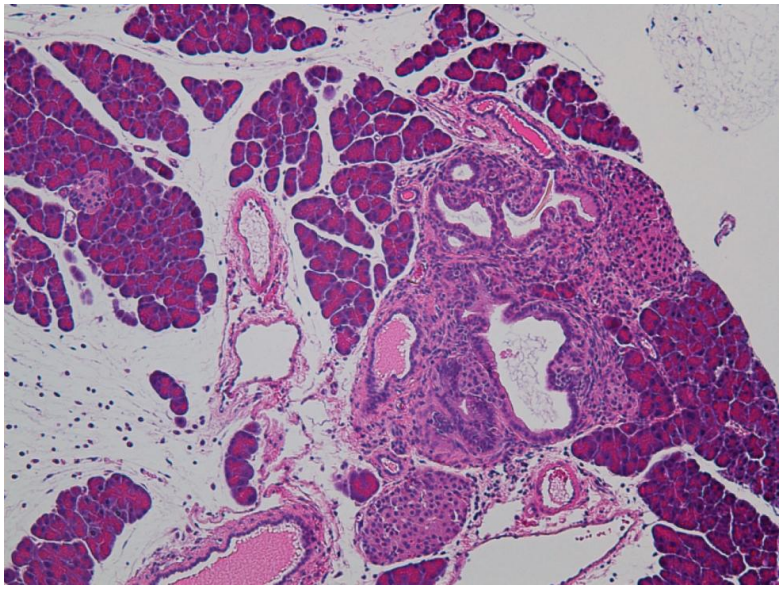
12d)



12c. IHC – 20X magnification of a transgenic KPC mouse focusing a moderately stained S100P tissue depicting slight expression of S100P

12d. IHC - 20X magnification showing comparatively better expression of SMAD4/DPC4 due to better stain adhesion.

13a)



13b)

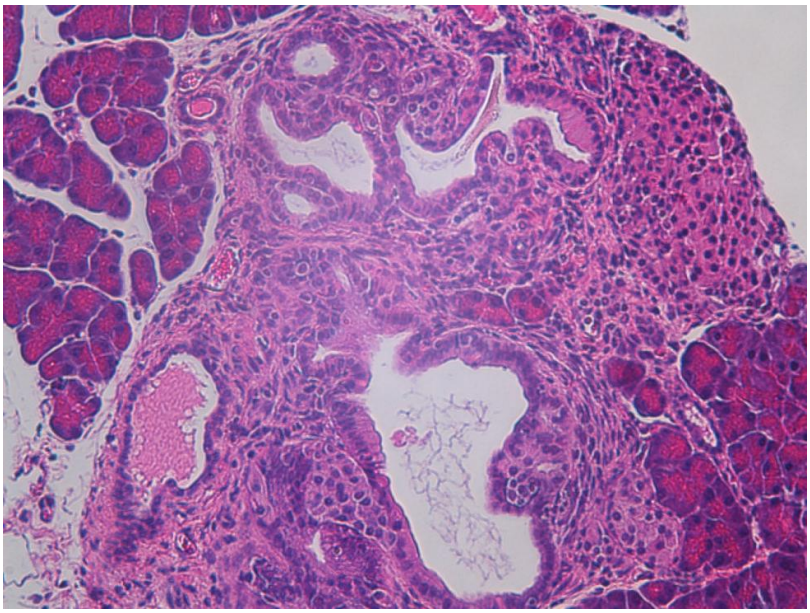
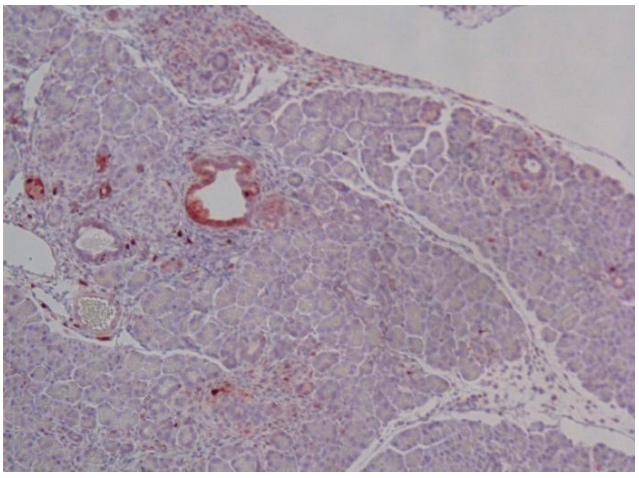
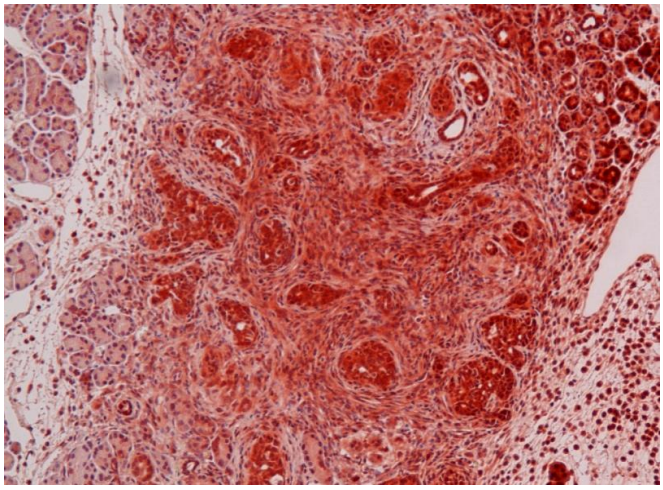


Figure 13: Microscopic regions of a transgenic KPC mouse given a 5% OPP diet and administered with Gemcitabine (KPG) - 13a.H&E staining at 10X magnification focusing affected exocrine area with mPanIN lesions; 13b. 20X magnification focusing PanIN-2 and PanIN-3 lesions with basally located flat nuclei and supra-nuclear mucin in the exocrine region.

13c)



13d)



13c. IHC – 20X magnification confirming normal expression of S100P protein due to less stain adherence;

13d. IHC – 20X magnification showing normal expression of SMAD4/DPC4 due to excessive stain adherence.

CHAPTER 4

DISCUSSION

Pancreatic cancer is the fourth leading causes of death in the United States and occurs either because they are inherited or acquired as we age. In most cases, it is diagnosed only at an advanced stage because of the inaccessibility, unavailability of resources and because it is non-responsive to many chemotherapeutic drugs. Most of the environmental factors like diet, smoking, and exposure to radiations are all a direct result of mutated genes which may become more permanent and result in the tumor formations and cancer. This problem can be minimized to a great extent by consuming more foods that are rich in antioxidants and bioactive components, which can scavenge away the free radicals produced by natural oxidative stress and other environmental factors. The transgenic LSL-$Kras^{G12D/+}$;LSL-$Trp53^{R127H/+}$;Pdx-1-Cre (KPC*)* mouse model was used to study the histopathology of pancreatic cancer. Kras protooncogene is found in 90% of human pancreatic cancers with a Pdx-1 pancreas specific promoter and results in pancreatic cancer precursors called pancreatic intraepithelial neoplasia (mPanINs)**[23**]. These lesions develop into invasive and aggressive metastatic adenocarcinomas. This transgenic mouse type is a clinically relevant model for studying pancreatic cancer as it provides very clear information by correlating with the human pancreatic ductile adenocarcinoma**[29].**

The mice were fed with isocaloric purified AIGN-93G diets at the age of 6-8 weeks for the control groups and a modified OPP diet for the treatment groups. The diets and water were provided throughout the entire study twice weekly for 6 weeks. Both food and water intake of the mice was well tolerated among both the control and treatment groups. There were no signs of

toxicity produced by the 5% OPP treatment diet in the control mice given an OPP diet (CP). The average consumption of food per week was 20-22g by all groups. There were no significant differences between the different groups in food and water consumption during week 1 and week 5 ($p<0.05$). All the control mice on regular purified diets survived the entire duration of the study, while the percentage survival rate of the KC, KP and KPG transgenic mice were at 87% and the KG mice at 75%.

The KC (Cancer+Regular diet) control group showed a significantly high number of PanIN-I,II,II and also total number of PanIN lesions when compared to the KP,KG and KPG treatment groups. This is because there was no source of therapy or treatment for the mice to recuperate from the severe damage caused by the destruction of cells. Among the treatment groups, KP (Cancer+ OPP diet) group had a significantly low number of PanIN-1 lesions and a high number of PanIN-III lesions. However, when the total counts of PanIN lesions were taken, there were a significantly high number of total PanIN lesions compared to the treatment groups KP, KG and KPG as expected. Among the treatment groups, the KP groups showed a significantly low number of total PanIN lesions compared to the KG and the KPG groups. This confirms that KP that was given a 5% OPP therapeutic diet was significantly able to reduce the total number of PanIN lesions compared to the KC group which was given a regular purified diet. Although the KG group performed better by reducing the total number of lesions significantly compared to the KP group, it can be found that when OPP and Gemcitabine were given in combination to the KPG group it showed a significant reduction in the total PanIN counts. This is because OPP is a mixture of several components and does not function as well as Gemcitabine which is a potent drug, but when given in combination, it was able to reduce the total number of PanIN-1 lesions in the KPG mice to a significant amount by arresting them and

retarding the progression to further PanIN-III lesions that eventually develop into full range carcinoma.

Furthermore, there was absolute reduction in the presence of adenocarcinomas in the epithelial tissue of the exocrine gland. Our MRI data suggested that several cystic neoplasms were detected in the the transgenic mice but a great reduction in the size of the tumors were observed specially in the KPG mice that were on a combination treatment diet. This conclusion can also be correlated with the gene expression data (not shown here) which showed decrease in MMP9, CCND1 and BCl_2 expression especially in the KPG mice when compared to the other groups. Damage to MMP9 gene is involved in the breakdown of extracellular matrix in normal physiological processes and diseases such as development, reproduction, tissue remodeling, tumor progression, metastasis and angiogenesis. NF-κB targets BCL-2 and BCL-X(L) by stimulating the anti-apoptotic signaling that plays a major role in cancer by slowing down apoptosis. Since all these genes are down-regulated in the KPG group, the histological findings are correlated with this analysis.

Immuno-histochemisty was performed using DPC4/SMAD4 and S100P antibodies. These anti-bodies were selected because over-expression of S100P protein contributes to the development of a number of tumors especially in Pancreatic adenocarcinoma **[30].** Positive expression of this calcium binding protein shows that the overall survival rate of the mice was reduced. These are clear biomarkers for analyzing and confirming the presence of cancer cells. The PanIN lesions were confirmed based on the intensity of the stain adhesion where deeper staining was observed in the order of KC>KP>KG>KPG groups. DPC4 on the other hand, is a tumor suppressor gene on chromosome 18q21. The deletion can happen due to a loss of one allele or deletion of both the alleles **[31].** All the pancreatic adenocarcinomas in this study

accounted to the loss of DPC4 expressions and is clearly evident in the microscopic visuals of the tissues where the clear indicator is the disappearance of the staining. Since this gene is associated with the TGFβ signaling pathway, its receptors, TGFβRI and TGFβRII interact with the DNA binding proteins and regulate the transcription of genes that initiate pancreatic cancer. Loss of expression was observed in the KC group when compared to the KPG group with deeper stain adhesion confirming the normal presence of the DPC4 gene in some regions of the tissue.

In conclusion, although OPP by itself was not as beneficial as gemcitabine alone in terms of lowering the number of mPanIN 3 lesions, the potential as part of combinational therapy with the current drug is quite evident from our data. Thus the combination of OPP and gemcitabine, due to their synergistic effect may be investigated further as a chemopreventive agent against cancer. Human trials are yet to be performed using this treatment method. However, this could be used as a possible chemo-preventive approach for patients suffering with pancreatic cancer once human clinical studies show similar positive results.

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ABSTRACT

ANTI-CANCER EFFECTS OF OIL PALM PHENOLICS ON PANCREATIC CANCER – HISTOLOGICAL EVIDENCE

by

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Oil palm phenolics, a water soluble portion is obtained from the milling of palm oil. It has been proven to be a remarkable anti-oxidative agent which reduces the overall oxidative stress in the body and cardiovascular disease in mice. The aim of this study was to see whether OPP has any beneficial effects on the development or progression of pancreatic cancer. A total of 30 male LSL.Kras^{G12D}/+; p53^{R172H}/+; PdxCretg/+ were categorized into control (CP, KC=6) and treatment (KP, KG, KPG=6) groups. The control groups received the regular purified diet and the treatment groups received a modified 5%OPP chow diet. 5µg/body weight of saline was give every alternate day and the treatment groups KP and KPG received the chemotherapeutic drug, Gemcitabine. At the end of the study, the animals were sacrificed, tissues collected and stored in 10% formalin for histological studies. Hematoxylin &Eosin staining was done to study the epithelial lesions caused by the cancerous cells in the exocrine pancreatic tissue. Immunohistochemistry using DPC4/SMAD4 & S100P antibodies was also done to observe the DNA damage of each mouse. The study shows the positive synergistic correlation of OPP and

Gemcitabine together which reduce the overall incidence of pancreatic adenocarcinoma in the KPC mice.

AUTOBIOGRAPHICAL STATEMENT

I have been introduced a lot to cancer studies in the last year during my course in Dr. Gupta's lab. I would love to study further about the developments and improvements in the diagnosis and treatment of cancer. I believe that my Master of Science degree at Wayne State has helped me gain exposure and will definitely help me in future when I pursue further with my studies.