Testing Mice at Risk of Pancreatic Cancer for Altered Protein Pathways Found in Diabetes

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Henley Cheung
University of Central Florida

TESTING MICE AT RISK OF PANCREATIC CANCER FOR ALTERED PROTEIN PATHWAYS FOUND IN DIABETES

by

HENLEY CHEUNG

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ABSTRACT

Pancreatic cancer is nearly asymptomatic, which can result in extensive grow and even metastasis to other organs before detection. When diagnosed at a late stage, the survival rate is 3%. Early detection is therefore the key to treating pancreatic cancer. Diabetes was identified as a risk factor for the development of pancreatic cancer, but the mechanism remains unknown. In this project, the objective was to delineate a link between diabetes and pancreatic cancer by examining their shared protein signaling pathways. In a previous study, hyper-activation of AKT1 resulted in a pre-diabetic phenotype and also increased upregulation of downstream phosphorylated mTOR and phosphorylated p70S6 kinase. More recently, mice with mutations that hyper-activated AKT1 and KRAS showed a significantly higher blood glucose level compared to littermate matched wild-type, mutant AKT1, or mutant KRAS mice. Interestingly, mice with a combination of mutations that hyper-activated AKT1 and KRAS also showed faster development of pancreatic cancer compared to these other groups of littermate mice.

Toward determining a molecular basis for the crosstalk between AKT1 and KRAS, pancreas and liver tissues were collected from all four groups of mice including wild-type, mutant AKT1, mutant KRAS, and mice with dual AKT1/KRAS hyper-activation. One strategy was to examine expression and/or phosphorylation of downstream protein signaling crosstalk by analysis of p70S6K using Western Blots. Erk 1/2 proteins were also tested as downstream proteins of KRAS to provide a molecular view of the individual and cooperative roles of AKT1 and KRAS in the mouse models. A potential feedback mechanism to affect insulin receptor signaling in the pancreas was examined using enzyme-linked immunosorbent assays (ELISA). A significant decrease in insulin receptor phosphorylation, possibly contributing to insulin

resistance, was found when mice had mutant hyper-activated KRAS. Contrary to the original expectations, mice with combined mutations of AKT1 and KRAS may contribute to the accentuated diabetic phenotype by targeting two different points in the AKT and KRAS protein signaling pathways. The information can help understand the relationship between glucose metabolism, diabetes, and pancreatic cancer development. By thoroughly studying the interactions between targets in the AKT1/KRAS signaling pathways, key molecular events that induce metabolic changes and potentially early biomarkers may lead to an improved understanding of risk and/or detection of pancreatic cancer.

DEDICATIONS

I dedicate my Honors in the Major thesis to my family and my girlfriend.

Thank you to my parents and sister, for their unconditional support of my academic career.

Thank you to Jennifer Wong, for all her love and encouragement.

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INTRODUCTION

Pancreatic Cancer Background

Pancreatic cancer is one of the most lethal cancers worldwide. It is the fourth-leading cause of cancer deaths in 2016 for both males and females in the United States (Figure 1). Patients suffered from pancreatic cancer have only 8% of overall 5-year relative survival rate, which is the lowest among all major types of cancers. The American Cancer Society estimated that over 40,000 of pancreatic cancer patients would die in 2017. This number of deaths is approximately the same as the annual number of reported new cases.

MALE		FEMALE	
Lung & Bronchus	84,590	**Lung & Bronchus**	71,280
Colon & Rectum	27,150	**Breast**	40,610
Prostate	26,730	**Colon & Rectum**	23,110
Pancreas	22,300	**Pancreas**	20,790
Liver & Intrahepatic bile duct	19,610	**Liver & Intrahepatic bile duct**	14,080
Leukemia	14,300	**Leukemia**	10,920
Esophagus	12,720	**Esophagus**	10,200
Urinary bladder	12,240	**Urinary bladder**	9,310
Non-Hodgkin Lymphoma	11,450	**Non-Hodgkin Lymphoma**	86,90
Brain & Other nervous system	9,620	**Brain & Other nervous system**	7,080

Figure 1. Estimated Cancer Deaths in 2017. (American Cancer Association, 2017)

The high mortality rate of pancreatic cancer is primarily due to the lack of a reliable early detection method, since resection of the tumor during a local stage is the only effective cure (Surgical Treatment of Pancreatic Cancer, 2016). Pancreatic cancer patients rarely display distinctive symptoms in the early stage. Symptoms such as back pain, abdominal pain, and jaundice are commonly observed in other diseases, which results in delayed diagnosis or even misdiagnosis. Currently, cancer antigen 19-9 (CA 19-9) is the best available tumor marker but it

cannot be used as a diagnostic or a screening test due to the suboptimal accuracy and specificity (only 79-81% sensitivity) (Ballehaninna, 2011).

Consequently, the tumor remains unnoticed while continuing to grow and eventually metastasizes to nearby organs, such as liver and small intestine. At this point, treatments like surgery, chemotherapy, and radiation therapy are no longer effective. The 5-year relative survival rate decreases substantially to 3% if the malignancy is detected in the advanced stage, which again demonstrates the pressing need to develop a reliable diagnostic tool.

Types of Pancreatic Cancer

Pancreatic cancer has essentially uncontrolled cell growth due to genetic mutations that accumulate in the tumors cells arising in the pancreas. The pancreas is an elongated and narrow J-shaped organ located behind the stomach and between the duodenum. It is anatomically divided into the head, the body, and the tail of pancreas to function as both an endocrine and exocrine gland, which could then lead to the formation of different pancreatic cancer. The diagnosis between endocrine tumor and exocrine tumor is crucial to determine the risk factors and proper treatment plan.

Pancreatic Neuroendocrine Tumors

Pancreatic neuroendocrine tumors (Islet cell tumors) are rarely occurring endocrine pancreatic cancer (Kaur et al, 2012). The endocrine portion of the pancreas contains clusters of hormone producing cells called the “islets of Langerhans”, which are small circular cells named

alpha cells and beta cells. These two cell types are essential in the regulation of glucose homeostasis. Alpha cells secrete glucagon to elevate blood glucose levels by reducing the speed of glycolysis and promoting gluconeogenesis. Beta cells produce insulin that increase cellular intake of glucose and glycogen synthesis to lower blood glucose levels. The islet cell tumor (ICT) is classified as "functional" if the neoplasm affects pancreatic hormone secretion and causes hormonal deregulation symptoms, while it would be "nonfunctional" if the neoplasm has no impact on the hormone secreting cells (Schwarz, 2009).

Pancreatic Ductal Adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is the predominant form of pancreatic cancer and the focus of this study. It is accountable for 94% of all reported cases in 2016 (American Cancer Society, 2017). PDAC is originated from the exocrine portions that compose 85% of the pancreas. Acinar cells in the exocrine pancreas produce digestive enzymes such as protease, lipase, and amylase. The secreted pancreatic juice enters the duodenum through the pancreatic duct to aid in digestion.

Current Treatments for Pancreatic Ductal Adenocarcinoma

Surgery

Although surgical resection is the only cure for PDAC, only 10-20% of patients are eligible for the operation. In the American Joint Commission on Cancer (AJCC) staging system, the surgically removable and localized tumors in these patients are considered to be in the

"resectable" stage (Katz et al., 2008). The rest of the patients are "borderline resectable" or "unresectable" because the tumor had already locally advanced or metastasized due to the lack of early detection method. However, 30% of patients may suffer from major complications after the procedure, such as gastroparesis (partial paralysis of the stomach) and they could only live about 12-18 months longer than patients with an unresectable tumor (Chari, 2007).

Chemotherapy & Radiation Therapy

Chemotherapy is considered to be a supplementary treatment of PDAC. The length and the type of treatment is based on the patients' conditions, which can be given to patients before or after the surgical resection to increase the success rate and survival rate (Pancreatic Cancer Treatment, 2016). Anti-cancer drugs such as Gemcitabine raises the 1-year survival rate for unresectable PDAC patients from 2% to 18%, and the survival rate can be further extended to 27% if coupled with Cisplatin (Heinemann, 2001).

Radiation therapy is sometimes used along with chemotherapy to support the treatment. This treatment can be given to "borderline resectable" tumor in order to shrink the tumor. It can also be given after surgery to lower the chance of recurrence. However, the effect of this therapy is still uncertain because it could have positive or negative overall outcome on different patients.

Molecular Basis for Pancreatic Ductal Adenocarcinoma Development

PDAC arises from the epithelial cell of the pancreatic duct. Pancreatic intraepithelial neoplasia (PanIN) is the precursor lesions of PDAC that is classified from PanIN-1 to PanIN-3 based on the structural and nuclear distortion. PanIN-1 is subdivided into flat epithelial lesion (PanIN-1A) and papillary epithelial lesion (PanIN-1B). PanIN-2 lesion begin to display nuclear abnormality such as some loss of polarity and enlarged nuclei. PanIN-3 is papillary with complete loss of nuclear polarity (Klein et al., 2002). It would take approximately 17 years to fully develop from PanIN-1 lesion into an invasive PDAC. The symptoms are likely to appear after PDAC reaches regional or distant stages. Patients may suffer from abdominal pain, back pain, weight loss, jaundice, and gallbladder or liver enlargement (Stark & Eibl, 2015).

KRAS

Epidemiological studies point to several risk factors that lead to PDAC progression. Certain genetic mutations are associated with the development of PDAC, such as KRAS, BRCA-1 & BRCA-2, and CDKN2A. Specifically, the KRAS oncogene is present in 99% of PanIN-1 and 95% of PDAC (Makohon-Moore & Iacobuzio-Donahue, 2016). KRAS is a 21 kDa GTP-binding protein that mediates cell growth by cycling through the active GTP-bound and inactive GDP-bound states (Cox et al. 2015). The mutation of KRAS gene leads to the formation of constitutively active GTP-bound KRAS and uncontrolled cell proliferation, which serve as an important PDAC initiation factor. KRAS also plays a role in inducing a chronic inflammation environment associated with PDAC (Liu et al., 2016).

Chronic Inflammation

Patients who suffer from chronic inflammatory diseases such as pancreatitis are also susceptible to PDAC development. These patients have higher chance of experiencing undesirable mutations because of the constant cycle of damage and repair in the pancreas. Smoking cigarette can cause DNA damage by ingesting carcinogens and obesity induce a pro-inflammatory state that favors genetic mutation. Although PDAC generally arise spontaneously, about 2% of the cases are hereditary (Windsor, 2007). Mutations in germline variant the that maintains genomic stability, such as BRCA 1 and BRCA 2, would increase the rate of mutations in cells during homologues recombination or DNA double-strand break repair machinery. The incidence and death rate of PDAC increase with aging since pancreas lesion persist for a long time (American Cancer Society, 2017). The accumulation of intrinsic mutations over the years in both oncogene and tumor suppressor genes along with extrinsic factor like smoking, would eventually drives the tumor to an exponential growth phase.

Diabetes

The progression of PDAC continues once the mutated cells are fixed in the epithelial cell population after passing through the surveillance of apoptosis, senescence, and immunoresponse. According to prior epidemiology studies, two-thirds of the PDAC patients are diabetic or glucose intolerant and 75% of the cases are recent onset diabetes (Munira & Chari, 2012). The increase in glucose uptake or glucose accumulation correlates with more rapid tumor progression as well as reduced response to treatments. Diabetic phenotypes could arise before or after PDAC formation. The cause and effect conundrum makes it difficult to sort out the relationship between the

morbidities. The Warburg effect suggests that cancer cells favor aerobic glycolysis instead of oxidative phosphorylation which is used in normal cells (Vander Heiden, 2009). Although aerobic glycolysis is not as efficient in ATP production, this process conserves carbon sources to produce biomass that is essential for cancer cells to rapidly proliferate. As such, glycolytic enzymes were found strongly increased in pancreatic tumor cells compared with normal pancreatic cells (Blum & Kloog, 2014).

In type II diabetes patients, their blood glucose accumulates and generates a hyperglycemic environment that cancer cells thrive in. The regulation of blood glucose is highly dependent on glucagon and insulin secreted by the endocrine pancreas to interconvert between glucose and glycogen. Although normal insulin secretion is observed in type II diabetes patients, their cells are resistant to insulin which fail to respond to the hormonal regulation (Classification and Diagnosis of Diabetes, 2015). Insulin resistance of the cell leads to hyperinsulinemia and hyperglycemia, which are both associated with an increased risk of PDAC despite of obesity (Cui & Anderson, 2012) (Stattin et al., 2007).

AKT

AKT, also known as protein kinase B, has 3 highly conserved isoforms (AKT1, AKT2, and AKT 3) that regulate cell growth and proliferation. The isoform AKT2 is known to play a critical role in glucose metabolism through insulin mediation since AKT2 knock-out mice are glucose intolerant and insulin resistant. Recent study has shown that AKT1 regulate glucose metabolism through glucagon instead, whereas the AKT1 mice remain sensitive to insulin stimulation (Albury

et al., 2016). Hyper-activated AKT1 mice exhibit a pre-diabetic phenotype that can be turn on and off by doxycycline treatment and is often found activated in PDAC tumor.

Remarkably, the combination of hyper-activated AKT1 and KRAS leads to a more severe diabetic phenotype and faster PDAC development compared to other groups of mice (Albury et al., 2015). We hypothesize that the crosstalk between AKT1 and KRAS upregulates the production of downstream proteins in the corresponding protein signaling cascades (Figure 2). One potential method to delineate the mechanism is to examine cells' protein signaling pathway, which is often considered as the language of cells.

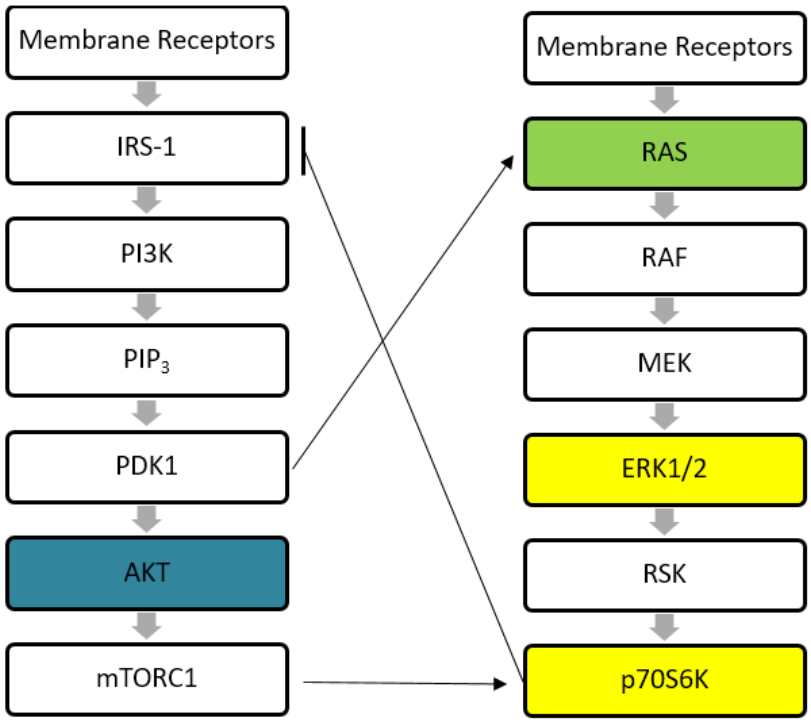

Figure 2. AKT and KRAS Signaling Pathway

Insulin receptor substrate 1 (IRS-1), phosphatidylinositol 3-kinase (PI3K), phosphatidylinositol 3,4,5-trisphosphate (PIP3), 3-phosphoinositide-dependent protein kinase-1 (PDK1), AKT1, mammalian target of rapamycin complex 1 (mTORC1), KRAS, Rapidly Accelerated Fibrosarcoma (RAF), MEK, Extracellular signal-regulated kinase (ERK) 1/2, p90 ribosomal protein S6 kinase (RSK), ribosomal protein S6 kinase beta-1 (p70s6K).

Study Design

In the study of PDAC, the *KRAS* gene is being investigated because of its essential role for cancer development observed in PanIN-1 and PDAC tumors. In addition, the *AKT1* gene alone has the effect of generating a pre-diabetic phenotype in mice while speeding up the PDAC development if coupled with constitutively activated KRAS. To study the link between diabetes and PDAC, wildtype, AKT1, KRAS, and AKT1/KRAS mice groups were selected in this project. The AKT1 transgenic mice are used to mimic diabetic patients because of its function in glucose metabolism. This also provides a more controlled model since the AKT1 mice generally acquire diabetes before tumor development, and it progresses with advanced age. The KRAS transgenic mice are used to mimic PDAC patients since it is a prevalent oncogene in PDAC patients.

We will focus on a junction protein, p70S6K, to validate the crosstalk between AKT1 and KRAS using western blots. Another protein of interest, ERK1/2, is also examined to determine the expression of KRAS under the crosstalk effect. Additonally, enzyme-linked immunosorbent assay (ELISA) is used to study membrane receptors that initiate the pathways by binding with total and phosphorylated insulin receptors. This could potentially reveal the mechanism of diabetes and PDAC development in AKT1/KRAS mice. The study of convergent points in the pathways of AKT1 and KRAS could potentially lead to a greater understanding of the relationship of glucose metabolism and the progression of pancreatic cancer.

MATERIALS AND METHODS

Genetically Engineered Mice

The animal studies were in compliance with the UCF Institutional Animal Care and Use Committee at the Association for Assessment and Accreditation of Laboratory Animal Care International accredited(AAALAC) UCF Lake Nona Animal Facility. Mice with TetO-MyrAKT1, but lacking the knock-in Pdx-tTA, were classified as normal or wild-type litter mates. Mice with Pdx-tTA and TetO-MyrAKT1 were classified as AKT1Myr mice having constitutively active AKT1 in the pancreas. Mice with Pdx-Cre; LSL-KKRASG12D were classified as KKRASG12D. Mice with Pdx-tTA, TetO-MyrAKT1, and Pdx-Cre; LSL-KKRASG12D were classified as AKT1Myr/KKRASG12D. All mice were euthanized according to American Veterinary Medical Association guidelines.

Blood Glucose Measurement

At least 6 individuals from each group of ~5 month old mice (WT, Akt1, KKRAS, and Akt1/KKRAS) were tested for blood glucose using a Contour glucometer (Bayer, Mississauga, Ontario, Canada) at 20 weeks.

Protein Homogenize

17 mice from the various groups were euthanized after fasting overnight and then blood and various tissues, including pancreas and liver, were collected and frozen. Tissue samples were homogenized mechanically using tubes with pestles (Kimble Biomasher II) in Tissue PELB (G-

Biosciences) and Protease & Phosphatase Inhibitor (Pierce). Another set of pancreas samples were homogenized mechanically in liquid nitrogen using ceramic mortar and pestle in RIPA Buffer (Santa Cruz) and Protease & Phosphatase Inhibitor (Pierce). The supernatants were collected after centrifugation at 20,000 *g* for 15 minutes at 4°C and the pellets were disposed. The concentrations of the extracted total proteins were determined using Coomassie Assay compared to a known reference protein, Bovine serum albumin (BSA).

Western Blots

For western blot analysis, 60 µg of each protein extract was combined with 4x Laemmli Sample Buffer and denatured in a boiling water bath for 5 minutes. Chameleon Duo Pre-Stained Protein Ladder (LI-COR) and protein samples were separated on 4% sodium dodecyl sulfate stacking gel and 12% sodium dodecyl sulfate resolving gels using a Mini-Protean Tetra Cell (Bio-Rad) unit. The gel was running at 80V for 15 minutes and then 100V for 1 hour. Proteins were transferred to immobilon-P PVDF Transfer Membrane (Millipore) at 100V at 4°C for 1 hour and 30 minutes using a Mini-Protean Tetra Cell (Bio-Rad) unit. Antibodies for western blots were anti-Total-p70S6K, anti-Phospho-p70S6K, anti-Phospho-ERK1/2, and anti-Beta-Actin antibodies (all from Cell Signaling Technology). Secondary antibodies were anti-mouse (DyLight, Thermo) and anti-rabbit (IR Dye; LI-COR Biosciences, Lincoln, NE), and signals were visualized using an Odyssey Infrared Imaging System (LI-COR).

ELISA

Four solid phase sandwich ELISA kits (all from Invitrogen): AKT (Total), AKT (pS473), Insulin Receptor (Beta-subunit), and Insulin Receptor (pY1158) were used.

The samples and the specific standard provided by the ELISA kit were added to the antibody-coated wells for 2 hours of incubation at 25°C. Detection antibody was added after 4 washing steps and incubate for 1 hour at 25°C. After washing steps, secondary antibody (streptavidin-HRP) was added to the wells for 30 minutes of incubation. After washing and 30 minutes of incubation with stabilized chromogen, stop solution was added and the absorbance was read at 450 nm.

RESULTS

At 20 weeks, WT, AKT1, KRAS, and AKT1/KRAS mice (n=30) blood glucoses were tested in by a glucometer. A portion of AKT1/KRAS mice (n=3) have significantly higher non-fasting blood glucose levels (600+ mg/dl) than the wild-type, AKT1, and KRAS mice. The rest of the AKT1/KRAS mice (n=8) have non-fasting blood glucose levels similar to AKT1 mice (Figure 3 & 4).

The level of activities of AKT and insulin receptor is determined by running ELISAs. AKT (Total), phosphorylated AKT(pS473), and insulin receptor (β - subunit) demonstrated persistent level of activity across the board (Figure 5, 6, and 7). However, there is a substantial variance among AKT1 and KRAS mice in the level of the phosphorylated insulin receptor (pY1158) (Figure 8).

Western blot analysis shows inconsistent bands of p70S6K (70 kDa) for the tissue samples extracted with the Tissue PELB. The image has strong background noise for all four groups of mice on the 12% SDS-Gel (Figure 9) and unknown heavy bands are observed universally at the top of the gel. After switching from PELB to the standard RIPA buffer, the result for ERK1/2 (44/42 kDa) bands are consistent but the strength of the signal is not ideal (Figure 10). The control, Beta-actin (43 kDa) bands, are shown in the gel with unknown heavy bands at the top of the gel again (Figure 11).

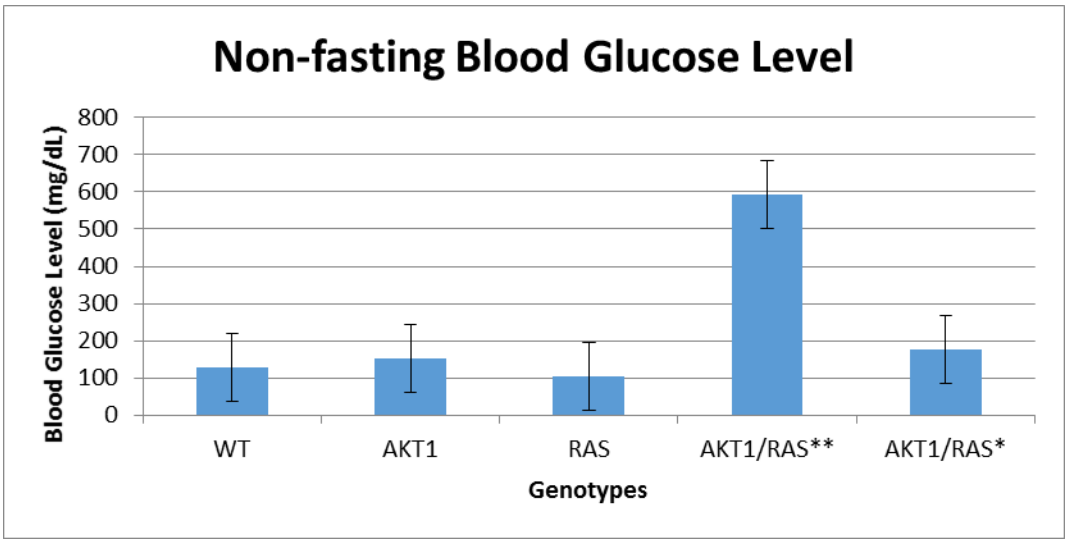

Figure 3. Non-fasting Blood Glucose Level in 6 months old Mice

*Blood glucose levels were measured in 6 months old mice using a glucometer. AKT1/KRAS** mice are found to have blood glucose level higher than 500 mg/dL. This group of mice have a more severe diabetic phenotype compared to AKT1 and are suspected to be at risk of PDAC development.*

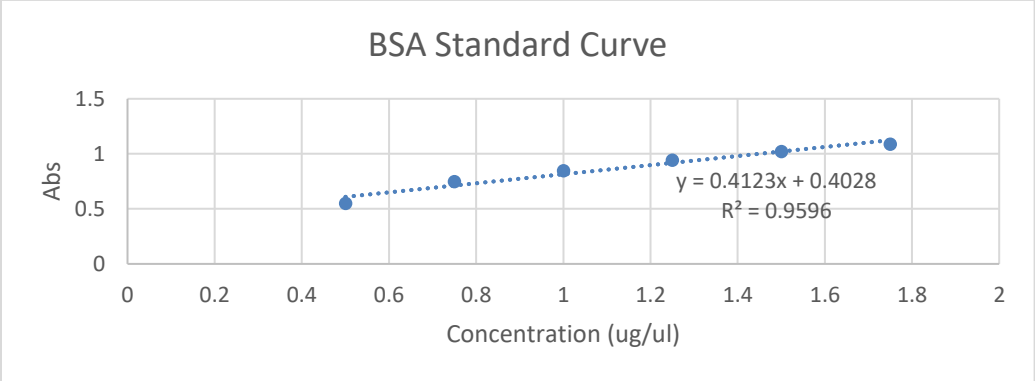

Figure 4. Total Concentration Determination using Bradford Coomassie Assay

A known reference protein, Bovine serum albumin (BSA), was used during the Coomassie Assay to determine the total concentrations of the protein.

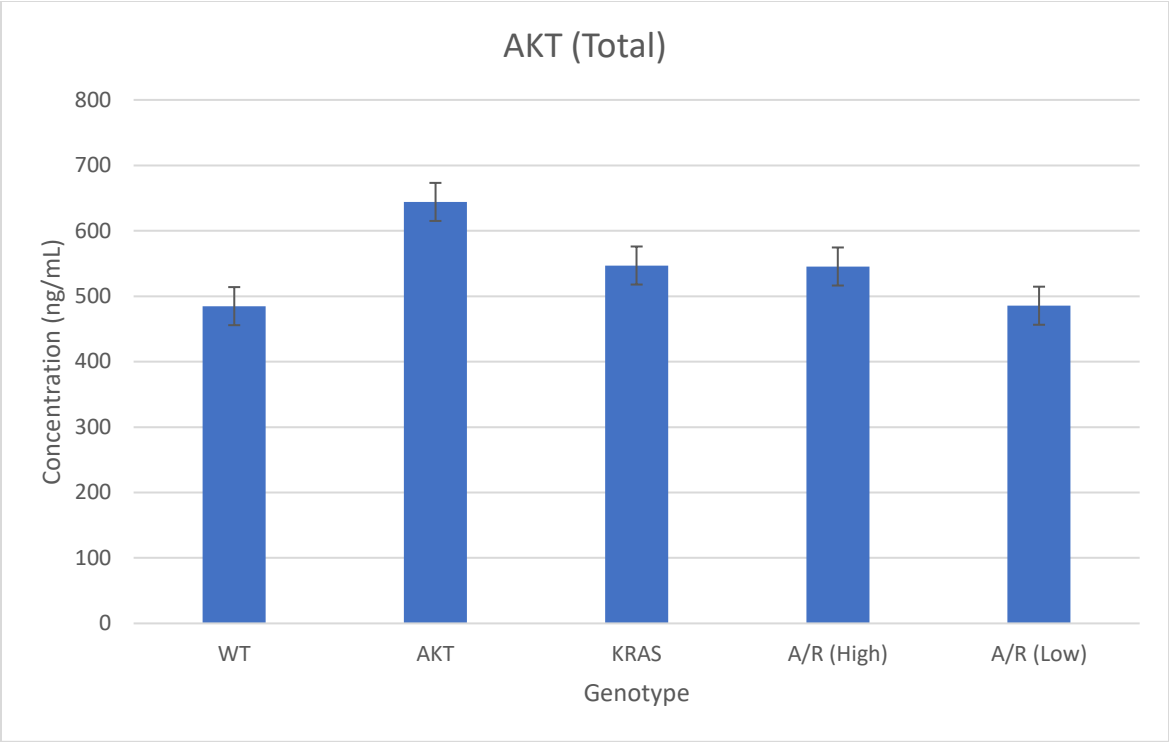

Figure 5. ELISA Assay for Total AKT

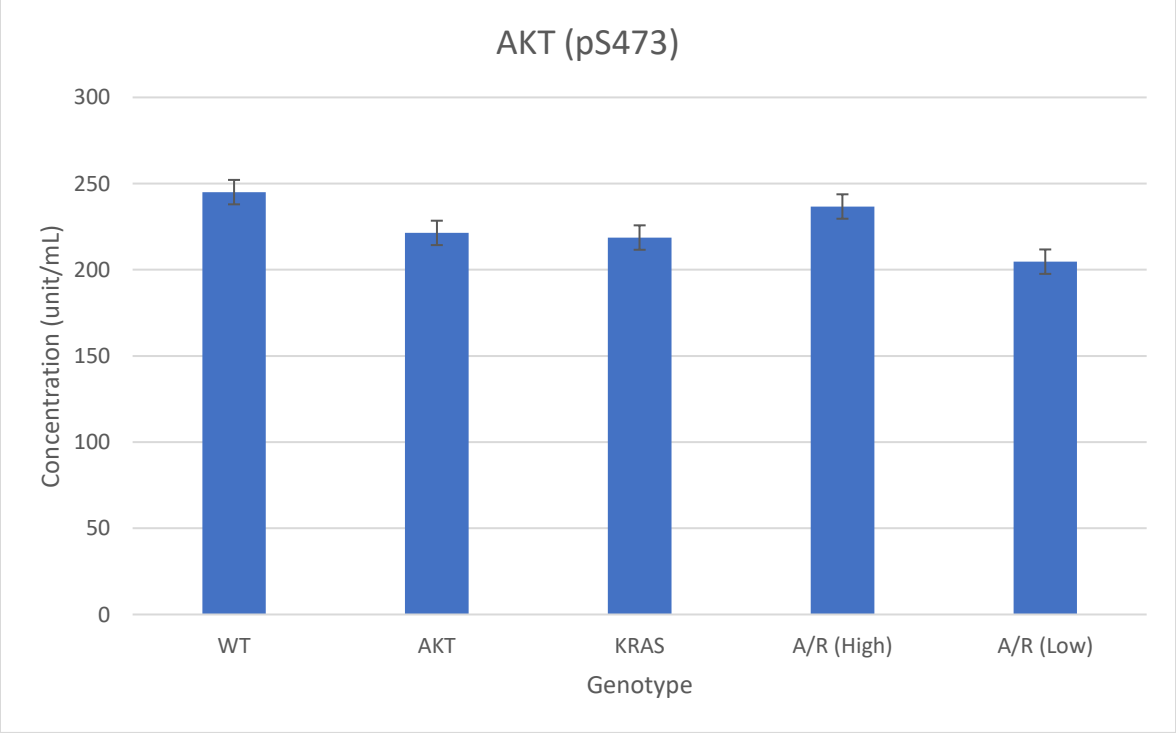

Figure 6. ELISA Assay for AKT (pS473)

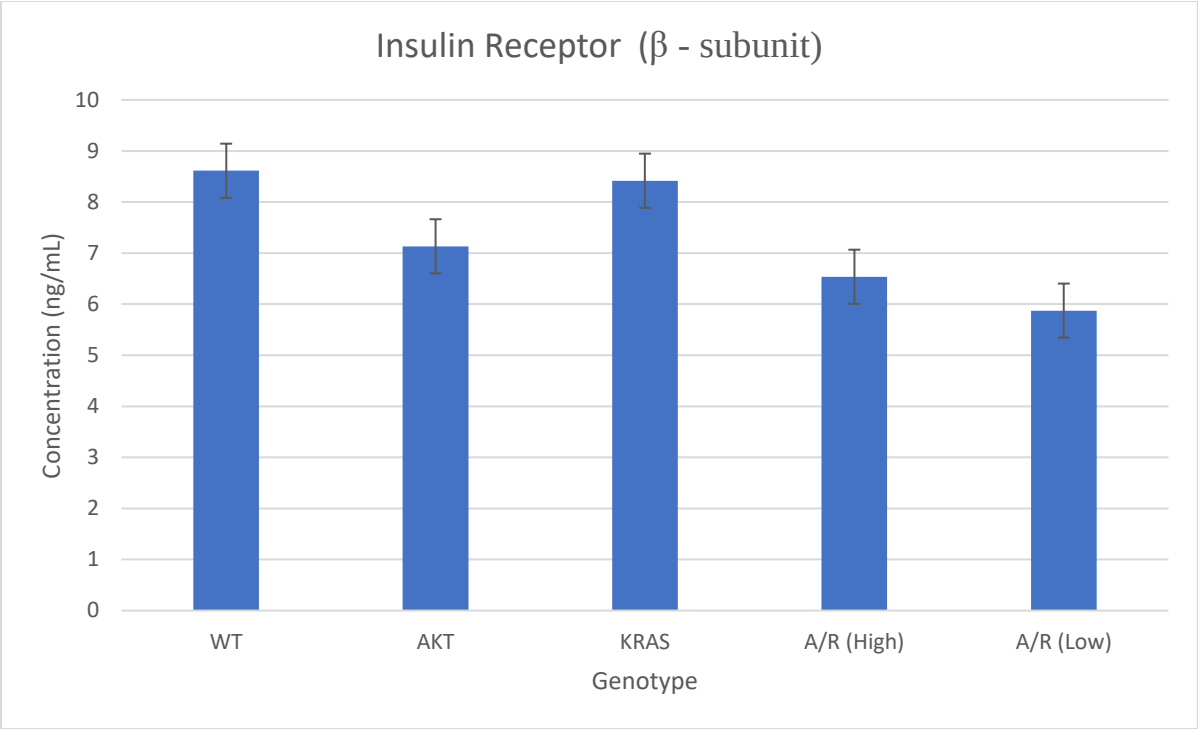

Figure 7. ELISA for Insulin Receptor (β - subunit)

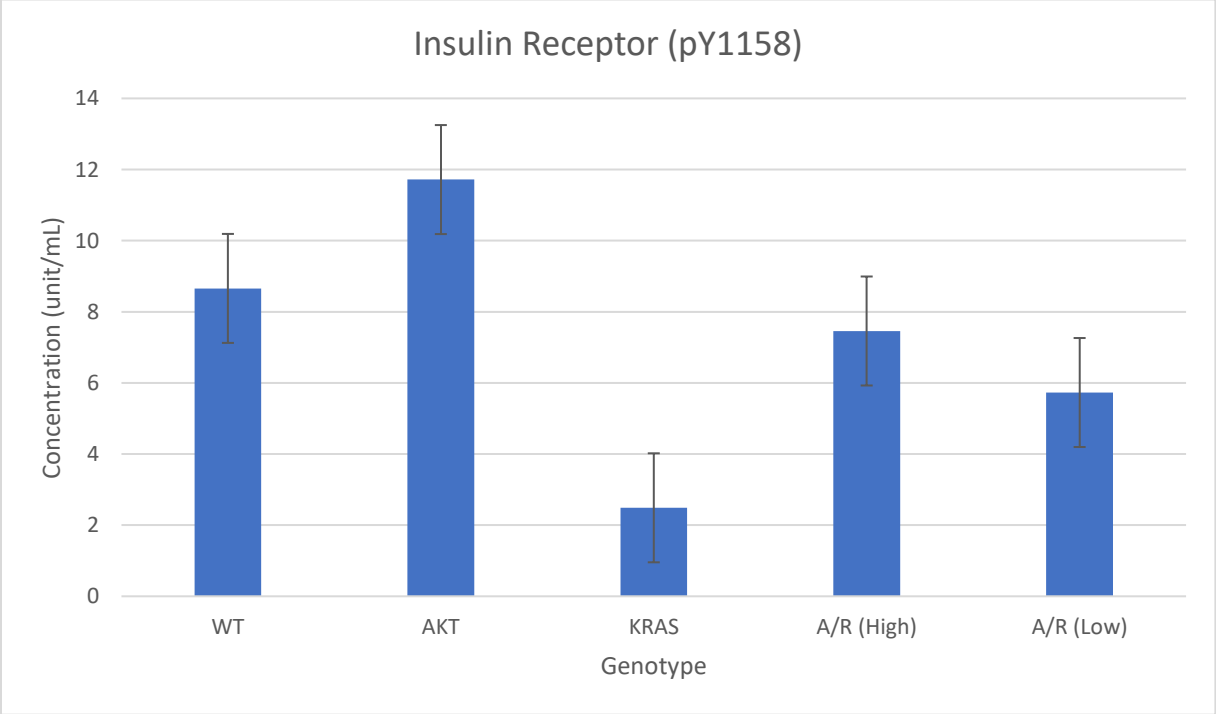

Figure 8. ELISA for Insulin Receptor (pY1158)

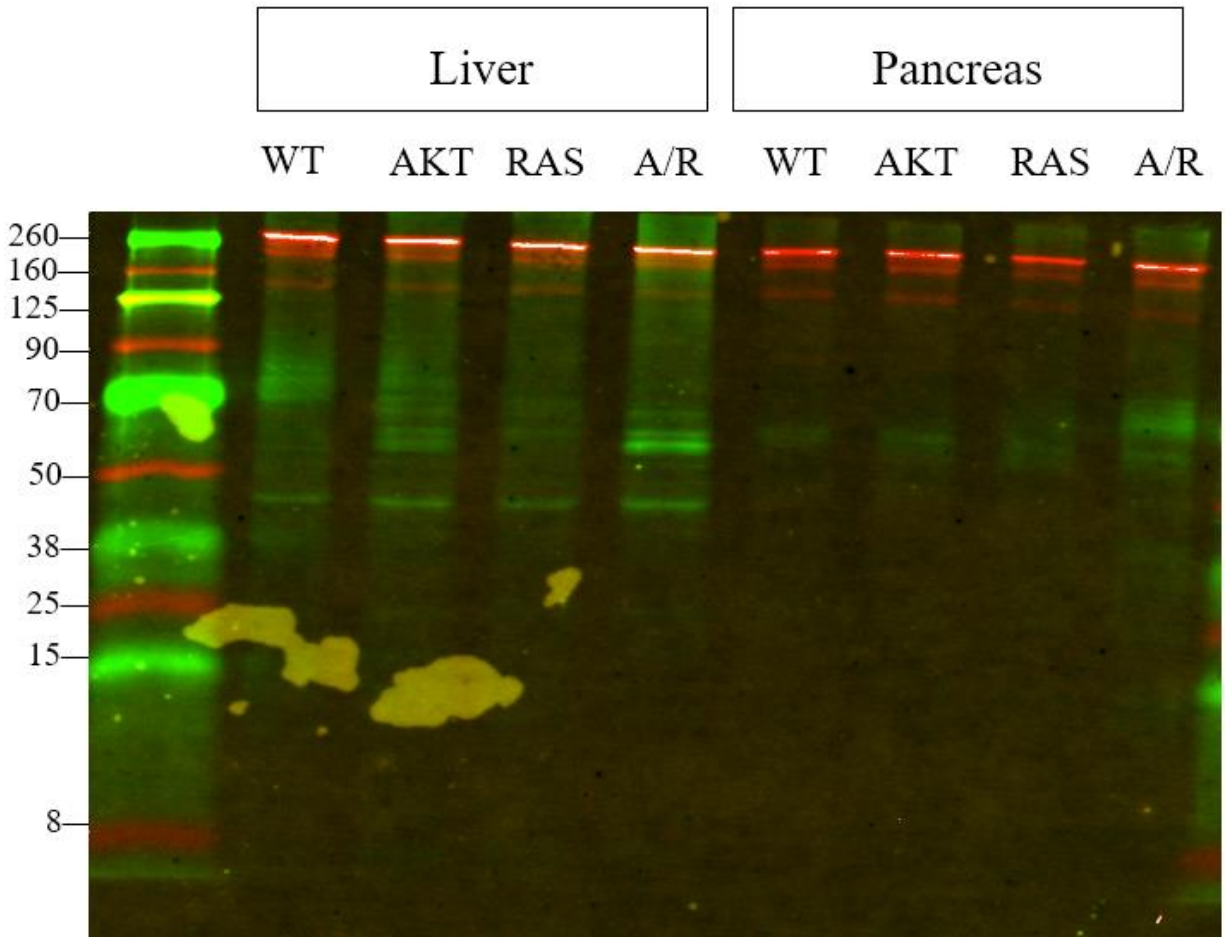

Figure 9. 12% SDS- PAGE, anti-Total and anti-Phospho p70S6K

Results of western blot analyses of p70S6K (70 kDa) in homogenized pancreas and liver tissues from mice 5790 (Wild-type), 5767 (AKT1), 5792 (KRAS), 5778 (AKT1/KRAS). p70S6K is detected using 12% gel and anti-Total and anti-Phospho p70S6K. Unknown heavy bands are also found at the top of the gel.

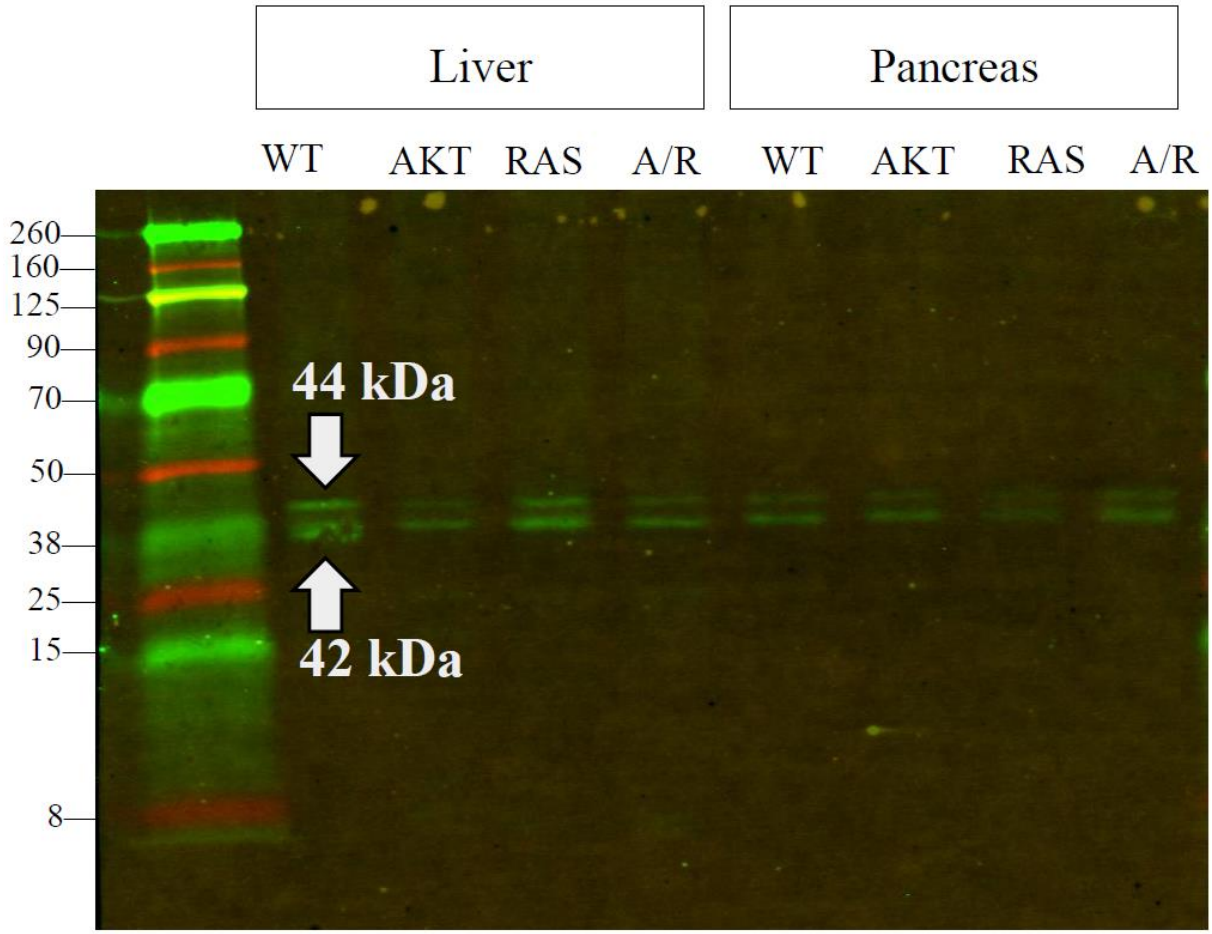

Figure 10. 12% SDS- PAGE, anti-ERK1/2

Results of western blot analyses of ERK1/2 (44/42 kDa) in homogenized pancreas and liver tissues from mice 5790 (Wild-type), 5767 (AKT1), 5792 (KRAS), 5778 (AKT1/KRAS). Erk1/2 is detected using 12% gel and anti-ERK1/2.

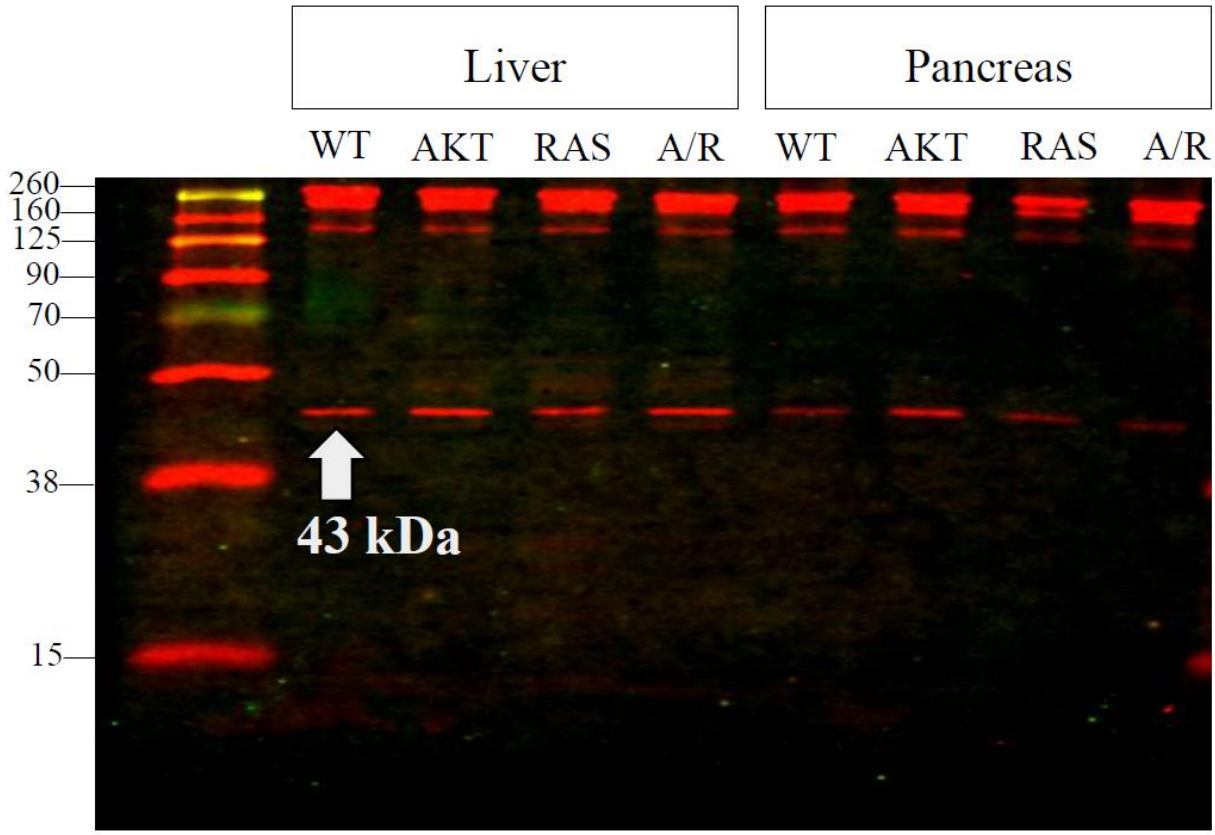

Figure 11. 12% SDS- PAGE, anti-Beta-actin

Results of western blot analyses of Beta-actin (43kDa) in homogenized pancreas and liver tissues from mice 5790 (Wild-type), 5767 (AKT1), 5792 (KRAS), 5778 (AKT1/KRAS). Beta-actin (43 kDa) is detected using 12% gel and anti-B-actin. Unknown heavy bands are also found at the top of the gel.

DISCUSSION

PDAC is the fourth leading cause of cancer death in the United States. Diabetes has been shown to be a risk factor for the development of this deadly malignancy. This project aims to investigate the mechanism of the deregulated glucose metabolism at the molecular level. In this experiment, the wild type mice had an average glucose level of 128.1 mg/dl. The non-fasting blood glucose test demonstrated the severe glucose deregulation in the 20 weeks old transgenic AKT1/KRAS mice, which can be further divided into a high glucose level group (600+ mg/dl) and a relatively low glucose level group (174.75 mg/dl).

The high glucose level group is suspected to be at risk of PDAC development. PDAC is an age-related malignancy that takes 17 years in human to be fully established from panIN-1. Therefore, the low glucose level group of AKT1/KRAS is expected to progress into more deregulated blood glucose levels and eventually merge with the high glucose level group.

Total and phosphorylation specific sites of AKT and insulin receptor are an indication of the molecular response to the genetic mutation that could lead to sequential molecular activities. The total AKT ELISA demonstrated the effect of the transgenic myristoylated AKT as the mice with mutant AKT mice have a significantly higher concentration (644.1 ng/mL) compare to the wildtype mice (484.8 ng/mL) in the pancreas. The serine phosphorylation at amino acid number 473 is one of two sites that need to be phosphorylated for full activity of the AKT protein kinase. However, the level of activities of phosphorylated AKT at serine residue 473 in AKT1/KRAS mice is remain persistent across all 4 genotypes. AKT activation is stimulated by growth factors and hormones such as insulin at the upstream receptor. The endogenous AKT activation is more

dependent on those stimulations rather than genetic modification, which shows that hyper-activation of AKT1 is not the sole contributor to the amplified glucose deregulation.

On the other hand, the KRAS mice have a substantial variance in the level of Insulin Receptor at the one of the three phosphorylation sites, namely tyrosine residue 1158 (Y1158), in the pancreas. This site is known to be functionally important to the autophosphorylation of the two intracellular beta-subunits of the insulin receptor and acts as a stabilizer (Hubbard, 2013). A reduced phosphorylation of the Y1158 site will decrease the kinase activity of the insulin receptor. This finding can be related to the insulin resistance displayed in the KRAS mice that contribute to further deregulate the glucose metabolism. There may be an outside pathway of KRAS that directly/indirectly represses the phosphorylation of the insulin receptor, which work independently from the AKT signaling pathway. We will conduct ELISAs again with a larger sample size of KRAS mice to confirm this finding.

In the meantime, we have yet to identify the crosstalk effect between the AKT and KRAS pathway. The initial attempt to homogenize the mice tissues using tubes with pestles (Kimble Biomasher II) in Tissue PELB (G-Biosciences) was unsuccessful. We intended to use a milder detergent to minimize the damage and preserve the structures of the protein of interest during homogenization. However, the plastic pestles did not provide enough rigidity to grind the samples into the appropriate form. The Tissue PELB used was also not strong enough to fully lyse and extract the protein from the mammalian cells. We believe that certain amount of protein remained in the insoluble protein pellet and was disposed after centrifugation, whereas the lysate contained cells that has not been completely lysed. To continue with the project, we decided to switch to the ceramic mortar and pestle to have a stronger grinding force. Tissues were frozen in

liquid nitrogen during the homogenization to prevent degradation. We also used the standard RIPA buffer to ensure the cells lyse completely. The unknown heavy bands found at the top of the 12% SDS-Gel were the result of the expiration of the 2-mercaptoethanol the Laemmli's loading buffer to fully denature the protein samples, which was easily solved by adding fresh 2-mercaptoethnol in the loading buffer.

In this study, we demonstrated the severe deregulation of glucose metabolism in AKT1/KRAS mice could be the result of the independent action of AKT1 and KRAS pathways. Although the hyper-activated AKT1 mice has shown to have elevated blood glucose level and establishment of a diabetic phenotype, it is not the only contributor to the severely deregulated AKT1/KRAS mice group.

AKT1 and KRAS pathways may have different unique mechanisms to affect the blood glucose levels. The AKT1 hyper-activation mutation leads to excess secretion of glucagon, which results in the breakdown of glycogen to elevate blood glucose level. Insulin is less effective at activating insulin receptor through mutant hyper-active KRAS due to the insufficient phosphorylation at site Y1158. These cells are found to be insensitive to glucose uptake in KRAS mice and blood glucose level continues to elevate.

Activated KRAS may contribute to the metabolic reprogramming of the cells and pathways such as glucose transport. RRAD (Ras-Related Associated with Diabetes) is a small GTPase that is associated with type II diabetes. Recent studies have suggested that RRAD acts as tumor suppressor by inhibiting the expression of glucose transporters (Wang et al., 2014). Inactivation of RRAD by DNA methylation is commonly seen in tumor tissues, which increases

glucose uptake in tumor cells, which subsequently perform aerobic glycolysis known as the Warburg effect (Yan et al., 2016).

In conclusion, the findings of this study suggest that the combined effect of glucagon mediated prediabetic phenotype and the insulin resistance contributes to the severely deregulated blood glucose level in AKT1/KRAS mice (Figure 12). The phosphorylated insulin receptor ELISA would need to be replicated with greater sample size to confirm this finding. The search for the crosstalk effect between the pathways continues, as we have optimized the protein homogenization method for mouse pancreas tissue. The expression level of the p70S6K also could be examined more thoroughly as a mechanism to indirectly lead to the inhibition of the glucose transporter type 4 (GLUT 4) that essentially contributes to the glucose uptake. Collectively, the study of the interaction of AKT1 and KRAS signaling pathways could lead to a greater understanding of the linkage between diabetes and PDAC, as well as potential markers for early detection of PDAC.

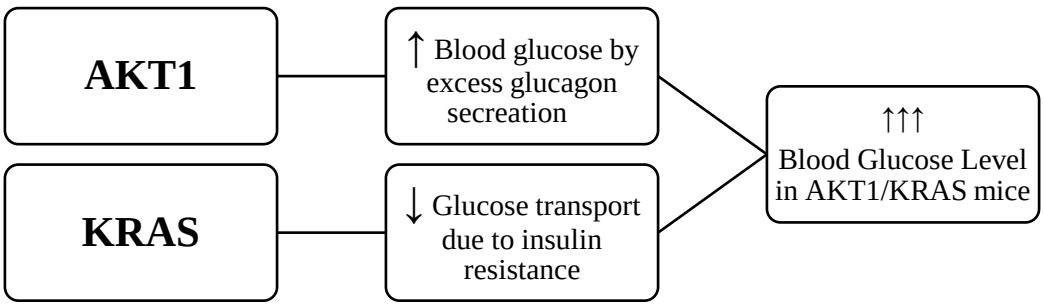

Figure 12. Model of AKT1 and KRAS combined effect on glucose deregulation.

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