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Analysis of gene expression in early-stage pancreatic cancer and investigation of 1,2,3,4,6-Penta-O-galloyl-β-D-glucopyranoside as a therapeutic

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ANALYSIS OF GENE EXPRESSION IN EARLY-STAGE PANCREATIC CANCER AND INVESTIGATION OF 1,2,3,4,6-PENTA-O-GALLOYL-β-D-GLUCOPYRANOSIDE AS A THERAPEUTIC

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Table of Contents

List of Tables

List of Figures

Abstract

Pancreatic cancer (PC) is one of the deadliest cancers among both men and women. However, if it is detected early, there is a significant increase in the 5-year survivability of the disease[1]. Currently, there are no truly efficient detection mechanisms available to detect early-stage PC when treatment options are more viable. The first objective of this project was to determine the expression levels of AHNAK2, IGHG3, EPPK1, CTHRC1, CEL, PLA2G1B, and GNMT—all known to be aberrantly expressed in PC cell lines—in early-stage PC tissue and assess the feasibility of their use as potential biomarkers for PC. To accomplish this, total RNA was extracted from the tumor samples and their expression was analyzed using RT-qPCR. The second objective was to determine the effects that the compound 1,2,3,4,6-Penta-O-galloyl-β-D-glucopyranoside (PGG) has on PC cell lines by counting the number of viable cells remaining after exposure to varying concentrations of PGG. It was found that the genes IGHG3 and CTHRC1, if taken together, may be able to identify the majority of early-stage PCs. Additionally, it was discovered that PGG has a significant effect on PC cell survivability. The EC_{50} value for BxPC-3 cells treated with PGG was determined to be 0.012 mg/mL and PGG was shown to significantly increase the expression levels of GNMT. Additional analysis of the expression levels of IGHG3 and CTHRC1 in blood samples will help provide further information about their viability as potential biomarkers for PC.

Introduction

Pancreatic Cancer (PC) is an extremely deadly disease with no truly efficient detection mechanisms. As of 2013, PC was the fourth deadliest cancer for both men and women, claiming the lives of more than 51,000 people across the United States alone[2]. This high mortality rate is principally due to difficulties in detecting PC during its earliest stages of development. This difficulty in detection largely stems from how PC produces very few symptoms early on making it extraordinarily challenging to diagnose[3]. If PC is detected early, however, there is a significant increase in survivability of the disease.

Between 2008 and 2013, the 5-year relative survival rate for PC diagnosed as localized, regional, and distant was 34%, 12%, and 3%, respectively[1]. Therefore, if PC is discovered earlier prior to metastasis, patients are nearly three times as likely to survive five years than when compared to a later diagnosis with tumor invasion of distant tissues already underway. Developing reliable and effective testing is critical to increasing the early detection of PC when treatment options are still possible and mortality rates are significantly lower.

Currently, there are limited options for the early detection of PC. One technique, a blood test called "CancerSEEK," has produced promising results for the detection of PC as early as stage 1 using a panel of biomarkers[4]. However, detection of early-stage PC is still difficult, and blood tests such as these are not commonly used for its detection. By identifying additional genes whose expression levels are significantly altered in early-stage PC, the ability to detect PC could be improved and survival rates could be increased.

Previously, research has been conducted in our laboratory on identifying novel genes that are either considerably upregulated or downregulated in PC to isolate potential biomarkers and targets for treatments. The genes AHNAK2, CTHRC1, IGHG3, and EPPK1 have been

determined to be upregulated in PC cell lines[5]. Additionally, GNMT, CEL, and PLA2G1B have been identified as being significantly downregulated in PC cell lines[6]. However, little to no research has been conducted into the expression levels of these genes in early-stage PC tissue samples; therefore, quantifying the expression of these genes in early-stage PC tissue could provide new insights into potential biomarkers as well as further information regarding gene regulation in early-stage PC.

While identifying genes that are aberrantly regulated in PC could help to increase the disease's early detection rate, discovering methods to renormalize or alter the expression of these genes is also important. If a gene is found to be critical to the progression of PC, having the ability to alter its expression could lead to potential therapeutic interventions. Previously, work in our laboratory has shown that the gene GNMT (Glycine N-methyltransferase) is highly downregulated in PC cell lines[6]. Furthermore, its expression in hepatocellular carcinoma has been shown to be upregulated in the presence of 1,2,3,4,6-Penta-O-galloyl-β-D-glucopyranoside (PGG), which is a potent inhibitor of these cells[7]. Understanding the effects of PGG on GNMT and PC cell survival could help to provide additional information pertaining to its possible role in the therapeutic treatment of PC.

Purpose

While PC is one of the lesser diagnosed cancers among American individuals, it has one of the highest mortality rates of all cancers. Early detection of PC, while difficult, could help decrease its mortality rate and save countless lives. Previously, the genes EPPK1, CTHRC1, IGHG3, and AHNAK2 have been shown to be highly upregulated in human PC cell lines[5] while the genes GNMT, PLA2G1B, and CEL have been shown to be highly downregulated in PC cell lines[6]. Along with this, the potent inhibitory effects of the compound PGG against hepatocellular carcinoma has been linked to increases in GNMT expression levels[7]. This project aims to determine the expression levels of the aforementioned genes in early-stage PC tissue samples and identify the impact of PGG on cell viability and GNMT expression in the PC cell line BxPC-3. Identification of genes significantly upregulated or downregulated, specifically in early-stage PC, could provide new therapeutic targets for treatment as well as potential biomarkers to help increase the early detection of PC and disease survivability.

Research Questions to be Answered

- What is the impact of PGG on BxPC-3 cell survivability?
- How does BxPC-3 cell exposure to PGG impact GNMT expression?
- Are the genes AHNAK2, IGHG3, CTHRC1, and EPPK1 significantly upregulated in early-stage PC tissue samples?
- Are the genes GNMT, PLA2G1B, and CEL significantly downregulated in early-stage PC tissue samples?

Literature Review

Altered gene regulation—where genes become either upregulated or downregulated in cells—is a predominate pathway leading to the development of cancer. However, if genes that are critical to the continued growth of cancer cells are identified and their regulation renormalized, it is possible that the afflicted cell could take corrective action and undergo apoptosis. Identifying these genes and mechanisms to renormalize their regulation, especially in the early stages of cancer growth, is an important step in the continued quest to decrease the mortality rates associated with all cancers, including PC. While multiple genes have been identified to either be upregulated or downregulated in PC, many of their expression levels in early-stage tissue samples have yet to be quantified.

Genes Associated with Being Upregulated in PC

AHNAK nucleoprotein 2 (AHNAK2) is a protein-coding gene that is responsible for the production of a nucleoprotein thought to be important in the calcium signaling process by associating itself with calcium channel proteins[8]. AHNAK2 was first shown to be upregulated in PC through a bioinformatic analysis of microarray datasets conducted in our laboratory[5]. This result was later confirmed when the gene was shown to be significantly upregulated in PC cell lines[9]. Later, multiple studies analyzed the expression of AHNAK2 in tissue samples from patients with PC and have shown that AHNAK2 may be a potential prognostic biomarker for patients with PC Ductal Adenocarcinoma[7,10]. Additional research into the expression levels of AHNAK2 in early-stage PC will help to provide more information about its feasibility and utility as a potential PC biomarker.

Epiplakin 1 (EPPK1) is a protein-coding gene located on chromosome 8 within the human genome. This gene is responsible for encoding a protein which is involved in the organization of the cytoskeletal architecture of human cells[11]. Furthermore, the protein encoded by EPPK1 "may function to maintain the integrity of keratin intermediate filament networks in epithelial cells"[11]. In a study conducted using microarray pancreatic cancer datasets, EPPKI was shown to be one of the genes most significantly upregulated when compared to normal pancreatic tissue[5]. After this discovery, EPPK1 was shown to be upregulated in pancreatic cancer cell lines using RT-qPCR[9]. However, research into the expression levels of EPPK1, specifically in early-stage PC tumors, is absent.

Collagen triple helix repeat containing 1 (CTHRC1) is another protein-coding gene associated with being upregulated in PC. The protein that CTHRC1 encodes for works in vascular remodeling, specifically when arterial damage has occurred[12]. In 2014, CTHRC1 was shown to be upregulated in PC microarray datasets[5]. Additionally, it has been shown to be aberrantly expressed in a variety of other types of cancerous tumors and even has been shown to promote PC progression[13]. Work to determine the expression of CTHRC1 in early-stage PC could be expanded upon to help further define its activity levels and its suitability as a potential biomarker for PC.

Immunoglobulin heavy constant gamma 3 (IGHG3) is a gene on chromosome 14 associated with PC. IGHG3 is a variant of Immunoglobulin 3 and is primarily associated with the immune system. The protein encoded by IGHG3 helps in recognizing foreign antibodies, initiates immune responses, and is found in the heavy chain of an immunoglobulin molecule[14]. Before being discovered as associated with PC, IGHG3 was shown to be associated with mesothelioma[15]. More recently, it has been shown to be significantly upregulated in PC

microarray datasets[5]. It is currently unknown why this gene, which is more commonly associated with the immune system, is specifically upregulated in PC cells. Again, however, data indicating the expression of IGHG3 in early-stage PC tissue is lacking.

Genes Associated with Being Downregulated in PC

Glycine N-methyltransferase (GNMT) is a protein-coding gene located on chromosome 6 of the human genome, and the protein that GNMT codes for is found in an enzyme located within the cytoplasm of cells as a homotetramer[16]. Furthermore, the protein helps to catalyze the conversion of S-adenosyl-L-methionine and glycine to S-adenosyl-L-homocysteine and sarcosine[16]. In 2015, GNMT was found to be abnormally methylated in PC tissue samples causing the enzyme it encodes for to be downregulated in 87% of cases[17]. More recently, GNMT was found to be downregulated in PC cell lines through the use of RT-qPCR[6]. Interestingly, GNMT expression has been determined to be affected by the compound PGG[7]. While PGG has been studied in a variety of different tissue types, mostly examining its potential for cancer treatment, it has not been significantly investigated in PC. If GNMT is found to be significantly downregulated in PC tissue, PGG could be a potential mechanism to therapeutically alter the expression levels of this tumor suppressor gene back to a more normal range.

Carboxyl ester lipase (CEL) is another protein-coding gene that is highly downregulated in PC. CEL codes for a protein that is secreted from the pancreas which helps in the absorption and hydrolysis of cholesterol and lipid-soluble vitamins within the digestive tract[18]. Phospholipase A2 group 1B (PLA2G1B) is a protein-coding gene that is also responsible for coding for an enzyme, secreted by the pancreas, that helps to catalyze the hydrolysis of membrane glycerophospholipids to release arachidonic acid and lysophospholipids[19].

In 2017, copy number variants of CEL were identified as risk factors for PC[20]. Later in 2018, CEL, along with PLA2G1B, was identified as being significantly downregulated in PC cell lines[6]. Most recently, CEL and PLA2G1B were both identified as being downregulated in tissue samples[21]. Further research into CEL and PLA2G1B and their expression levels in early-stage PC will help to provide additional insights into their potential feasibility as early-stage PC biomarkers and utility as targets for therapeutic intervention.

Methodology

Quantification of Gene Expression in Early-Stage PC Tumor Tissue

Isolation of Total RNA from Early-Stage Tumor Tissue

Seven paired early-stage PC tumor samples (mass ranges around 30 mg) were obtained from the University of Iowa Hospitals and Clinics. Total RNA isolation was conducted on these samples following a procedure using the Qiagen RNeasy Micro Kit and structured around the Qiagen RNeasy Handbook with some modifications[22]. These modifications were due to the methods development process conducted in our laboratory because of the significant presence of RNases in the pancreas and tumor samples.

First, in a cold room at 4°C, 900 µL of a solution containing 1 mL Buffer RLT to 50 µL of ß-Mercaptoethanol (RLT:ME) was pipetted into the 2-mL centrifuge tubes containing the tissue samples. Tissue homogenization was then conducted using a tissue homogenizer (Qiagen TissueRuptor II) set on low for 10 seconds and whose probes had been previously sterilized with a 70% ethanol solution and allowed to dry. This mixture was transferred to a plastic, round-bottomed test tube with an approximate size of 1.25 cm by 14 cm. The centrifuge tube was washed with another 900 µL of RLT:ME solution, and the solution was then transferred to the plastic test tube. Following this, tissue disruption was carried out using the tissue homogenizer set at full speed for 1 minute on the RLT:ME tissue sample mixture. In order to achieve complete disruption, the probe was moved in a circular, up and down type pattern during all tissue disruption events.

The sample was placed on ice and, outside the cold room, transferred to a 2 mL microcentrifuge tube and centrifuged at 13,200 rpm for 3 minutes. Next, the supernatant was transferred, in aliquots of 900 µL, to two new 2 mL microcentrifuge tubes. 900 µL of 70%

ethanol was added to each sample and mixed by pipetting. Next, 700 µL of the sample was then added to a RNeasy Spin Column and centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded and another 700 µL of the sample was placed into the spin column and centrifuged using the same settings as before. Finally, the flow-through was discarded, the remaining volume of sample was run through the spin column using the previous settings, and the flow-through of this final run was discarded.

After running the entire volume of sample through the spin column, 700 µL of Buffer RW1 was added to it, centrifuged for 15 seconds at 10,000 rpm, and the flow-through was discarded. Next, 500 µL of Buffer RPE was added to the spin column and the tube was centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded. Following this, an additional 500 µL of Buffer RPE was added to the spin column, centrifuged for 2 minutes at 10,000 rpm, and the flow-through was discarded. The spin column was again centrifuged for 1 minute at 13,200 rpm to ensure complete drying of the column and was then immediately placed into a 1.5 mL microcentrifuge collection tube. 30 µL of RNase-free water was added to the spin column membrane and centrifuged for 1 minute at 10,000 rpm. Immediately following RNA isolation, total RNA yields were quantified by UV Spectroscopy using a Nanodrop spectrophotometer (Thermo Fisher) calibrated with 2 µL of RNase free water.

Conversion of Isolated RNA to cDNA

Isolated RNA for each sample was converted to cDNA using a reverse transcription process. To accomplish this, the iScript cDNA Synthesis Kit (Bio-Rad) was used and the procedure was carried out following the suggested manufacturer protocol[23]. The only alteration to this procedure was in the amount of RNA used. Due to early difficulties in Real Time-

quantitative Polymerase Chain Reaction (RT-qPCR) reproducibility caused by high Ct values, the amount of RNA was increased to 2 µg for samples which had a sufficient amount of extracted RNA available for use. Samples with smaller amounts of extracted RNA were reduced as needed to accommodate the lower yields from these samples with an emphasis on using the most RNA possible to lower the Ct value for all samples and increase their reproducibility.

Analysis of gene expression using RT-qPCR

Gene expression in each paired PC tissue sample (tumor and normal) was quantified using RT-qPCR. RT-qPCR was carried out using iTaq Universal Sybrgreen Supermix and done following the manufacturer's suggested procedure[24]. Each reaction was conducted in quadruplicate with a total reaction volume of 20 µL. Furthermore, each gene tested was prepared with a master mix containing iQ SYBR Green Supermix, sterile nuclease-free water, template DNA, and forward and reverse primers as illustrated in Table 1. If multiple genes were to be analyzed, the volumes shown in Table 1 were multiplied by the number of genes being tested to calculate the total reagent volumes for each sample needed.

Reagent	**Master Mix For Each Gene (µL)**
iQ SYBR Green Supermix	46.8
Template DNA	3.8
Sterile Nuclease-Free Water	41.2
Primers (Forward and Reverse)	1.7
Total Volume	93.5

Table 1: Reagent volumes for RT-qPCR

Master mixes for each sample were split up by gene and then had the appropriate primer added to them. This process is shown in more detail below in Figure 1. It should be noted that primer volumes are always 1.7 µL and are added after the separation of the original master mix as indicated in the manufacturer's protocol[24].

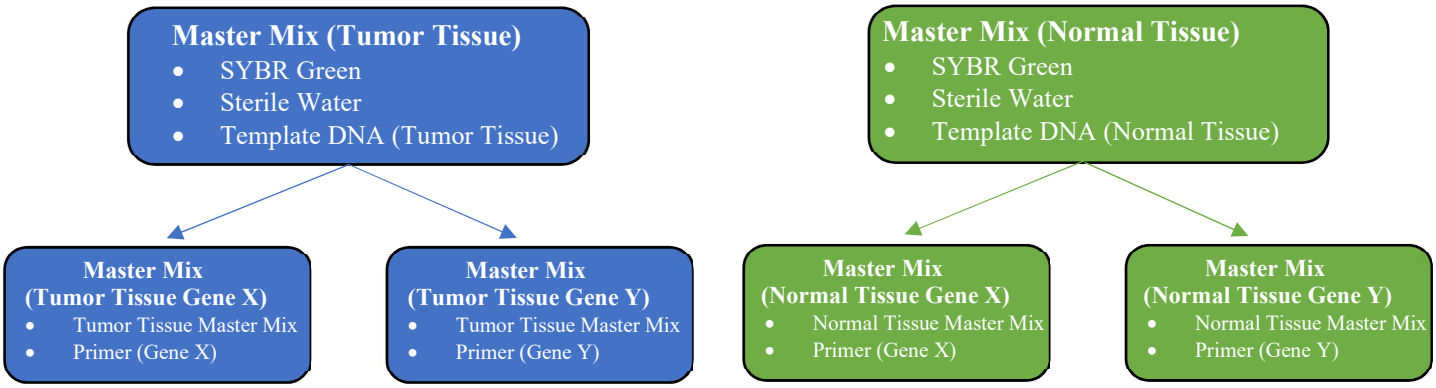

Figure 1: Master mix allocation procedure for RT-qPCR

The master mixes were then aliquoted into four RT-qPCR wells on a 96 well plate. It was found that flicking each master mix 10 times before each centrifuge step and mixing by pipetting before all master mixes were aliquoted produced the most consistent and reproducible results. After aliquoting all samples into the 96 well plate, the plate was sealed with a coverslip which was pressed firmly and evenly over the entire surface to remove all bubbles. Any bubbles in the solutions in the wells were removed by flicking and the entire plate was then centrifuged for 40 seconds at 3300 rpm. The set-up for the RT-qPCR process was as follows: initially, the temperature was set at 50.0°C for 2 minutes and then increased to 95.0°C for 10 minutes for initial denaturing and enzyme activation. Following this, 40 cycles of denaturing at 95.0°C for 15 seconds accompanied by an annealing and extension stage at 60.0°C for 45 seconds was performed. This procedure was the same for each sample and gene tested. The gene GAPDH was

used to normalize each paired sample. RT-qPCR primers for all genes tested are shown in Table 2 below. Finally, these results were then analyzed for significance using the student's t-test.

Gene	Forward Primer	Reverse Primer
CTHRC1	5'-CAATGGCATTCCGGGTACAC-3'	5'-GTACACTCCGCAATTTTCCCAA-3'
IGHG3	5'-AGGACTCTACTCCCTCAGCA-3'	5'-GGCATGTGTGAGTTGTGTCA-3'
EPPK1	5'-GTACGAGCGGTTGGAGCAT-3'	5'-TGTTTGCTTTTGGGTCGATGA-3'
GAPDH	5'-CCATGTTCGTCATGGGTGTG-3'	5'-CAGGGGTGCTAAGCAGTTGG-3'
AHNAK2	5'-GATGTGCGACTGCTTCCAC-3'	5'-CAGCCTCAGTCGTGTATTCG-3'
GNMT	5'-ACTGGATGACTCTGGACAA-3'	5'-ACTGAGGATGTGGTCGT-3'
CEL	5'-GTCACCTTCAACTACCGTGTC-3'	5'-GGCCGCGATATTCCTCTTCAC-3'
PLA2GB1	5'-AAATGATCAAGTGCGTGATCC-3'	5'-TTGCTGCTACAGGTGATTGC-3'

Table 2: Forward and reverse primers for RT-qPCR

PGG Impact on PC Cell Line BxPC-3

Cell Maintenance

The PC cell line BxPC-2, previously acquired from the American Type Culture Collection, was reconstituted from frozen stocks and grown in RPMI Medium 1640. The cell media was additionally supplemented with 1% Penicillin-Streptomycin solution along with 10% Fetal Bovine Serum. The cells were kept at 37°C in an atmosphere of 5% CO_2 and were split as needed to ensure that cell confluency never exceeded 80%.

Determination of the EC_{50} Value

When determining the impact that PGG has on BxPC-3 cell survival, initially 300,000 cells were added to each well in a 6-well tissue culture plate conducted in triplicate. For example, three 6-well tissue culture plates were used each containing 300,000 cells/well along with 1.5mL of cell media. After 24 hours of uninterrupted growth, PGG was added in concentrations of 0.1mg/mL, 0.05mg/mL, 0.025mg/mL, 0.012mg/mL, and 0.006mg/mL along with a control well

containing no PGG, all in triplicate. The PPG solution was made by initially diluting the compound in solid form to a concentration of 5x in a 1:1 ethanol to water solution which was subsequently diluted to a final volume containing 10% ethanol. After vortexing, small aliquots of 100 µL were created from this original master solution and used. The addition of PGG was carried out by removing all cell media, washing each well with 1.5mL of Phosphate-buffered saline (PBS), and lastly adding 1.5mL of fresh cell media containing the appropriate concentrations of PGG.

The cells grew uninterrupted for 72 hours. After the specified time period, the wells were washed with 1.5mL of PBS and the remaining cells were counted using a Bio-Rad TC10 Automated Cell Counter following the manufacturer's suggested procedure[25]. In our laboratory, we observed that in the range of 50,000 cells/mL – 1,000,000 cells/mL, the results from the TC10 Automated Cell Counter were consistent with those from a manual hemocytometer.

Quantification of PGG Impact on GNMT Expression

A 6-well cell culture plate seeded with 300,000 cells of BxPC-3 and 1.5mL of cell culture media was allowed to grow for 24 hours. Next, PGG was introduced into the wells using the same concentrations and procedure as described above. After growing in the PGG/cell media solution for 24 hours, the cells were harvested, and total RNA extraction was conducted on each sample per the Qiagen RNeasy Handbook using the Qiagen RNeasy Mini kit[22]. Isolated RNA was converted to cDNA using 1µg of RNA and following the procedure outlined for the tissue samples. Finally, the gene expression of GNMT for each concentration of PGG was analyzed using RT-qPCR following the same procedure described previously.

Results and Discussion

The clinicopathological characteristics of all seven tissue samples are shown in Table 3 below. Based on the stage and grade identified in Table 3, all patients were considered to have early-stage PC. As shown in the table below, the PC tissue samples were comprised of a nearly even mix of samples from both male and female patients. Furthermore, the patients span a large age range from 51 to 85 years of age.

Identifier	Age	Gender	Stage[1]	Grade	Size (cm)
S1	72	M	T3N1	G3-poorly differentiated	3.2 x 3 x 2.8
S2	64	F	T3N1	G2-Moderately differentiated	3 x 2.2 x 1
S3	85	M	T4N1	G2-Moderately to poorly differentiated	4 x 3 x 1
S4	76	F	T3N1	G2-Moderately differentiated	3 x 2.6 x 2
S5	65	F	T3N1	G2-Moderately differentiated	2.3 x 2 x 1.3
S6	63	M	T3N1	G3-Poorly differentiated	5.5 x 4 x 3.6
S7	51	F	T3N1	G2-Moderately differentiated	4.8 x 3.3 x 2.9

[1] American Joint Committee on Cancer TNM staging system.

Table 3: Clinicopathological characteristics of all patients that were utilized in this study. Additionally, the grade and total size of the pancreatic tumor is also shown.

Genes IGHG3, CTHRC1, EPPK1, and AHNAK2

The expression levels of IGHG3 in the early-stage PC tissue samples, as determined by RT-qPCR, is shown below in Figure 2. IGHG3 was determined to be significantly upregulated ($p<0.001$) in 6 of the 7 tissue samples. Furthermore, 3 of these samples were upregulated by more than 15x with sample S4 30x overexpressed when compared to normal pancreatic tissue from the sample patient. These results are consistent with previous research focused on the expression of IGHG3 in PC cell lines[5].

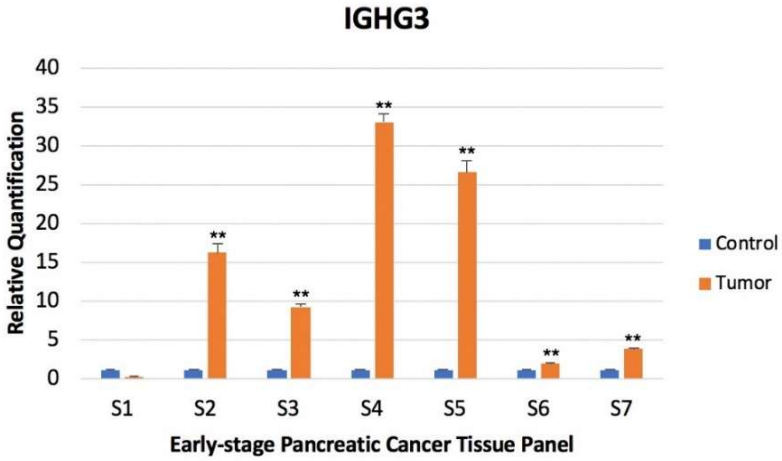

*Figure 2: Expression levels of IGHG3 in early-stage PC tissue samples. ** indicates a p value <0.001*

CTHRC1 also had samples where its expression was drastically upregulated. As shown in Figure 3, CTHRC1 was upregulated ($p<0.001$) in 5 of the 7 samples tested. Similar to IGHG3, CTHRC1 was also approximately 30x overexpressed in one of the samples when compared to the control. Most importantly, when taken together, IGHG3 and CTHRC1 were overexpressed in 6 of the 7 samples with expression levels nearly 10x greater than that of the control. These results indicate that IGHG3 and CTHRC1 could potentially function as early-stage PC biomarkers for most types of PC.

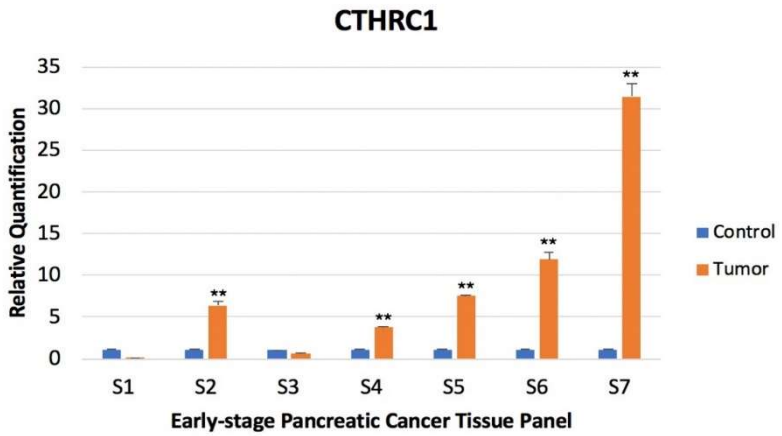

*Figure 3: Expression levels of CTHRC1 in early-stage PC tissue samples. **p<0.001*

When compared to the results from IGHG3 and CTHRC1, EPPK1's expression was not as consistent. Figure 4 indicates that EPPK1 was significantly upregulated ($p<0.001$) in 3 of the 7 samples tested. These samples were overexpressed between 1.5x and 2x which is significantly less than the overexpression observed for genes CTHRC1 and IGHG3. Additionally, EPPK1 was significantly downregulated in 4 of the 7 samples. While EPPK1 was overexpressed in a panel of 7 PC cell lines, it did not exhibit similar behavior in early-stage PC tissue as nearly half of the samples exhibited overexpression while the other samples were underexpressed[9].

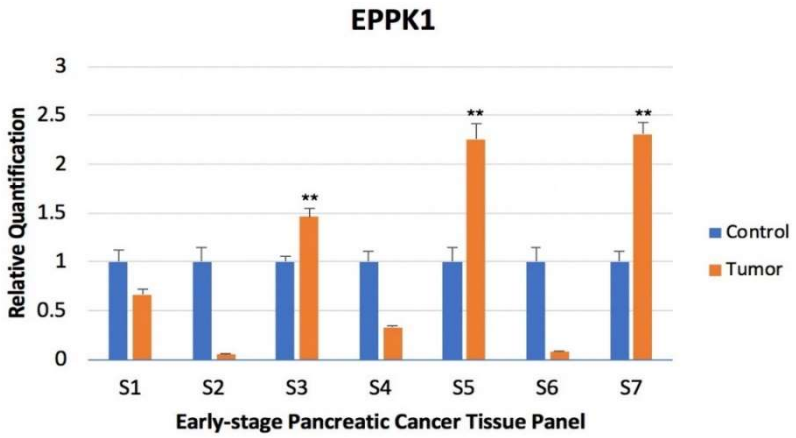

*Figure 4: Expression levels of EPPK1 in early-stage PC tissue samples. **p<0.001*

While AHNAK2 was highly upregulated in PC cell lines, its expression levels in this experiment were too low to enable accurate detection by RT-qPCR. Looking forward, AHNAK2's expression level in early-stage PC tissue samples could be evaluated using techniques more sensitive than RT-qPCR. This could more accurately assess its regulation, potential for use as a biomarker, and feasibility as a target for therapeutic treatment.

Genes CEL, PLA2G1B, and GNMT

As shown in Figure 5, CEL mRNA was significantly underexpressed in 4 of the 7 samples and significantly overexpressed in the other three samples. While CEL was downregulated in four samples, it was surprisingly overexpressed in the other three samples. Previously, it had been shown in cell lines that CEL was significantly and consistently underexpressed[6]. This result indicates that there are some early-stage PC tissues where CEL is overexpressed. These findings impact its potential role as an early-stage PC biomarker due to its inconsistent expression levels.

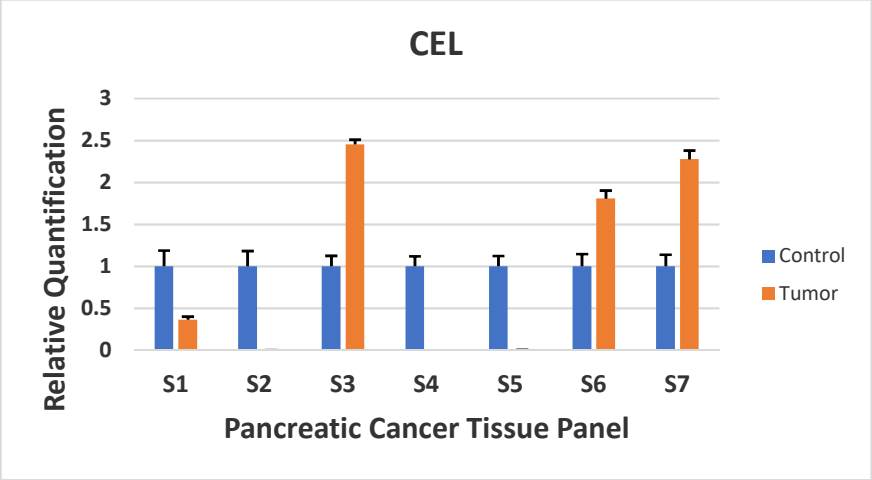

Figure 5: Expression levels of CEL in early-stage PC tissue samples.

Along with CEL, Figure 6 shows that PLA2G1B was significantly underexpressed in 4 of the 7 samples tested and overexpressed in only one sample. This result, like that from CEL, is not in complete agreement with previous results analyzing the expression levels of PLA2G1B in PC cell-lines[6]. This indicates the PLA2G1B would need to be paired with other genes in order to act as a comprehensive early-stage biomarker.

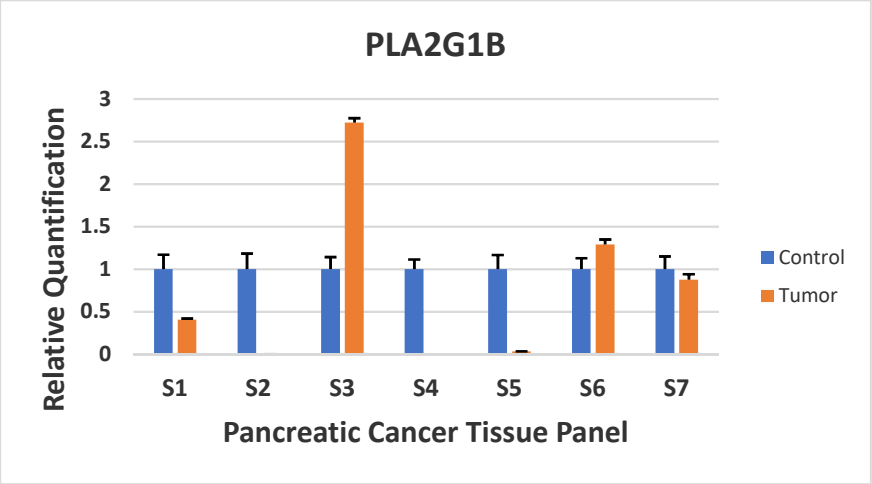

Figure 6: Expression levels of PLA2G1B in early-stage PC tissue samples.

Finally, as illustrated in Figure 7, GNMT was significantly underexpressed in four samples and overexpressed in the other three. This result, like that from the other genes PLA2G1B and CEL, highlights their ability to detect approximately half of the early-stage PC samples tested. However, all these genes would need to be strategically paired with other genes that are capable of detecting the remaining samples to develop a comprehensive biomarker test that can accurately identify a large majority of early-stage PCs.

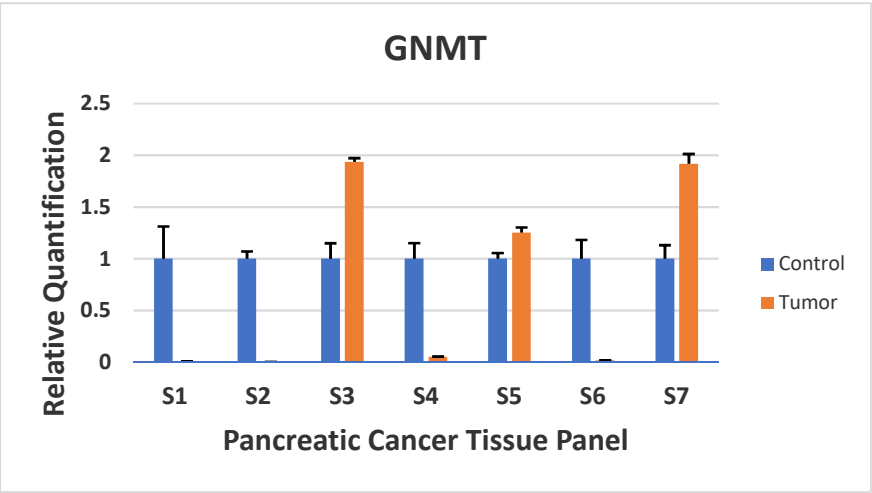

Figure 7: Expression levels of GNMT in early-stage PC tissue samples.

Interestingly, there seems to be a pattern in the expression of CEL, PLA2G1B, and GNMT. PLA2G1B was underexpressed in the same four samples (S1, S2, S4, and S5) as CEL. Furthermore, they were both overexpressed by nearly the same amount in sample S3. Finally, the expression levels of GNMT seemed to be strikingly similar to the results of CEL. Specifically, both CEL and GNMT were underexpressed in samples S1, S2, and S4 while both being significantly overexpressed in samples S4 and S7. Finally, all three genes were overexpressed in sample S4. This result suggests some sort of underlying similarity between samples S1 through S5 with respect to the genes CEL, PLA2G1B, and GNMT. The mechanism causing the similar expression of these genes in early-stage PC could be investigated in the future and may reveal more information about a common way that early-stage PC impacts gene regulation in the pancreas.

Overall, these results indicate that IGHG3 and CTHRC1, when taken together, may be able to identify the majority of early-stage PCs. In addition to this, further testing needs to be conducted, specifically concerning the expression of these genes in blood or plasma samples. If these genes could be isolated and shown to be significantly upregulated in patient blood samples, the prospect of a biomarker test utilizing both IGHG3 and CTHRC1 would be significantly increased.

GNMT and the Effect of PGG

As explained previously, the PC cell line BxPC-3 was treated with varying concentrations of PGG and the impact that this had on the cell's viability was determined. The results from this are shown below in Figure 8 where the EC_{50} value is 0.012 mg/mL.

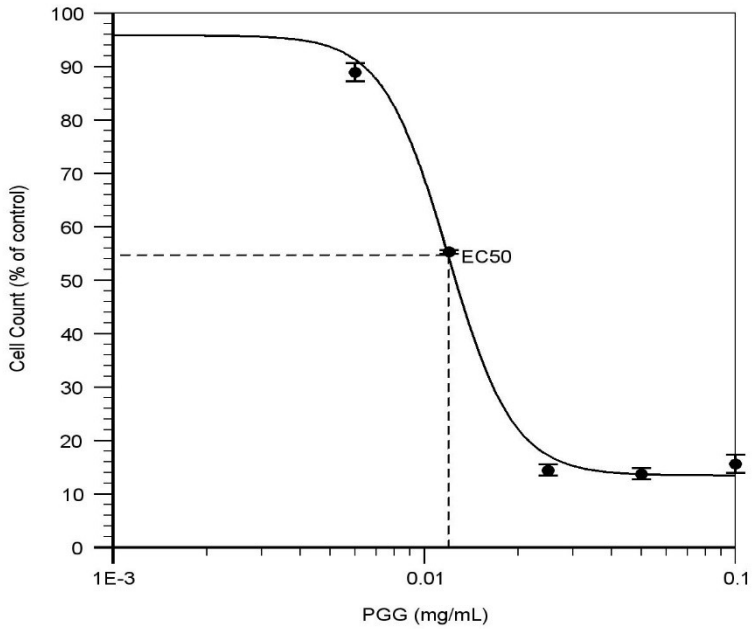

Figure 8: Impact of PGG on the survivability of PC cells BxPC-3. The EC_{50} value is 0.012 mg/mL.

After identifying that PGG was indeed responsible for the death of PC cells, the impact that PGG had on the expression levels of GNMT was analyzed using RT-qPCR. As shown in Figure 9, a PGG concentration of 0.012 mg/mL nearly doubled the expression level of GNMT. Furthermore, when the concentration of PGG was doubled again, the expression levels of GNMT approximately doubled from the previous concentration. These relationships show that PGG is having a significant impact on the expression levels of GNMT in PC cell line BxPC-3.

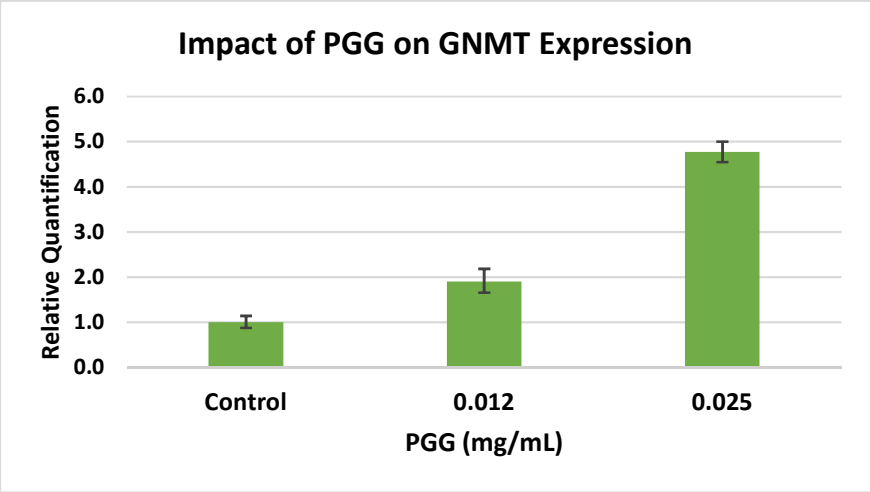

Figure 9: Impact of PGG on the expression levels of GNMT mRNA in BxPC-3 cell line.

Based on these results, it seems that PGG has a significant ability to alter the gene expression of GNMT in PC cells while also promote cell death. These results help to show that PGG may have potential as a therapeutic treatment for PC. However, more testing needs to be conducted on the impact of PGG on regular pancreatic cells, isolation of GNMT as a plasmid vector to conclusively determine that PGG is directly affecting its expression levels, and further gene analysis to see other effects that PGG is having on the PC cells.

Conclusions

The purpose of this project was to identify genes aberrantly expressed in early-stage PC and evaluate the effectiveness of PGG as a possible therapeutic for the treatment of PC. The genes IGHG3 and CTHRC1, when taken together, may be able to identify a large majority of early-stage PC. Due to the limitations of this project, blood and plasma samples were not able to be tested for the expression levels of IGHG3 and CTHRC1. However, additional research focused on assessing their expression levels here would provide further information about their ability to act as early-stage PC biomarkers. The genes PLA2G1B, CEL, and GNMT exhibited a remarkable similarity in expression levels in 5 of the 7 early-stage tumor tissues analyzed. Increased research into the cause of this similarity in expression levels may help reveal more information about the impact that PC has on gene expression in the pancreas. Additionally, PGG was shown to have a significant impact on the survivability of PC cells with an EC_{50} value of 0.012 mg/mL. PGG significantly increased the expression levels of the gene GNMT in the PC cell line BxPC-3. This result may indicate a possible pathway that causes the cytotoxic effects that PGG has on the PC cells and should be further explored in the future. Overall, this research provides more information pertaining to gene regulation in early-stage PC, indicates a possible mechanism that could be used to alter the expression of GNMT in PC cells, and could potentially lead to new biomarker testing capable of identifying early-stage PC. This information may help to provide new insights and diagnostic capabilities for the treatment of PC in the future which would ultimately decrease the mortality rate associated with the disease.

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