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Analysis of protein expression for potential biomarkers in human pancreatic tumor cell lines

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ANALYSIS OF PROTEIN EXPRESSION FOR POTENTIAL BIOMARKERS IN HUMAN PANCREATIC TUMOR CELL LINES

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Abstract

Pancreatic cancer is an aggressive cancer of the pancreas that has been shown to have extremely low survival rates. It can be quite lethal because of its asymptomatic early stages and inability to detect cancer at the cellular level. Once diagnosed, the cancer will have usually spread or metastasized beyond surgical resection. Thus, research is focused on finding potential biomarkers in tumor cell lines to help in earlier diagnosis of this disease leading to better treatment options. Previous work using bioinformatics data suggest several cellular genes including, CTHCR1, EPPK1, AHNAK2, and IGHG3, can be potential biomarkers for pancreatic cancer. The goal of this project was to determine if protein expression of these genes was altered in a variety of human pancreatic tumor cell lines. Protein isolation and quantification followed by Western blot analysis was used. Our results showed a statistically significant increase of CTHCR1 protein expression in SW 1990, a pancreatic tumor cell line, compared to the HPDE-6 control cell line. This data adds to the current evidence that CTHCR1 can act as a prospective pancreatic cancer biomarker. There was also increased CTHCR1 protein expression in four other human pancreatic tumorigenic cell lines, but they were not found statistically significant. EPPK1 was another potential biomarker studied; however, its large molecular weight was a challenge and caused insufficient transfer of proteins. No bands were visualized.

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Introduction

The diagnosis of cancer is a life changing event. There are always many concerns and questions such as: has it spread, what is the prognosis, what are possible therapies, and how long is the life expectancy? Unfortunately, these questions usually have poor responses with regard to pancreatic cancer. Pancreatic cancer is an aggressive cancer called a "silent killer." It typically has no symptoms in the early stages when surgical resection would be a preferred option. Currently, diagnostic detection strategies are not specific or universal for all patients. By the time of diagnosis, the cancer has usually metastasized beyond surgery, and other treatment options still have low survival rates. Research suggests that there is significant need to discover new specific pancreatic cancer biomarkers which are a measurable substance in an organism whose presence is indicative of a certain disease or infection. New biomarkers specific to pancreatic cancer would help in earlier diagnosis. Based on previous work by Goonesekere et al. (2014), four genes, CTHCR1, EPPK1, AHNAK2, and IGHG3, have been suggested as potential biomarkers for pancreatic cancer. The purpose of my thesis project is to attempt to determine if these proteins can act as potential biomarkers. The project seeks to determine if the level of protein expression of the four proteins is altered in several known human pancreatic tumor cell lines and verify their status as novel biomarkers in pancreatic cancer. After determining protein expression, we will be able to evaluate and better understand these genes as potential biomarkers for pancreatic cancer. This thesis work will add to the current literature about pancreatic cancer and potentially help in earlier diagnosis of this deadly disease leading to better prognosis, targets for therapeutic intervention, and treatment options.

Review of Literature

Pancreatic Cancer

The pancreas is a large organ deep in the abdomen with 2 glands: exocrine glands that secrete digestive enzymes into the duodenum and endocrine cells that release hormones into the blood stream. Exocrine glands and the duct where the enzymes are released make up more than 95% of the pancreas while endocrine cells make up less than 4% (American Cancer Society, 2015). The endocrine hormones produced, such as insulin and glucagon, are important in controlling blood sugar levels. As reviewed in Singh et al. (2015), the majority of pancreatic tumors come from exocrine cells. Cancer cells can be described as growing out of control and invading other tissues due to their damaged DNA (American Cancer Society, 2015). The tumor cells can grow around a population of cancer stem cells; these are cells that have the ability to differentiate and self-renew. Even though not all tumors are cancerous, the exocrine and endocrine cells of the pancreas have different types of tumors which have different treatment and prognosis. Pancreatic ductal adenocarcinoma (PDAC) makes up approximately 90% of pancreatic tumors which consequently starts in the ductal epithelium of the pancreas (Singh et al., 2015). Other less common types of exocrine cells include adenosquamous carcinomas, squamous cell carcinomas, signet ring cell carcinomas, undifferentiated carcinomas, and undifferentiated carcinomas with giant cells (American Cancer Society, 2015). Solid pseudopapillary neoplasms and ampullary cancer are other less common types, but more commonly treatable. Endocrine tumors are rarer and are commonly known as neuroendocrine tumors or islet cell tumors (American Cancer Society, 2015). They also generally have better prognosis than exocrine tumors. Since pancreatic cancer is typically asymptomatic in early stages and due to the metastasis of the disease, chances for cure at the time of diagnosis becomes

very difficult (McKinney et al., 2011). Pancreatic cancer (PC) is one of the most aggressive types of cancer in the United States with a median survival rate of less than six months according to McKinney et al. (2011). It is the fourth most common cause of cancer death in North America (Ouyang et al., 2000).

Pancreatic cancer is associated with an extremely poor prognosis due to the advanced stage in which it is usually detected; many times the cancer has metastasized and is unable to be resected surgically (Park et al., 2013). The location of the pancreas deep in the abdomen behind the stomach eliminates the ability to palpate tumors, and almost acts as a hidden, sanctuary site. Pancreatic cancer also can seem to come by "surprise" due to little to no known association with family history or other diseases that increase the risk of PC (Chakraborty et al., 2011). Pancreatic cancer is hereditarily complex. Age, however, is positively correlated and a majority of pancreatic patients are over the age of sixty (Singh et al., 2015). Approximately 10% of PC patients are thought to have a familial susceptibility with only a minority of these patients having an inherited component and usually the responsible gene cannot be identified (Sclafani et al., 2015). There are also risk factors such cigarette smoking and alcohol abuse that are associated with the development of this cancer (Sclafani et al., 2015). Pancreatic cancer continues to be a struggle for clinicians and scientists in diagnosis, and little progress has been made in the treatment of the disease. There is a need to find the best route of care for each specific patient.

Diagnosis

Diagnosis has been difficult as not much progress has been made in biomarker identification in pancreatic cancer. A biomarker is a measurable indicator of a specific biological state, the presence or stage of a disease (Rifai et al., 2006). A biomarker can be the presence of a specific gene or protein, and they can be used to screen for, diagnosis, or monitor the activity of

diseases clinically. Clinically, the ultimate goal would be using a blood test or other readily accessible biological fluids to test to see if a certain biomarker indicative of a disease is present. These tests would be much faster and less invasive for patients and could be done at any time, making a possibility of earlier diagnosis available. Biomarkers are also used in molecularly targeted therapy (Rifai et al., 2006). However, their discoveries and implementations are challenging and slow. Currently, there a few genes that are found mutated in pancreatic cancer. Mutations in inherited genes such as BRCA2, p16, and the genes responsible for hereditary non-poly colorectal cancer can increase a patient's chances of developing PC (American Cancer Society, 2015). Another non-invasive type of diagnosis is done through the detection of serum markers in blood. CA19-9 is currently used and the most common to help detect pancreatic cancer; however, this molecular biomarker has low specificity, is not expressed in high levels in pancreatic cancer, and is also not constantly expressed in all PC patients (McKinney et al., 2011). Some pancreatic cancers do not secrete CA 19-9 even at the later stages (Haab et al., 2015). A variety of other serum markers have been reported to be elevated in pancreatic cancer; however, they too have low sensitivity and specificity, and currently very few of these serum markers have shown encouraging results (Bünger et al., 2011). Thus, there is great need for identifying more specific markers that can detect pancreatic cancer while the disease is in its early stages and that are found more consistently in PC patients (McKinney et al., 2011). The heterogeneity of pancreatic cancer and range of mutations identified is likely to account for its poor response to treatments and aggressiveness (Sclafani et al., 2015). The discovery of biomarkers is needed to detect the full spectrum of pancreatic cancer cases (Haab et al., 2015). Better PC screening would give patients earlier and better treatment options; therefore improving survival ratings.

Treatments

Treating pancreatic cancer is usually unsuccessful for the patient with only 10-15% of patients amenable to surgery (Park et al., 2013). Other than tumor resection, most patients often have to turn to more intensive treatments such as conventional radiation and chemotherapy, which also can have adverse effects. The resistance to cytotoxic drugs and resection surgery along with PC's asymptomatic and metastatic nature make treatment extremely difficult leading to high annual death rates. These annual mortality rates are even comparable to breast cancer which is diagnosed at a rate 5 times greater than pancreatic cancer (Howlader et al., 2011). PC patients have an approximate 5-year survival rate of less than 5% (Lee et al., 2010). In summary, pancreatic cancer is one that that needs better methods of detection and screening while the tumor is still at an early stage. Even though therapies have improved over the past ten years, overall survival rates are still significantly low and new strategies are necessary.

Detection of potential new biomarkers

As stated previously, CA-19 is the biomarker currently used in pancreatic cancer diagnosis and progression; however, its sensitivity to pancreatic cancer is not high (McKinney et al., 2011). Therefore, research into finding other genes and markers of pancreatic cancer has been a high priority. In a meta-analysis of microarray datasets by Goonesekere and colleagues in 2014, research focused on finding genes that were significantly upregulated in pancreatic cancer. The study found four genes that were upregulated that were not previously associated or had a known role with a cancer, specifically pancreatic cancer; AHNAK2, collagen triple helix repeat containing-1 (CTHCR1), immunoglobulin heavy constant gamma-3 (IGHG3), and epiplakin (EPPK1) were the four proteins. This thesis project was designed and based on this work.

CTHCR1

The work by Goonesekere et al. (2014) suggested collagen triple helix repeat containing-1 or CTHCR1 as a significantly up-regulated gene. CTHCR1 is a secreted protein approximately 30 kilo Daltons (kDa), and expressed at high levels during vascular remodeling and tissue repair (Beachy et al., 2004). In a study performed by Tang et al. (2006), expression of CTHCR1 was investigated in twenty solid cancer types through western blotting and CTHCR1 expression in cancer cDNA arrays. Human CTHCR1 was detected and identified by western blot analysis in melanoma cell lines, and not in normal melanocytes showing its potential as being expressed in cancer but not normal cells. In the other part of the study by Tang et al. (2006), they used a CTHCR1 specific gene probe to hybridize around 300 samples of cDNA from clinical tumor biopsies along with a matching benign tumor biopsy. Results showed that CTHCR1 gene levels were increased or upregulated (abnormally higher) in a majority of the cancers represented such as cancers of the rectum, small intestine, colon, liver, lung, ovary, pancreas, and breast, compared to the normal, control tissues (Tang et al., 2006). This can give optimism and perhaps makes this gene an attractive option for developing a potentially new biomarker for various cancers.

While CTHCR1 has been previously reported to be present in other carcinomas with poor survival rates such as melanoma, breast cancer, colorectal, and gastric cancer, CTHCR1 has only been associated with pancreatic cancer in a limited number of reports (reviewed in Goonesekere et al., 2014). CTHCR1 also seems to play a role in tumors that are aggressive in nature. Mice injected with CTHCR1 were found to have an increased number of tumor metastases. Results also showed CTHCR1 expression was strongly detected in pancreatic adenocarcinoma cells (Park et al., 2013) and that CTHCR1 expression enhances tumor progression, metastasis, tumor

cell migration, and adhesion. According to Tang et al. (2006), in metastatic melanoma, CTHCR1 function is unclear, but results showed increased levels of tumor cell migration and tumor invasion by regulating the surrounding environment. There are indications that CTHCR1 may have a significant role in PC tumors, but it may not be required and all the functions are not completely understood (Park et al., 2013). Based on this work, the current project evaluated if increased CTHCR1 protein levels can serve as a potential biomarker for pancreatic cancer.

EPPK1

EPPK1, epiplakin, was another gene found to be significantly upregulated in the microarray meta- analysis of pancreatic cancer by Goonesekere et al. (2014). Epiplakin is an unusual protein compared to other members in the plakin family of cytolinker proteins that are associated with the cytoskeleton and junctional complexes. EPPK1 may participate in supporting intermediate filaments however specific function of this very large 552 kDa protein is not precisely known. It has been found to be expressed in the pancreas and pancreatic ductal adenocarcinomas and may be a useful biomarker for pancreatic cancer (Yoshida et al., 2008). This increased expression in pancreatic tissue provides potential for this gene and protein product to be evaluated as a potential biomarker of pancreatic cancer.

IGHG3

Immunoglobulin heavy constant gamma-3 (IGHG3) was another potential PC biomarker cited by Goonesekere et al. (2014). This gene was significantly upregulated. It is known that IGHG3 is a secreted antigen binding protein, but has no assigned function at this time (Goonesekere et al., 2014). IGHG3 has not been directly associated with specific cancer development or progression.

AHNAK2

AHNAK2 is a large protein like EPPK1, with an estimated size of approximately 600 kDa. AHNAK2 is the second protein in the AHNAK family along with AHNAK. AHNAK2 was found to be significantly upregulated gene in pancreatic cancer in the meta-analysis (Goonesekere et al., 2014). Numerous studies have contributed to finding the function of AHNAK2; however the specific function is unknown. The protein AHNAK has been associated with forming part of multi-protein complexes, most likely acting as a structural support, which AHNAK2 may share similarities with (Davis et al., 2014). AHNAK has been linked to several muscular diseases and may be functional component to the costameric network (Goonesekere et al., 2014).

There are limited studies linking the role of AHNAK2 in cancer (Davis et al. 2014); however, there was discovery that AHNAK was significantly enriched in purified pseudopodia of six different metastatic cancer cell lines and another study showed that knockdown of AHNAK resulted in decreased migration and invasion of the cancer cells. In contrasting studies, AHNAK was described as a protein that functions as a repressor of cancer, that is, it acts as a tumor suppressor (Davis et al., 2014). While the exact function of AHNAK2 and AHNAK may be unclear, they seem to have a large diversity of roles possibly pertaining to cancer, especially pancreatic cancer. The meta-analysis suggested both IGHG3 and AHNAK2 need further investigation to understand their association to pancreatic cancer, as their increased protein expression could be a prospective marker for PC.

Cell Lines

The current project was designed to evaluate levels of protein expression of these potential biomarkers in a variety of tumor cell lines compared to a control cell line. We have used immortal human pancreatic duct epithelial (HPDE) cells as a control pancreatic cell line. HDPE cells demonstrate a similar gene expression pattern to normal, non-tumorigenic pancreatic cells as compared to a cancerous cell line (Ouyang et al., 2000). This research proposes to use the immortal human pancreatic duct epithelial (HPDE) cells as a control for our future studies on pancreatic cancer.

Summary

Pancreatic cancer will continue to be a deadly diagnosis if we are unable to diagnose the disease in its early stages. The current biomarkers and screening tools have not been successful in detecting the tumors early enough for successful resection and recovery. Chemotherapy and radiotherapy have also not guaranteed success. The necessity for new biomarkers or target genes is ongoing. Previous studies have recognized CTHCR1, EPPK1, IGHG3, and AHNAK2 as potential new biomarkers; these genes have been found to be expressed at higher levels in pancreatic cancers. However, as there is still limited literature and knowledge, more experimentation is needed. The goal of this thesis research project is to specifically quantify the protein levels of the suggested genes to determine if the proteins are expressed at higher levels in pancreatic cancer cell lines compared to normal pancreatic cells. This work can then add to the current literature for finding new potential biomarkers for pancreatic cancer to improve early detection of this lethal disease.

Materials & Methods

Cell culture

A panel of six pancreatic tumor cell lines of human origin were obtained from the American Type Culture Collection (ATCC). The cell lines included PANC-1, BxPc-3, AsPC-1, Capan-2, HPAF-II, and CFPA-1. Human Pancreatic Duct Epithelial cells, HPDE-6, is a non-tumorigenic cell line and was used as a control cell line. All cells were grown to confluence at 37°C in a humidified 5% CO2 incubator and were passaged every 2-4 days depending on cell growth. Cell culture medium varied for all cell lines. HPDE-6 cells were grown in Keratinocyte SFM supplemented with EGF and bovine pituitary extract and with 1% antibiotic-antimycotic. ATCC-formulated RPMI-1640 Medium was used for AsPC-1 and BxPC-3. ATCC-formulated Dulbecco's Modified Eagle's Medium was used for PANC-1. ATCC-formulated McCoy's 5a Medium was used for Capan-2. ATCC-formulated Eagle's Minimum Essential Medium was used for HPAF-II. ATCC-formulated Iscove's Modified Dulbecco's Medium was used for CFPAC-1. ATCC-formulated Leibovitz's L-15 Medium was used for SW 1990, and SW 1990 was the only cell line grown in a free gas exchange with atmospheric air incubator due to the L-15 medium formulation. All cell line mediums had fetal bovine serum (FBS) to a final concentration of 10% and 1% penicillin/streptomycin added.

Protein isolation and quantification

Total protein was harvested from a 100 mm tissue culture dish containing cells that were 80-100% confluent. Cell lysates were prepared according to the protocol provided from Santa Cruz Biotechnology utilizing RIPA buffer (1X Lysis Buffer: 1X TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide). The RIPA buffer was supplemented

with PMSF (2 mM), sodium orthovanadate (1 mM), and protease inhibitor cocktail. To solubilize cellular proteins, 0.5 mL of complete RIPA buffer /100 mm dish was added with the plates on ice. The plates were gently rocked while on ice for 15 minutes, and then a cell scraper was used to remove the adherent cells which the resulting lysate was transferred into a micro-centrifuge tube. The lysate was passed through a 25-gauge needle to shear the DNA, and then the tube of lysate was incubated on ice for 30 minutes. After incubation, the cell lysate was centrifuged at 10,000 rpm for 10 minutes at 4° C. The supernatant fluid, or whole cell lysate, was then transferred to a new micro-centrifuge tube, and the pellet is discarded. The cell lysate was subsequently quantified by the modified colorimetric Lowry assay (Bio-Rad) or stored at -80C and quantified at a later date.

A modified Lowry Assay was used to quantify the proteins. Specific amino acids of a protein interact with a dye producing a blue color which can be measured at 750nm. Absorbance is positively correlated to the amount of protein present. To create a standard curve, known dilutions of bovine serum albumin (BSA) from 0 to 2mg/ml were placed into a 96-well plate. Five replicates of each standard concentration (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0mg/ml) were added to the plate. Additionally, a dilution of 1:4 or 1:5 of each cell lysate in RIPA buffer was also included in the analysis. Five replicates of 5ul of each sample were added to the 96-well plate. Next, reagents A and S (Bio-Rad) were combined and 25ul were added to each well. 200ul of reagent B (Bio-Rad) was added into each well. The plate was then incubated at room temperature for 15 minutes. During this time, any bubbles present were removed to prevent inaccuracies when the absorbance was read. After 15 minutes, absorbance of the solutions in the 96 well-plate was read at the wavelength of 750 nm in a spectrophotometer. A linear regression

line was generated using the SoftMax Pro 4.3.1 LS program. The correlation coefficient (R^2) had to be above 0.97 in order for the data to be used for further analysis.

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis

After protein isolation and quantification, protein extracts from each cell line were separated on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). CTHCR1 with a molecular weight of 25 kDa migrates farther through the gel than Epiplakin-1 which has molecular weight of 552 kDa. This extremely large protein takes longer to pass through the gel. To separate CTHCR1 protein from the cell lysate, 22 micrograms of protein were added to a 12% gel. Before loading the samples containing protein and 6X loading buffer, the protein samples were denatured with heat for 5 minutes. The gel was run at room temperature for 55 minutes at 120 V in 1X Tris-glycine running buffer (25mM Tris base, 190mM glycine, 0.1% SDS with pH around 8.3). To separate EPPK1 protein from the cell lysate, a 4-20% gradient acrylamide gel was loaded with 30 micrograms of whole cell lysate (protein).The gel was run at 200V for two hours in 1X Tris-glycine running buffer at 4˚C. After gel electrophoresis, the proteins were transferred to a PVDF membrane using the iBlot 2 Dry Blotting System. The membrane was then subjected to immunoblot analysis, described in the following section, or stored at 4 degrees Celsius until analysis at a later time.

Immunoblot analysis

Immunoblot analysis was used to identify the target protein on the PDVF membrane. Blocking the membrane prevents non-specific binding of antibodies; the primary antibody binds to the specific target protein, while the specific secondary antibody binds to the primary antibody. The secondary antibody has an enzyme conjugated to it which allows a detectable

signal to be produced upon addition of substrate. In these experiments, the protein containing PVDF membrane was incubated at room temperature in 5% milk in TBS blocking solution (5 grams per 100 ml of Tris Buffer Saline) for one hour with rocking followed by a TBS (Tris Buffer Saline) wash for ten minutes, TBS-T (Tris Buffer Saline Tween20) wash for ten minutes, and then TBS wash again for ten minutes. Next, the PVDF membrane was incubated with a primary antibody targeted against the specific proteins (either anti-CTHCR1 or anti-EPPK1) at various dilutions (1:50 – 1:300) in TBS-T or TBS buffer overnight at 4 degrees Celsius. Following overnight incubation with rocking, blots were washed with TBS and TBS-T as stated above, and subsequently probed with a secondary antibody in TBS-T or TBS buffer for one to three hours gently rocking at room temperature. Following exposure to the secondary antibody, the unbound secondary antibody was removed and a wash series of TBS and TBS-T as stated previously was performed to the membranes.

Visualization of the Protein Bands

After the final wash, proteins were visualized using a chemiluminescence kit (Western Lightning). Western Lighting is a non-radioactive, enzymatic reaction light-emitting system designed to detect proteins immobilized on a membrane using an imaging system. Membranes were incubated with gentle agitation for 30 to 40 seconds in equal parts of the Enhanced Luminol Reagent and the Oxidizing Reagent. The membranes were then removed from the solution and excess reagent was drained off. The STORM 860 phosphoimager was used immediately after to scan the membranes. The blue fluorescence channel was used with normal sensitivity, pixel size of 200 microns, and a PMT voltage of 800nm. After the membranes were scanned, they were washed as stated previously in TBS and TBS-T, and then were ready to be stripped. Visualization of bands used the Image Quant 5.2 program. Using the Image Quant 5.2 program,

each specific protein band was individually selected and then the pixel volume was computed. These volumes were entered into a spread sheet for analysis.

Actin Normalization

In order to normalize the concentrations of the targeted proteins from each of the blots, each blot was stripped of its previous antibodies and reprobed with an antibody to actin, a protein found in similar concentrations in most cells. The blots were stripped in stripping buffer (62.5 mM Tris-HCL H 6.8, 2% SDS, 100 mM 2-mercaptoethanol). Blots were incubated for 2 hours with gentle agitation at 50° C. Then the membranes were washed in TBS-T for 5 minutes, 6 times. The chemiluminescence kit (Western Lightning) was then used to make sure all bands had disappeared. Then membranes were washed again in TBS-T as explained previously. At this point, the blots were ready for reuse and incubated for 1 hour at room temperature with gentle rocking in 5% milk TBS-T blocking solution followed by TBS wash for ten minutes, TBS-T wash for ten minutes, and then TBS wash again for ten minutes. Blots were re-probed with anti-actin primary antibody (Santa Cruz Biotechnology, 1:200) followed by a wash cycle and then a complementary secondary antibody (Santa Cruz Biotechnology, 1:2,000), then visualization and quantification as previously described.

Normalization is commonly used when measuring gene expression relative to a reference sample such as actin. Actin is a “house-keeping gene” found in all cells and is required for basic cellular functions. To evaluate fold change determination, the intensity values from CTHCR1 and corresponding actin intensity values were used. However, ratios of the intensity of the protein CTHCR1 in the tumorigenic cell line compared to the control HDPE-6 intensity values were first calculated. As there were two HPDE-6 intensity values from each gel, the values were averaged and used to calculate the ratios. This was done for the CTHCR1 protein and for the

actin protein. In order to normalize the values of the CTHCR1 to the level of actin, the calculated ratio (tumorigenic cell line/HDPE-6) of the CTHCR1 protein was divided by the actin ratio (tumorigenic cell line/HDPE-6) to determine the normalized fold change. If this ratio was greater than one, there was overexpression of the protein in tumorigenic cell lines compared to control, if the ratio was below one, tumorigenic cell lines showed decreased expression of CTHCR1 compared to control.

Statistical Analysis

Quantification of bands calculated the expression of the target protein in tumorigenic cell lines compared to control HDPE cells. In the analysis, three different samples from each cell line were used in the analysis. As described above, the band intensity values were normalized with an actin control and compared to the control cell line, HDPE. One way ANOVA with a two sided Dunnett's t-Test for post-hoc analysis was performed using the normalized fold values to determine statistical significance where $p<0.05$ was considered statistically significant.

Results

Currently, pancreatic cancer has a poor prognosis which could be explained by the asymptomatic progression, late detection, and lack of effective treatments. This research was based on an effort to provide new, potential biomarkers to help with earlier detection of pancreatic cancer. Protein extracts from several human pancreatic cell lines were evaluated for the expression of several potential protein biomarkers and compared to levels found in non-tumorigenic pancreatic cell line that served as control. Western blot analysis was used in this comparison. We were able to test two potential biomarkers: CTHCR1 and EPPK1.

CTHCR1

CTHCR1, collagen triple helix repeat containing-1, is a secreted protein approximately 30 kilo Daltons (kDa), and expressed at high levels during vascular remodeling and tissue repair (Beachy et al., 2004). It was suggested as a significantly upregulated protein in work by Goonesekere et al. (2014). This thesis work through western blot analysis found levels of CTHCR1 to be significantly increased in one of the seven human pancreatic cancer cells that were tested (Figure 1). The other six cell lines tested did not have any significant change in levels of CTHCR1. Figure 1 is a representative gel for CTHCR1. The gel order is shown with the molecular weight marker in the first lane, two unique samples of control HPDE-6, and one sample from each of the 7 tumorigenic cell lines. Three separate gels with this order were run and analyzed. The protein samples were each distinct on each gel, that is, each tumorigenic sample was from 3 different cell passages of each cell line. There are non-specific bands visualized on the blot as well. In the blot, SW 1990, in the far right lane, has the greatest amount of CTHCR1 as quantified by measuring the band intensity using Image Quant software.

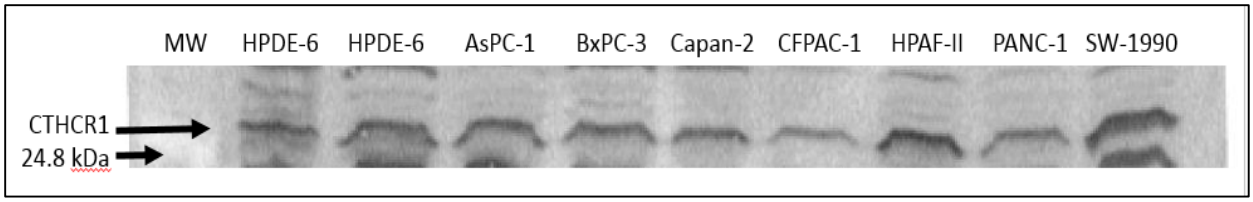

Figure 1. *Western blot analysis shows protein extracted from HPDE-6 cells (control) and one individual, distinct sample from each of the seven pancreatic tumor cell lines. A band at the expected region for CTHCR1, 26 kDa, can be seen in western blots. A molecular weight marker is present on the left. This is a representative blot of the 3 replicates used in the analysis.*

Figure 2 shows the previous blot after stripping it of anti-CTHCR1 antibodies and re-probing with anti-Actin antibody. Actin is a housekeeping gene involved in cell structure and motility, and found in almost all cell types; it was used to determine a normalized fold change for the CTHCR1 in tumorigenic cell lines compared to the HDPE-6 control cells. To make sure that intensity values were different based on altered expression and not based on unequal amounts of total protein loaded in each well, comparisons were made with actin since this protein is not changed in the tumorigenic cell lines. The band intensity was used to normalize the results of CTHCR1. Actin appeared in all the cell types and produced very consistent bands.

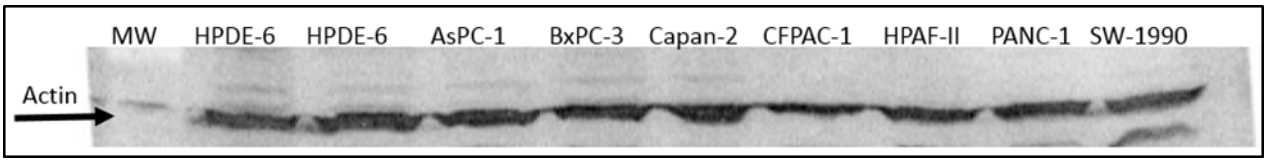

Figure 2. *This is the corresponding blot from figure 1 after stripping CTHCR1 antibodies and re-probing with anti-actin. Western blot analysis shows protein extracted from HPDE-6 cells (control) and one individual, distinct sample from each of the 7 pancreatic tumor cell lines express the protein, actin, which is expected. A molecular weight marker is on the left.*

Table 1 in Excel spreadsheet outlines the calculation process of determining the various values of each blot. The volumes from each of the CTHCR1 bands and Actin bands were determined by measuring the band intensity by Image Quant software. The two control HPDE-6 volumes were averaged from each study; the ratio of "Sample Vol. / Control Vol." was calculated between control and the samples from the tumorigenic cell lines by dividing the tumor cell line volume by the average HDPE-6 volume. This was also performed for the actin protein. The "Sample Vol. /Actin vol" column in the table shows the sample volume divided by the corresponding actin volume ratio. "Normalized fold change" of the table shows the normalized fold change by dividing the ratio of the sample volume/ actin volume ratio by same ratio of the control HPDE-6 sample . This process was repeated for the three replicate blots.

CTHCR-1 Lane	Cell Type	Volume of Band	Volume of Band with Avg. HPDE-6	Sample Vol. / Control Vol.	Sample Vol. / Actin vol.	Normalized fold change
1	MW marker					
2	HPDE-6	180349.81				
3	HPDE-6	332229.61	256289.71	1	0.482373328	1
4	AsPC-1	247537.33	247537.33	0.965849663	0.49534263	1.026886443
5	BxPC-3	272646.32	272646.32	1.063820783	0.544797343	1.129410172
6	Capan-2	201473.05	201473.05	0.786114472	0.418828643	0.868266588
7	CFPAC-1	122261.3	122261.3	0.477043343	0.306756115	0.635930922
8	HPAF-II	286937.11	286937.11	1.119581079	0.686705824	1.423598247
9	PANC-1	185162.56	185162.56	0.722473641	0.436486412	0.90487261
10	SW-1990	469643.48	469643.48	1.832471073	1.158479222	2.401623713
ACTIN						
1	MW marker					
2	HPDE-6	479893.49				
3	HPDE-6	582726.25	531309.87	1		
4	AsPC-1	499729.51	499729.51	0.940561315		
5	BxPC-3	500454.57	500454.57	0.94192598		
6	Capan-2	481039.33	481039.33	0.905383764		
7	CFPAC-1	398561.9	398561.9	0.750149625		
8	HPAF-II	417845.75	417845.75	0.786444547		
9	PANC-1	424211.51	424211.51	0.798425804		
10	SW-1990	405396.55	405396.55	0.763013399		

Table 1. *Analysis of CTHCR1 band intensities to calculate fold change of protein in tumorigenic pancreatic cell lines compared to control cells.*

Cell line	Normalized fold change ratios	Average of Normalized Ratios	Standard Error of the Mean
HPDE6	1	1	0
HPDE6	1		
HPDE6	1		
HPDE6	1		
HPDE6	1		
HPDE6	1		
AsPC1	1.026886443	0.909089779	0.201558259
AsPC1	0.516319976		
AsPC1	1.1840629		
BxPC3	0.868266588	0.635674284	0.181087922
BxPC3	0.759803007		
BxPC3	0.278953255		
CAPAN	0.868266588	1.05537445	0.198856934
CAPAN	1.452861313		
CAPAN	0.84499545		
CFPAC	0.635930922	1.32077718	0.342497379
CFPAC	1.650848583		
CFPAC	1.675552035		
HPAF-II	1.423598247	1.206709816	0.216888432
HPAF-II	0.989821384		
Panc-1	0.90487261	1.091981275	0.14079838
Panc-1	1.367786293		
Panc-1	1.003284923		
SW-1990	2.401623713	1.817707951	0.420680245
SW-1990	2.050342668		
SW-1990	1.001157473		

***Table 2**. CTHCR1 statistical analysis using One-way ANOVA.*

Table 2 shows the calculated normalized ratios for all of the samples. The table also shows the average of each cell line's normalized ratios and the standard error of the mean of each sample. For cell line HPAF-II, there are only two samples as there was an error in the loading process and a third replicate was not available. Table 2 shows that pancreatic cell lines AsPC1 and BxPC3 have decreased expression of the protein CTHCR1 as compared to the control cell line HPDE-6. The other five pancreatic tumor cell lines (Capan-2, CFPAC-1, HPAF-

II, PANC-1, and SW 1990) show increased expression compared to HPDE-6 cell lines. SW 1990 appears to have the greatest overexpression of CTHCR1, almost a two-fold increase. Table 3 below shows the results of the ANOVA multiple comparison Dunnett's t-test. Only one cell line showing statistical significance was SW 1990 with p value of 0.044, showing it was significantly altered compared to HPDE-6. The other samples were not statistically significant with values above 0.05. Figure 3 below shows the results from Table 2 and Table 3 in graphical format.

Dunnett t (2-sided)[a]

Variable	Control	Mean Difference	Std. Error	Significance
AsPC1	HPDE6	-.09091	0.271644176	1.000
BxPC3	HPDE6	-.36433	0.271644176	0.698
Capan-2	HPDE6	.05537	0.271644176	1.000
CFPAC-1	HPDE6	.32078	0.271644176	0.800
HPAF-II	HPDE6	.20671	0.313667677	0.986
PANC-1	HPDE6	.09198	0.271644176	1.000
SW 1990	HPDE6	.81771*	0.271644176	0.044*

*. The mean difference is significant at the 0.05 level. *. Significant when below 0.05

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table 3. *ANOVA Dunnett's t-test results for CTHCR1. Compares protein expression of the panel of human pancreatic tumor cell lines to the control cell line. SW 1990 shows statistical significance with a value below 0.05.*

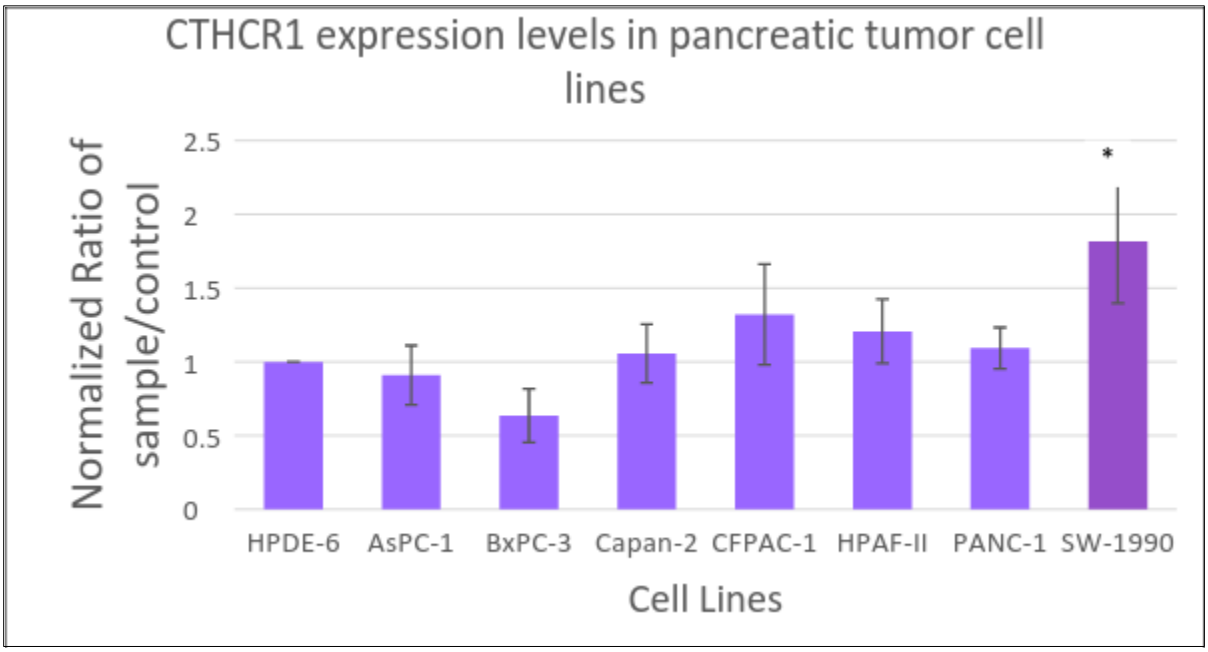

Figure 3. *CTHCR1 expression levels in a panel of human pancreatic tumor cell lines. Each cell line was normalized with actin and formulated a normalized ratio of sample volume over control volume. Error bars represent the standard error of the mean for each cell line. HDPE-6 is the control cell line. Ratios above 1.0 show increased expression compared to control and those below 1.0 show decreased levels of this protein. SW 1990, indicated with an asterisk, is the only statistically significant cell line with increased CTHCR1 expression.*

In Figure 3, the horizontal axis is the specific cell line and the vertical axis is each cell lines' average of normalized fold change ratios (From Table 2). Error bars represent the standard error of the mean of each cell line. The HPDE-6 is the control cell line with a baseline ratio of 1. AsPC-1 and BxPC-3 show decreased expression of CTHCR1 while the other cell lines have increased expression. However, these alterations in expression did not prove statistically significant except for SW 1990. SW 1990 has an almost two- fold increase of expression of CTHCR1 over the control HPDE-6 (Figure 3).

EPPK1

EPPK1 was another protein found to be significantly upregulated in the previous analysis performed by Goonesekere et al. (2014). It is a larger in molecular weight at 552 kDa and is associated with the cytoskeleton proteins. Western blot analysis on the protein EPPK1 was attempted and it proved difficult to obtain results. With EPPK1's molecular weight at 552 kDa, results from gel after transfer showed that the protein did not fully transfer from the gel to the membrane. As shown in Figure 4 there was a significant amount of protein left in the wells of the gel where EPPK1 and higher molecular weight proteins would be located. The darker coloration of purple indicates more protein.

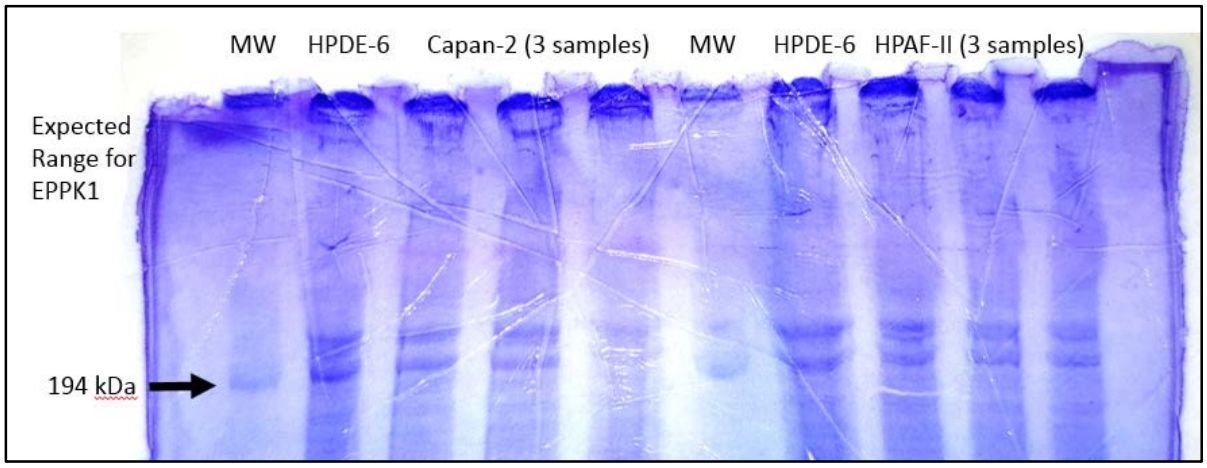

Figure 4. *A gel for EPPK1 after SDS-Page and transfer of proteins to membrane stained in Coomassie Blue stain and dried showing a significant amount of protein left over in the wells of the gel.*

Incomplete transfer leads to less protein on the membrane. When the membranes were probed with anti-EPPK1 antibodies, no bands were visualized as shown in Figure 5. In one instance, there was an experiment when a faint band could be seen near the top of the membrane

as seen in Figure 6 that was suspected to be EPPK1; however, the band located in this region of the membrane was never visualized more than once even though several modifications of methods were attempted such as increasing the transfer time, increasing the intensity of voltage, varying the dilutions of primary and secondary antibodies and the medium of diluent. Additionally, the amount of protein loaded onto the gel was also increased from 22ug to 50ug; however, EPPK1 could not be visualized more than one time.

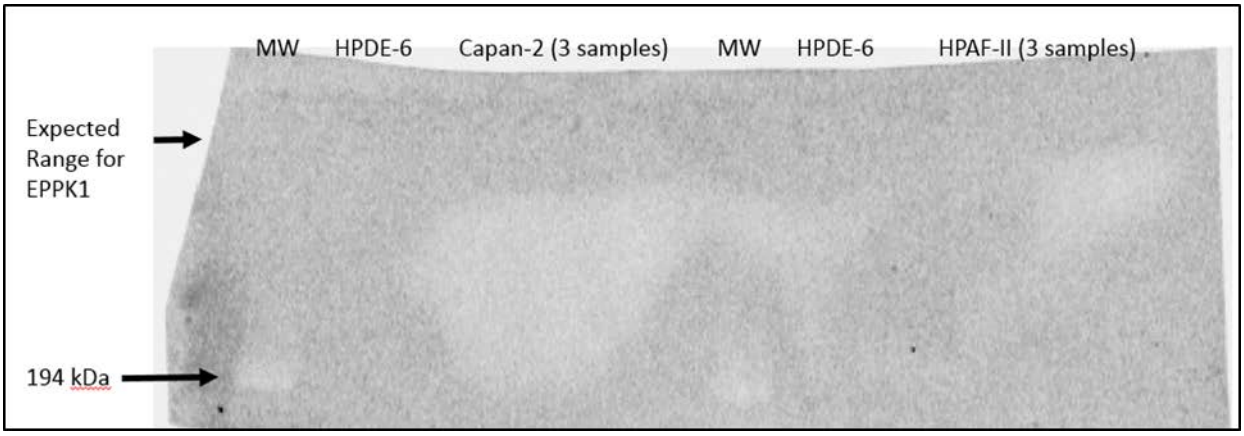

Figure 5. *Example of a Western blot with no visible bands in the EPPK1 region probed with anti-EPPK1 antibody. A molecular weight protein is seen on the far left and in the 6th lane.*

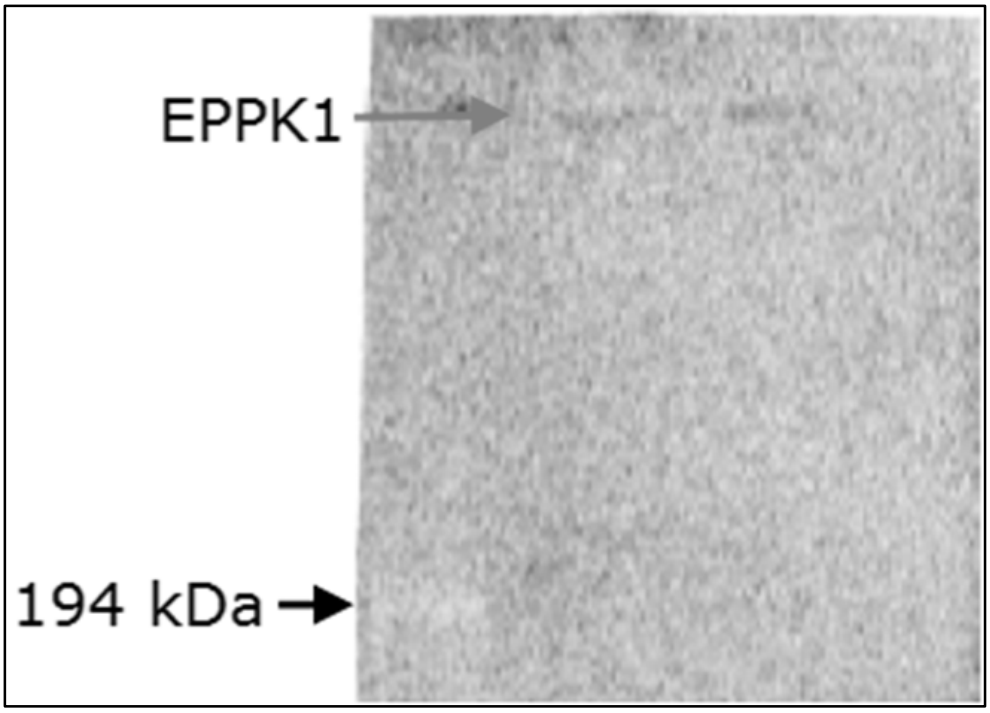

Figure 6. *Western blot analysis shows HPDE-6 (on left) and BxPC-3 (on right) cell lines showed protein expression of EPPK1. Molecular weight marker is on the left. The bands were detected at the expected region for Epiplakin-1, 552 kDa.*

IGHG3 and AHNAK2

Due to restricted time and focus on completing CTHCR1 and EPPK1 analysis, evaluation of IGHG3 and AHNAK2 protein levels was not performed at this time. CTHCR1 and EPPK1 required an extensive amount of time to optimize the western blot methods.

Discussion

This thesis project was based on a previous meta-analysis study to suggest several cellular genes including, CTHCR1, EPPK1, AHNAK2, and IGHG3, as potential biomarkers for pancreatic cancer (Goonesekere et al., 2014). Our goal was to determine if protein expression of these genes was altered in a variety of human pancreatic tumor cell lines. Significant changes in protein expression in tumor cell lines compared to control could disclose a new potential biomarker to help in earlier diagnosis of pancreatic cancer leading to better treatment options. Cell culture, protein isolation and quantification followed by Western blot analysis was performed. The results showed that there was a significant increase, nearly two-fold, in expression of CTHCR1 in the tumorigenic cell line SW 1990 (Figure 3). The human pancreatic tumorigenic cell line, SW 1990, was obtained from a 56 year old male who had adenocarcinoma of the pancreas derived from the spleen metastatic site. The cell line obtained from the American Tissue Cell Collection is epithelial and adherent in nature and did not require 5% CO_2 for growth, unlike the other cell lines used in this study. There was also a trend of increased expression in CTHCR1 in four other pancreatic tumor cell lines (Table 2), there was no statistically significant increase in protein expression. In contrast, two pancreatic tumor cell lines that had decreased expression of the protein CTHCR-1, however, these levels were also not statistically significant.

CTHCR1, also known as collagen triple helix containing 1, has been associated with multiple other cancers other than pancreatic cancer. CTHCR1 is a secreted protein around 30 kDa and its function is related to tissue repair (Goonesekere et al., 2014). A prior study looked at CTHCR1's presence and role in melanoma and other human solid cancer cells. The potential biomarker was found to be expressed at the mRNA and protein levels in melanoma cancer cells

(Tang et al., 2006). Although the study did not find a significant function of CTHCR1, CTHCR1 was found at much higher levels in invasive melanoma compared to a noninvasive type which suggests CTHCR1 may play a role in migration and metastasis. The same study by Tang et al. found that the majority of 19 human solid cancers had increased expression of CTHCR1, such as cancers of the pancreas, rectum, colon, small intestine, cervix, thyroid gland, lung, ovary, and breast; however, testis, prostate, and trachea tumors were the exception and showed no significant increase (Tang et al., 2006). These results add to our finding that CTHCR1 was upregulated in the pancreatic tumor cell line compared to control. The meta-analysis study showed that CTHCR1 was greater than a 1,000 fold increase in protein expression (Goonesekere et al., 2014). While in our hands, there was only a two-fold significance of CTHCR1 in a cancerous cell line to control there was still an increase and thus suggests that CTHCR1 can still be a potential biomarker with future research.

Challenges faced in the research

Western blot analysis can be a challenging technique. The CTHCR1 and EPPK1 studies took much longer than expected. Optimizing the western blot protocol to visual bands was a long process and took many different trials and variations. There were 40 total western blots run over the span of doing research with several blots divided off into small segments meaning even more trials. For CTHCR1, the amount of isolated total protein loaded onto the gel ranged from 22 to 35 micrograms (ug) of protein with the attempt to get a stronger, visible signal. For EPPK1, total isolated protein ranged from 22 ug to 50 ug. Transfer time of the protein was also adjusted many times. A wet transfer method of transfer was first used, then the iBlot system was used; this system was much more efficient not only in the time of transfer, but the amount of protein that was transferred. The temperature conditions, dilution solution such as TBS, TBS-T, or 5% milk

in TBS, or 5% milk in TBS-T, and length of time of the primary and secondary antibody incubations were also modified. CTHCR1 ranged ratios of primary from 1:100 to 1:300 and secondary from 1:2,000 to 1:15,000. EPPK ranged ratios of primary from 1:100 to 1:500 and secondary from 1:1,500 to 1:10,000. Western blot analysis is time-consuming considering that each blot takes two to three days and then if stripping and reprobing the blot, another two days must be added. Western blot analysis takes time, persistence, and longevity to optimize the protocol.

Future Suggested Work

Several areas of future work can be expanded from this thesis. More trials of western blots looking at CTHCR1 could be obtained for a stronger statistical analysis. In this current study, there were only three replicates. Other than SW 1990 showing significant expression the protein CTHCR1, tumorigenic cell line, BxPC3, appeared to have only half the expression of the control cell line. This was not found significant at this time; however, with more trials, it may be found significant. Another way to enhance this study would be to remove the background bands. Several of the blots of CTHCR1 show excess signal of non-specific bands even after numerous runs. The multiple bands can come from excess of primary or secondary antibodies; multiple optimization experiments to adjust concentrations would need to occur. In future studies, more time could be allowed for this. More optimization experiments to find the lowest concentration of isolated protein needed to detect a band would also be constructive.

EPPK1 was very challenging to visualize. EPPK1 was one of the higher molecular weight proteins at 552 kDa along with AHNAK2 at approximately 600 kDa. Incomplete transfer of the protein from the gel to the membrane would be an area to focus on in future work. Even though 17 western blots were run for EPPK1, there was only one faint band visualized once.

Unfortunately, this visualization was never repeated. This is a very small victory, but shows there is room for more improvement with more time.

Due to time restrictions and the difficulties of visualizing CTHCR1 and EPPK1, IGHG3 and AHNAK2 were not evaluated using western blot analysis at this time. Our lab has frozen down samples of the pancreatic tumor cell lines and control cell line and stored the isolated protein samples. An antibody used to detect IGHG3 has been purchased and is ready to use. IGHG3 has a smaller molecular weight, similar to CTHCR1, and with more time, IGHG3 should be easily visualized on western blots. In contrast, I do expect the AHNAK2 will be difficult to transfer similar to EPPK1 due to its high molecular weight of approximately 600 to 630 kDa. This research will hopefully be continued in the future.

Another aspect of future work would be comparing the results of protein expression to the results from the RNA expression study performed. As mentioned earlier, this research was part of a two-part study with half looking at expression of the target gene at the RNA level through qPCR and the other half at the protein level through western blot analysis. Comparing these results from both parts of research could add supporting evidence that one of the four genes could be a potential biomarker for pancreatic cancer.

Conclusions

Pancreatic cancer is commonly discovered and diagnosed at a later stage when the cancer has spread or metastasized beyond surgical resection. Even with traditional therapies, a patient with advanced stages of PC has a very poor prognosis and life expectancy. PC is asymptomatic in early stages; finding a new, alternative way to detect pancreatic cancer early on is inevitable for better treatment options. Therefore, research has been focused on finding new biomarkers which are specific indicators of a certain disease. The current project was based on work using bioinformatics data by Goonesekere et al. (2014). The investigators suggested CTHCR1, EPPK1, AHNAK2, and IGHG3 as potential biomarkers for pancreatic cancer. The goal of this thesis was to determine if protein expression of these genes was altered in a variety of human pancreatic tumor cell lines through western blot analysis. CTHCR1 results showed a significant increase of protein expression in SW 1990, a pancreatic tumor cell line, compared to the HPDE-6 control cell line. This suggests that this human tumorigenic cell line had an increased level of CTHCR1 protein when compared to amounts found in a cell line that appeared to be normal. This data adds to the current that evidence that CTHCR1 can act as a prospective pancreatic cancer biomarker. There was also increased CTHCR1 protein expression in four other human pancreatic tumorigenic cell lines, but they were not found statistically significant. EPPK1 was another potential biomarker mentioned significantly upregulated in a previous study (Goonesekere et al., 2014); however, its large molecular weight was a challenge and caused insufficient transfer of proteins. Several adjustments were made, but unfortunately no bands indicating present proteins were visualized. More time and research will be needed to look more into EPPK1, AHNAK2, and IGHG3, and also in repeating CTHCR1. In conclusion, these new findings about CTHCR1

will add to the literature of potential biomarkers for pancreatic cancer which in the future will lead to earlier detection and much better prognosis.

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