IDENTIFICATION OF POTENTIAL BIOMARKERS IN PDAC

Rebecca Lynn Whittlesey

A thesis submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master's of Science in the Department of Genetics in the School of Medicine.

Chapel Hill
2014

Approved by:

Jen Jen Yeh

William Kim

Jeff Sekelsky

© 2014
Rebecca Lynn Whittlesey
ALL RIGHTS RESERVED

ABSTRACT

Rebecca Lynn Whittlesey: Identification of potential biomarkers in PDAC
(Under the direction of Jen Jen Yeh)

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer deaths in both men and women in the United States, with a 5-year survival rate of approximately 6%. The poor prognosis associated with PDAC emphasizes the need to improve detection and screening, therefore we aimed to identify new biomarkers associated with PDAC progression.

137 cancer associated genes identified from our microarray data were screened for gene expression using 13 PDAC cell lines and 42 samples isolated from the blood of 21 PDAC and 4 control patients.

56 genes were found to have expression in >70% of PDAC cell lines and low expression in control buffy coat samples. 16 of these genes showed significant survival differences in patients with low expression when analyzed in our microarray data. Two of these genes, *GJB3* and *MFI2*, showed a trend of higher expression in metastatic PDAC patients compared to benign or local PDAC patients.

GJB3 and *MFI2* may have clinical relevance for patient survival and be useful as metastatic biomarkers for PDAC, but improved sample isolation techniques and screening of more samples is needed to determine their significance.

TABLE OF CONTENTS

LIST OF TABLES ..... **VI**

LIST OF FIGURES ..... **VII**

CHAPTERS

I. PANCREATIC CANCER 1

INTRODUCTION 1

BIOMARKERS 3

CIRCULATING TUMOR CELLS AND NUCLEIC ACIDS 6

METHODS 9

PATIENT SAMPLES 9

REAL TIME PCR ANALYSIS 10

MICROARRAY AND STATISTICAL ANALYSIS 10

RESULTS 11

IDENTIFYING CANCER ASSOCIATED GENES 11

ANALYSIS OF QPCR DATA 12

MICROARRAY ANALYSIS 14

SURVIVAL DATA 16

QPCR OF PATIENT SAMPLES 17

DISCUSSION 19

REFERENCES 24

LIST OF TABLES

Table 1. qPCR Analysis of 137 Candidate Genes 13

Table 2. Composite Scores of Genes in Microarray Expression 15

Table 3. Genes Associated with Survival 16

Table 4. Patient Sample Data 17

LIST OF FIGURES

Figure 1. Differential Expression in PDAC samples 18

CHAPTER 1: PANCREATIC CANCER

Introduction

The pancreas is a small organ located deep within the abdomen, under the stomach, which is responsible for the production of digestive enzymes and hormones, such as insulin. Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer accounting for 90% of cases. Around 46,000 people are diagnosed with pancreatic cancer each year in the United States (Siegel, Naishadham et al. 2013). It is the 9th and 10th diagnosed cancer in men and women, respectively, yet is the 4th leading cause of cancer deaths. Survival rates are around 20% after the first year of diagnosis and reduce to 6% after 5 years (DeSantis, Lin et al. 2014). The incidence of PDAC increases with age; with a median age of 71 years at diagnosis. The median survival for patients with advanced pancreatic cancer is around 6 months (Siegel, Naishadham et al. 2013). These statistics highlight the poor prognosis associated with PDAC and emphasize the need to improve early detection, develop better treatments, and better understand disease progression.

Pancreatic cancer occurs through genetic mutations caused mostly by environmental factors including smoking, alcohol, diet, infectious agents, pollution and radiation (Anand, Kunnumakkara et al. 2008). The accumulation of mutations over time eventually leads cells to become malignant, by gaining certain characteristics including uncontrolled cell growth; resisting cell death and evading the immune response; bypassing DNA repair pathways; and other mechanisms (Hanahan and Weinberg 2011). In PDAC, the progression of cancer via the activation of the *KRAS2* oncogene, inactivation of the tumor-suppressor gene *CDKN2A* followed by tumor-suppressor genes *TP53* and *SMAD4*, confers tumor growth

(Hong, Park et al. 2011, Herreros-Villanueva, Gironella et al. 2013). Up to 90% of PDAC have mutations in the *KRAS2* oncogene, compounded by a 95% mutation rate in *CDKN2A*, resulting in increased proliferation and resistance of cell death (Hong, Park et al. 2011, Herreros-Villanueva, Gironella et al. 2013). *TP53* mutations, occurring in 50-75% of tumors, allow cells to bypass DNA repair pathways, causing genomic instability (Hong, Park et al. 2011, Herreros-Villanueva, Gironella et al. 2013). Loss of *SMAD4* in 50% of tumors results in abnormal signaling of the transforming growth factor beta cell surface receptor (Hong, Park et al. 2011, Herreros-Villanueva, Gironella et al. 2013). Additional mutations have also been implicated, including, *MLL3, TGFBR2, ARID1A* and *SF3B1* (Biankin, Waddell et al. 2012). An accumulation of these mutations leads to a heterogeneous population of tumor cells, making it difficult to understand the biological changes of PDAC as well as poor response to treatment regimens.

Only 5-10% of PDAC cases are associated with a family history of this disease, making it problematic to study inherited types of this cancer (Anand, Kunnumakkara et al. 2008). Smoking has been shown to initiate these genetic changes in up to 30% of cases, but other factors including alcohol and diet do not show a strong correlation to an increased risk of developing pancreatic cancer (Blackford, Parmigiani et al. 2009, Bosetti, Lucenteforte et al. 2012, Chaudhry, Hall et al. 2013, Pericleous, Rossi et al. 2014). New research investigating the onset of diabetes and diagnosis of pancreatic cancer has shown only a mild correlation and the highest risk of being diagnosed with pancreatic cancer is within one year of developing diabetes (La Torre, Sferrazza et al. 2014). The inability to predict patients susceptible to PDAC, without a family history of disease, makes early screening for disease difficult.

Early detection is challenging because initial symptoms are non-specific, including abdominal discomfort, nausea, and loss of appetite. At the time of detection, most patients present with either

locally advanced or metastatic disease, in which treatment options are limited. Unlike many other malignant diseases, the metastatic spread of PDAC is thought to begin when the primary tumor is very small and is hard to detect through CT imaging scans or abdominal ultrasounds (Yachida and Iacobuzio-Donahue 2013). Early detection is also limited because of the lack of a disease specific marker. Currently, the best tumor marker for pancreatic cancer is carbohydrate antigen 19-9 (CA19-9) which provides suspicion of cancer or disease, but is not sensitive or specific enough for diagnosis (Cen, Ni et al. 2012). CA19-9 has been detected in chronic pancreatitis, and may be normally expressed in some patients with early stage PDAC, as well as other cancers (Goggins 2005, Duffy, Sturgeon et al. 2010). PDAC commonly metastasizes to multiple sites throughout the body, with a preference for some sites including the liver, lungs, peritoneum, and adrenal glands. In addition, this disease progression is associated with significant morbidity and is highly resistant to the current treatment therapies. The lack of diagnostic tools makes this disease very deadly and the need for more research to improve detection will benefit patient outcomes.

Biomarkers

Cancer detection is often performed using a blood test to look for known biological cancer markers. A biomarker is a characteristic that can be measured to give information about the state of a cell, biological or pathogenic processes, or a pharmacological response. Biomarkers can include specific cell types, genes or gene products, enzymes, or hormones. Information can be obtained from body fluids such as blood, serum, plasma, or urine as well as body tissues. There are three divisions of biomarkers: predictive, diagnostic, and prognostic. Some biomarkers may span all three types and can provide a wealth of information, helping us understand the biology of cancer and aiding in the development of better medical treatments.

In cancer, biomarkers are used to detect disease and monitor the progression and response to treatment. Predictive biomarkers help estimate the results of a therapeutic intervention in an individual patient and can categorize subpopulations of patients who are most likely to respond to certain therapies (Jazieh, Foote et al. 2014). Predictive biomarkers can also be targets for therapy. Measuring biomarkers, such as genes or proteins, in patients with a family history of pancreatic cancer helps physicians determine if a patient is at an increased risk of developing this disease. For example, patients positively screened for *BRCA2* mutations have a much higher chance of developing breast cancer (Harris, Fritsche et al. 2007). *BRCA1* and *BRCA2* mutational screens have been shown to alter risk assignment in patients with a family history of PDAC, therefore improving screening recommendations in patients with mutations (Lucas, Frado et al. 2014). Other genetic predictive biomarkers include mutations of *KRAS, TP53, EGFR*, and *ERBB2* for colorectal, esophageal, liver, and pancreatic cancer (Verma and Manne 2006). Continued research into the development of pancreatic cancer as well as currently identified predictive biomarkers in other cancers may lead to the discovery of a useful predictive tool for pancreatic cancer.

Diagnostic biomarkers can be measured to positively confirm the presence of a disease or cancer. A diagnostic biomarker must have a low incidence of false positives and negatives, be detectable in all patients, and be specific and selective for a disease. The FDA highly regulates tests used for diagnosis because of the potential harm that may be caused if the test is not accurate. Currently there is not an identifiable biomarker that meets these criteria to confirm a diagnosis of pancreatic cancer. Gold *et al.* has previously shown the high specificity for monoclonal antibody PAM4 to bind mucins specific to PDAC (Gold, Newsome et al. 2013). In a combination study of PAM4 and CA19-9, they showed the sensitivity level of CA19-9 in detecting stage I PDAC was 58% and 64% using PAM4 (Gold, Gaedcke et al. 2013). The specificity for detection using PAM4 was 96% suggesting that a combination test of CA19-9 and PAM4 may improve early detection of PDAC (Gold, Gaedcke et al. 2013).

Combinations of other biomarkers such as microRNAs with CA19-9 may have the potential to be useful as a diagnostic or prognostic tool for PDAC. One group sought to identify potential diagnostic biomarkers in patients at an increased risk for familial pancreatic cancer. In human patient samples, serum levels of miR- 196a and miR-196b were significantly higher in patients with sporadic and familial pancreatic cancer than in patients with chronic pancreatitis, early cancer and healthy controls (Slater, Strauch et al. 2014). Measurement of elevated levels of these two miRs may be used as a diagnostic tool in patients with an increased risk of pancreatic cancer. Currently, this study and many others have suggested different biomarkers as indicators or possible diagnostic value, but further research must be conducted to validate their clinical use. Utilizing a single biomarker or biomarker panel for detection of cancer through non-invasive methods is the most optimal in preventing unnecessary procedures for patients, but currently, for some cancers, is not an option.

Biomarkers can be measured longitudinally during the course of disease. Levels of biomarkers may correlate with progression, survival and drug response. Currently CA 19-9 is measured in PDAC patients to determine tumor burden and monitor disease progression (Ballehaninna and Chamberlain 2012). Elevated levels of CA 19-9 are associated with metastatic spread, adverse patient outcomes, and recurrence of this disease. Therefore CA19-9 is an effective tool in monitoring a patient's disease. Recently, SMAD4 has become a cancer marker of interest because it is lost in about 50% of PDAC (Yachida, White et al. 2012). New evidence suggests that SMAD4 downregulation is not only important for the development of PDAC, but also for the progression of the cancer. The loss of SMAD4 results in cancer cells that have an increased tendency for invasion and metastasis, leading to a worse prognosis (Jazieh, Foote et al. 2014). Numerous studies have suggested that loss of SMAD4 correlates with a higher incidence of metastasis and poor overall and disease free survival (Iacobuzio-Donahue, Fu et al. 2009, Singh, Srinivasan et al. 2012, Oshima, Okano et al. 2013). A combination study into the

effectiveness of monitoring both CA19-9 and SMAD4 levels in patients may better represent how patients are responding to treatment and their prognosis.

Biomarker research is receiving a lot of attention because of the potential they hold for personalized medicine and targeted therapy. Individualized medicine is tailored treatment for a patient based on specific molecular and biological traits of their disease. As we learn more about cancer cells and their surrounding environment, the number of subtypes of each cancer increases. A patient's cancer may be subtyped according to a biomarker that is present or absent, increased or decreased. Personalized treatment plans can then be developed to provide treatment that is appropriate and effective. Therefore, identifying and studying biomarkers will contribute to tailoring patient therapies.

Circulating Tumor Cells and Nucleic Acids

Metastatic disease is the main cause of death in cancer patients. Circulating tumor cells are tumor cells that are released or dissociate from a primary or metastatic tumor. This process is thought to occur through the epithelial-mesenchymal transition (EMT) in which epithelial cells are transcriptionally reprogrammed to lose their polarity and adhesion, and gain migratory and invasive properties. EMT-inducing transcription factors dynamically modulate cell adhesion and promote metastasis by regulating the expression of the cadherin family of proteins. E-cadherin expression is repressed with a switch to N-cadherin expression. This transition to mesenchymal gene expression allows the cell to migrate and enter into the blood stream (Yu, Bardia et al. 2013, Liu, Zhang et al. 2014). We know there are dynamic genetic and biological changes during the progression of cancer, and the expression of many genes vary as cells enter a mesenchymal state (EMT) and revert back to an epithelial state (MET)in metastatic disease (Liu, Zhang et al. 2014). CTCs can travel through the blood stream and exit; if the environment is suitable, these cells can undergo mesenchymal to epithelial transition and seed themselves in different organs (Liu, Zhang et al. 2014). Cell growth and proliferation then leads to

metastatic growth. CTCs have been termed a 'liquid biopsy' because they provide a window of insight into the genetics and biology of a tumor. Rhim *et al*. tracked cells through cancer progression using a pancreas specific Kras and p53 mutant mouse line (Rhim, Mirek et al. 2012). They found that circulating PDAC cells are mesenchymal, invasive and exhibit stem cell like features. Furthermore, their mouse models suggest that PDAC CTCs are detectable before primary tumor formation and may actually metastasize to distant organs very early on (Rhim, Mirek et al. 2012). More research to understand the dynamic properties that CTCs possess may help in understanding cancer progression.

The number of CTCs in blood can be as low as 1 cell per $1x10^6$ mononuclear cells, and their genetic heterogeneity makes detection extremely difficult (Harouaka, Kang et al. 2014). The development of new CTC capture and enumeration methods are published weekly and rely on a number of selection methods. Initially, researchers were only interested in counting these cells in the blood, which may be useful as a biomarker for disease. More recently however, we are learning that analyzing the biology and genetics of these cells may tell us much more about a patient's disease. To isolate these rare cells from blood, a system has to be specific and selective enough to disregard blood cells, yet capture as many CTCs as possible. There is not a single marker that will either detect all CTCs or all blood cells. Isolation methods often utilize antibodies against epithelial markers or blood markers. Other methods take advantage of the size of CTCs, with a range of 12-25 microns, for isolation (Zheng, Lin et al. 2007). Physical methods of isolation are also utilized to enrich CTC populations. A simple and fairly inexpensive method of isolation is by cell density using gradient centrifugation. This method isolates all the mononuclear cells from blood and downstream enrichment methods for CTCs are needed. Ellis *et al.* performed a negative selection method on cells isolated using density gradient centrifugation of blood from prostate cancer patients. Using both anti-CD45 and anti-CD61 magnetic beads to remove blood cells, they were able to identify prostate-specific antigen (PSA) expressing cells using qPCR (Ellis, Pfitzenmaier et al. 2003).

In addition to CTCs, circulating nucleic acids (cNA) have recently been proposed as a marker of disease. This includes DNA, mRNA and microRNAs that can be measured in the serum or plasma of patients. Cell free DNA (cfDNA) or circulating tumor DNA (ctDNA) levels have been shown to be increased in cancer patients, even in the absence of CTCs (Kopreski, Benko et al. 1999, Shinozaki, O'Day et al. 2007). BRAF mutation V600E, which is present in over 70% of metastatic melanomas, can be detected in cfDNA and has been shown to be useful in monitoring patients with melanoma who are receiving therapy (Shinozaki, O'Day et al. 2007). The physiological events that lead to the increase of cNA during cancer development and progression are still not well understood. It is believed that macrophages may release DNA into the tissue microenvironment when engulfing apoptotic or necrotic cells(Schwarzenbach, Hoon et al. 2011). Some evidence suggests that on average, the size of this DNA varies between small fragments of 70 to 200 base pairs and large fragments of approximately 21 kilobases (Jahr, Hentze et al. 2001). Analysis of ctDNA may allow for the detection of tumor related genetic and epigenetic alterations that are relevant to cancer development and progression.

In general, the studies of ctDNA as cancer biomarkers focus on monitoring the presence of promoter hypermethylation, aberrant tumor DNA mutation, microsatellite alterations, and mitochondrial DNA in blood circulation (Stroun, Maurice et al. 2000). Molecular characteristics of cancer including genetic and epigenetic alterations have been found in PDAC samples from tumor tissue, pancreatic fluid, and biopsies (Stroun, Maurice et al. 2000). These molecular characteristics are now being investigated in ctDNA from cancer patients. Dabritz *et al.* detected *KRAS* mutations in the plasma DNA of 36% of patients with PDAC, compared with no patients with pancreatitis. By combining *KRAS* mutations with CA 19–9 levels they were able to diagnose PDAC with a sensitivity of 91% (Dabritz, Preston et al. 2009).

In summary both CTCs and ctDNA carry tumor related genetic and epigenetic alterations that are relevant to cancer development, progression and resistance to therapy. Monitoring these alterations in a patient through minimally invasive blood tests for circulating tumor cells and cell-free nucleic acids can allow for repetitive real-time monitoring and will, therefore, contribute to the clinical utility in the determination of prognosis and treatment. Utilizing new isolation methods is promising for the study of CTCs and cNAs, but many hurdles still remain. The biggest obstacle is determining which biomarkers to use for each type of cancer that will react with as many CTCs or as much cNA as possible, while maintaining specificity and sample purity. Most of these tools and biomarkers need further validation to become acceptable forms of isolation and detection. We aimed to identify genes associated with metastatic disease that have potential clinical use as a prognostic biomarker. Using PDAC patient microarray data, we generated a list of genes that we tested on both cancer cell lines and PDAC patient samples to identify *GJB3* and *MFI2* as potential biomarkers for metastatic disease.

Methods

Patient Samples

Patient blood samples were obtained after informed consent. The white blood cell buffy coat was isolated via two methods of density gradient centrifugation. Blood was either layered on top of 5 mls of Ficoll at density 1.077 or a double gradient of Percoll (d= 1.092) and Ficoll (d=1.077) and centrifuged at room temperature for 20 min at 450g. The white blood cells formed a buffy coat band in the Ficoll gradient or separated into monocyte and lymphocyte layers in the double gradient and were collected separately. The cells were washed with PBS to remove residual density gradient media, frozen in RPMI-1640 with 10% DMSO and stored in liquid nitrogen. Some buffy coat samples had further cell isolation methods performed using the Miltenyi CD45 microbeads and magnetic positive selection

columns. Buffy coat samples from 6 metastatic PDAC patients were cultured on collagen matrix (Invitrogen) for one week, then washed, trypisinzed, and frozen in liquid nitrogen.

PDAC cell line pellets previously frozen were thawed and RNA isolation of cell lines and patient samples were performed using either the Qiagen MiniRNA kit or Zymo QuickRNA miniPrep kit. RNA concentrations were quantified using the Nanodrop. cDNA was created using the Applied Biosystems cDNA synthesis kit.

Real Time PCR Analysis

RPLP0, ACTB, and GAPDH genes were selected as reference genes for their consistent expression with low standard deviation (less than 0.05) in the cells lines used in this screen. 13 cancer cell lines (HS766T, BxPC3, PC3, Aspc1, Capan2, MiaPaca2, CFPAC-1, HPAC, HPAF-II, Panc1, HuPT3, RT4, MCF7), the immortalized HPNE pancreatic cell line, the THP1 blood monocyte line and a buffy coat pool from three benign control patients were screened. Genes were analyzed in triplicate. 0.667ng/ul cDNA was used for cell lines and 3.33ng/ul for patient samples. Invitrogen Taqman primers optimized for each gene and Taqman Universal Master Mix were used. Samples were added to 384 well plates and amplified using the Applied Biosystems Viia6 or Viia7. Triplicate values were averaged to define a cycle threshold (CT)value for each gene in each cell line. The geometric mean of CT values for ACTB, RPLP0, and GAPDH served as a normalization value. The ΔCT was calculated as: ΔCT = (CT – geometric mean) for each gene within each cell line. Significance was evaluated using an unpaired t-test.

Microarray and Statistical Analysis

Microarray data used in this study is publically available (Chaika, Yu et al. 2012). Survival and expression analysis was performed using the statistical software Prism Graphpad. Overall survival (OS) was analyzed using the Kaplan-Meier product-limit method and the significance of our variables was

measured by the log-rank test. The Gehan-Breslow-Wilcoxon Test was used to analyze associations between two variables.

Results

Identifying Cancer Associated Genes

To identify genes that play a contributing role in the development and progression of cancer, we identified genes differentially expressed in metastatic and primary tissues from 5 patients with matched tissues. Using our previously published microarray data set of PDAC patients, we identified a list of genes with greater than 2 fold higher expression in metastatic and primary tumors and a list of genes highly expressed in metastatic tumors, both compared to matched normal pancreas tissue. Metastatic samples were defined as having >30% tumor content in tissues. We then selected genes located at the plasma membrane that were differentially expressed in more than three metastatic sites. This resulted in 137 genes that were overexpressed in primary and metastatic tissues compared to normal tissue. We hypothesize that these genes are playing a role in tumorigenesis and metastasis and have the potential to be screened as biomarkers for PDAC.

Analysis of qPCR data

We screened our list of genes in cancer cell lines and control patient samples to analyze these genes as potential biomarkers. Low expression of each gene was defined as ΔCT ≥ 32 – geomean and high expression as ΔCT ≤ 32 – geomean. Each gene was assigned scores ranging from 0-3 for low or high expression levels in the buffy coat(BC) pool, cancer cell lines, and HPNE cell line; and a composite score was generated [Table 1A]. A score of 0 was assigned to genes with a CT value greater than 32 in the WBC pool. A score of 1 was assigned to genes with a CT between 30 and 32 in the WBC pool. A score of 2 was given to genes with a CT less than 30 in the WBC pool. Gene expression status in cancer cell lines was scored according to the percentage of high expression in cell lines. Genes with high expression in greater than 85% of cell lines were assigned a 0, high expression in greater than 70% of cell lines were assigned a 1, and high expression in less than 60% of cell lines were assigned a 2. HPNE was used as a surrogate for normal pancreas expression. A score of 1 was assigned to genes with low expression and 0 was assigned to genes with high expression in HPNE [Table 1C]. 80 genes with a score of 0-3 were kept for further analysis and 57 genes with a score greater than 3 were discarded from future analysis [Table 1B-D].

A:

	BC Pool	Cell Line Expression	HPNE Expression	
Score	raw CT	high expression= ΔCT ≤ 32 -geomean	high expression= ΔCT ≤ 32 -geomean	
0	>32	high ≥ 85%	high	**Composite Score Tabulated**
1	30-32	high ≥ 70%	low	
2	<30	high ≤ 60%		

B:

Composite Score	Gene
0 - 3	kept for further analysis
> 3	discarded from further analysis

C:

Gene	BC CT	Cell Line	HPNE	Score
MFI2	0	0	1	1
GJB3	0	1	0	1

D:

Composite Scores of Genes in Cell Line qPCR Analysis			
0-1	1-2	1-2	3
HPNE - = 1	HPNE - = 1	HPNE - = 1	HPNE - =
HPNE + = 0	HPNE + = 0	HPNE + = 0	HPNE + =
BC Pool = 0	BC Pool = 0	BC Pool = 1	BC Pool =
Cell Line = 0	Cell Line = 1	Cell Line = 0	Cell Line =
AMIGO2	ANO1	EPHB3	ICOSLG
CDH3	CEACAM1	EPHB4	
CEL5R1	COL17A1	F2RL1	
CLDN7	CPLX1	SPRED1	
EPCAM	GJB5	CAMK2N1	
FERMT1	GJC1	CNNM4	
IGSF9	PANX2	IRAK2	
ITGB4	PMEPA1	ITGA3	
KCNK1	TJP3	NT5E	
MAL2	UPK2	RRAS2	
MALL (BENE)	GJB3	TNFRSF21	
MFI2	HAS3	TPBG	
MST1R	MUC1	TSPAN5	
PHLDA3	OXTR	F7	
PPAP2C	SLC44A4	IL1RAP	
RHBDL2	SLC4A11	ATP1B3	
RHOV		SLC16A1	
SLC52A3			
CACNB3			
CDH1			
CGN			
CLDN12			
CLDN4			
DOK4			
DSG2			
EFNA4			
EFNB2			
EPHA2			
F3			
GPRC5A			
GRB7			
ITGA2			
LRP5			
MET			
MICA			
OSMR			
PERP			
PPP1R16A			
SCNN1A			
TM4SF1			
TSPAN15			
DCBLD2			
GPR126			
IQGAP3			
SPRY4			
MICB			

Table 1: qPCR Analysis of 137 Candidate Genes: Each gene tested from our list was assigned a score for three different criteria of expression: buffy coat pool, cancer cell lines, and HPNE (A). Composite scores were generated to exclude genes with expression in the buffy coat pool (B). *MFI2* and *GJB3* scores (C). Composite scores, 0-3, for the 80 genes included for further analysis (D).

Microarray Analysis

In order to further narrow down my list of potential biomarkers, I looked at the expression data in the PDAC patient microarray data set our lab has generated. This allowed us to confirm in a larger dataset what genes were overexpressed in primary and metastatic tumors compared to normal pancreas. An issue with the initial screen to create our list was that it had a very limited sample size of matched samples, including 14 normal pancreas, 5 primary PDAC and 28 metastatic PDAC tissues. An additional 33 normal pancreas, 161 primary and 28 metastatic samples were used to look at expression data. I was able to determine significant differences in the level of expression between the normal pancreas tissue and primary PDAC tumors, metastatic tumors, and cell lines. A separate scoring system was developed to prioritize candidate genes. A score of 0 was given to each gene with significance ($p < 0.05$) in the three comparisons: normal pancreas versus primary PDAC, normal pancreas versus metastatic tumors, and normal pancreas versus cancer cell lines [Table 2A, C]. A score of 1 was given to genes without significance for each comparison. 24 genes had a composite score greater than 2, and were excluded from further analysis [Table 2B]. 56 Genes had a composite score of 0-2 [Table 2D].

A:

Significance in Microarray data set: Normal Pancreas Tissue vs Primary PDAC, Metastatic Tissue Cell lines (p≤ 0.05)				
Score	Normal vs Primary & Met	Normal vs Met only	Normal vs Cell Lines	
0	<	<	<	**Composite Score Tabulated**
1	not significant	not significant	not significant	

B:

Composite Score	Gene
0 - 2	kept for further analysis
3	discarded for further analysis

C:

Gene	N vs P & M	N vs M	N vs Cell Lines	Composite Score
GJB3	0	1	0	1
MFI2	0	1	0	1

D:

Total Scores for Significance in Microarray data set: Normal Pancreas Tissue vs Primary PDAC, Metastatic Tissues, Cell lines (p≤ 0.05)			
1	1	2	2
HPNE+	HPNE+	HPNE+	HPNE+
HPNE-	HPNE-	HPNE-	HPNE-
Normal<	Normal<	Normal<	Normal<
Primary & Met	Met	Primary & Met	Met
Cell Lines	Cell Lines		
AMIGO2	CLDN12	IGSF9	SCNN1A
ANO1	SLC16A1	CEACAM1	CACNB3
CDH3	OSMR	KCNK1	GRB7
COL17A1	GPR126	PHLDA3	IRAK2
FERMT1	IQGAP3	CGN	LRP5
GJB5		DOK4	MICA
ITGB4		EFNA4	MICB
MAL2		MUC1	OXTR
MALL (BENE)		TPBG	SPRY4
MST1R		TSPAN15	
RHBDL2		CAMK2N1	
SLC52A3			
CELSR1			
MFI2			
DCBLD2			
TNFRSF21			
CLDN4			
CNNM4			
EFNB2			
EPHA2			
GJB3			
GPCR5A			
HAS3			
IL1RAP			
ITGA2			
ITGA3			
MET			
NT5E			
PERP			
SLC4A11			
TSPAN5			

Table 2: Composite Scores of Genes in Microarray Expression: Each gene from the previous analysis was scored according to significant overexpression in primary and metastatic tumors, metastatic tumors and cell lines compared to normal pancreas tissue (A). Composite scores were generated to exclude genes without significant expression (B). *MFI2* and *GJB3* scores (C). Composite scores, 0-2, of the 56 genes (D).

Survival Data

Finding a biomarker that also shows an association with outcome may also be prognostic, therefore we looking at survival data of 136 patients with primary PDAC tumors. For each of the 56 genes, patient samples were systematically split into two groups using a sliding cutoff of gene expression. The partition which gave the most significant survival difference via log rank test was used to determine median overall survival for the low and high expression groups. 16 of these genes showed associations with survival [Table 3]. Thus these 16 genes may have promise as cancer biomarkers.

Median Overall Survival in Primary PDAC samples by Gene			
Gene	**Expression**	**Median Survival (months)**	**P Value**
CACNB3	low	21 (0-54)	0.0005
	high	13 (1-59)	
MET	low	33 (1-53)	0.0014
	high	15 (0-59)	
FERMT1	low	22 (1-59)	0.0016
	high	13 (0-43)	
IRAK2	low	19 (1-59)	0.004
	high	11 (0-42)	
OSMR	low	20 (1-59)	0.005
	high	10 (0-52)	
HAS3	low	21 (1-53)	0.011
	high	14 (0-59)	
KCNK1	low	19 (0-59)	0.011
	high	13 (1-42)	
GPCR5A	low	22 (1-55)	0.015
	high	14 (0-59)	
MAL2	low	21 (0-55)	0.015
	high	15 (1-59)	
NT5E	low	19 (0-59)	0.016
	high	8 (4-30)	
PERP	low	21 (1-59)	0.017
	high	15 (0-55)	
GPR126	low	21 (0-54)	0.024
	high	13 (1-59)	
CGN	low	24 (1-54)	0.035
	high	15 (0-59)	
MFI2	low	19 (0-55)	0.038
	high	11 (1-59)	
GJB3	low	24 (2-53)	0.042
	high	17 (0-59)	
EFNA4	low	24 (1-55)	0.044
	high	17 (1-25)	

Table 3: Genes Associated with Survival. 16 genes showed significant ($p < 0.05$) survival differences in patients with low expression. Median survival in months is shown between the high and low gene expression groups.

qPCR of Patient Samples

To further investigate our 56 genes as potential biomarkers, 42 samples from 25 patients were screened. This included 4 patients with benign non-pancreatic diseases, 5 with local PDAC, 5 with advanced PDAC, and 10 with metastatic PDAC [Table 4]. The sample types included in this analysis were cells isolated from the buffy coat, monocyte and lymphocyte layers, CD45 selection from buffy coat and cultured samples. EpCAM (epithelial cell adhesion molecule) was used as a surrogate marker for epithelial cell identification.

Patient Status	Patient	Date Collected	Sample Type	Geomean	EpCAM Expression (ΔCT)
Control	75	12/19/2012	buffy coat	25.8	3.9
	106	4/23/2014	buffy coat	24.0	11.4
	108	4/23/2014	buffy coat	24.2	9.9
	111	4/23/2014	buffy coat	24.4	10.9
Local PDAC	45	12/7/2012	monocyte	23.6	11.6
	45	12/13/2012	monocyte	24.5	4.6
	45	4/16/2013	CD45+	22.3	11.2
	45	4/16/2013	lymphocyte	24.2	5.3
	62	11/9/2012	monocyte	23.9	14.2
	62	3/29/2013	CD45+	26.1	10.3
	66	12/7/2012	monocyte	24.8	9.9
	66	12/14/2012	monocyte	24.1	5.7
	74	12/14/2012	monocyte	25.2	10.6
	74	12/14/2012	monocyte	24.6	7.7
	86	3/27/2013	CD45+	25.4	10.0
Advanced PDAC	67	12/7/2012	monocyte	23.3	13.4
	67	3/22/2013	lymphocyte	26.9	10.5
	87	3/28/2013	CD45+	26.9	7.7
	87	3/28/2013	lymphocyte	25.7	13.8
	73	4/11/2013	CD45+	22.5	8.5
	73	4/11/2013	lymphocyte	27.2	5.1
	90	4/17/2013	CD45+	23.4	14.2
	90	4/17/2013	lymphocyte	23.6	8.1
	101	4/26/2013	buffy coat	24.3	8.2
Metastatic PDAC	20	7/6/2012	culture	24.6	3.5
	26	11/29/2012	culture	24.6	9.4
	26	2/28/2013	lymphocyte	28.4	4.3
	41	3/1/2013	CD45+	23.2	9.3
	47	9/28/2012	culture	22.2	7.8
	48	11/20/2012	culture	23.3	7.2
	48	11/30/2012	culture	22.5	9.4
	48	12/14/2012	culture	22.6	8.0
	48	12/14/2012	monocyte	25.8	11.1
	48	12/27/2012	culture	23.6	7.7
	49	11/20/2012	culture	24.3	5.9
	68	12/7/2012	monocyte	23.5	9.8
	68	2/8/2013	culture	25.0	4.0
	72	12/20/2012	monocyte	25.2	3.6
	95	4/18/2013	CD45+	22.8	9.1
	95	4/18/2013	lymphocyte	26.2	3.6
	138	7/2/2013	lymphocyte	28.2	0.3
	138	7/2/2013	monocyte	23.5	3.8

Table 4: Patient Sample Data. Samples collected are stratified by patient status, date collected and sample type. EpCAM expression and geomeans of reference genes are included for each sample analyzed.

We identified two genes, *GJB3* and *MFI2,* with significant differences in median overall survival for patients when split into low and high expression groups [Figure 1]. These genes showed trends toward higher expression in metastatic patient samples compared to benign and local PDAC, suggesting that they may be potential biomarkers of metastasis.

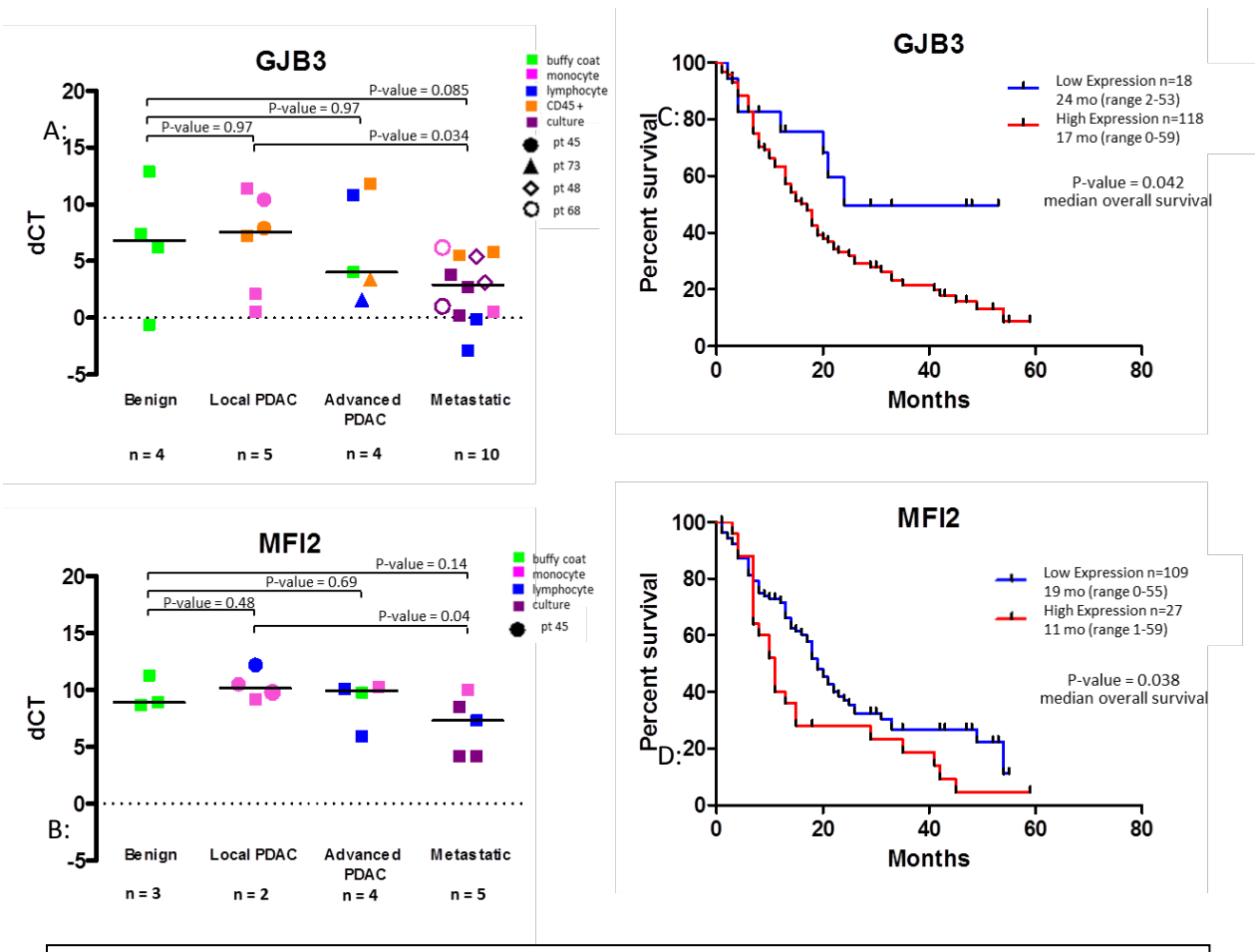

Figure 1: Differential Expression in PDAC samples. *GJB3* and *MFI2* qPCR expression data by patient status and sample type shows higher expression in metastatic compared to benign and local PDAC groups (A-B). Multiple samples from the same patient are noted in the legend. Kaplan-Meier survival plots of *GJB3* and *MFI2* show significant differences in survival (C-D).

Discussion

Much research is currently being conducted to identify genes involved in cancer initiation and progression in order to identify how these genes can be targeted for therapy. We sought to identify possible biomarkers specific to PDAC that could be used as cancer screening tools. Discovery of biomarkers indicative of metastatic disease could improve our understanding of PDAC and potentially be used to improve patient outcomes. In our screen, a large number of genes were shown to have high expression in cancer cell lines and some genes showed differences in expression in our PDAC patient samples. However, this does not guarantee the identification of a biomarker. Screening pancreatic cancer patient samples produced some promising candidates, but further evaluation using more samples will be needed. The information we gathered from this screen has provided us a starting point for identifying new biomarkers for PDAC.

The patient samples used to screen our list of candidate biomarkers presented some issues. Our results were limited by low RNA concentrations in some of our samples, allowing us to screen a fixed number of genes. Additionally, it is unclear whether RNA was derived from CTCs or cell free RNA. We attempted to use samples with high EpCAM expression, as this may be indicative of CTCs, but expression was variable within each patient status group. One benign control, patient 75, showed expression in EpCAM as well as 15 of the 56 genes screened. Contamination in this sample could be one possible reason as to why a non-cancerous patient showed expression in EpCAM and 15 cancer associated genes. This sample may have affected significant differences in expression levels between patient status groups. Increasing the number of control samples will reduce standard deviations and provide a more appropriate measure of expression. Furthermore, analyzing and understanding EpCAM expression in future benign control samples may also help us determine if it is an appropriate control for future studies and useful for identifying CTCs in PDAC patients. Another explanation for the variability

observed in benign control samples is the possibility that we isolated cell free RNA as opposed to RNA from CTCs. Cell free nucleic acids, including cell free RNA (cfRNA), have recently been found to exist in the bloodstream, making it unclear whether our isolated RNA was derived CTCs or cfRNA (Schwarzenbach, Hoon et al. 2011, Pucciarelli, Rampazzo et al. 2012). Determining the source of RNA in future analyses will help us better understand aberrant results such as this. Expression of reference genes in patient samples were also more variable than we observed in cell lines. Because some patient samples had higher expression of reference genes, we only used patient samples with a CT geometric mean less than 29 for analysis. Controlling these variables in future analysis and including more samples from each patient status group and benign controls may produce more robust results

Another confounding factor in interpreting our results is that some patients had multiple samples isolated on the same day or different days. An additional variable affecting our results is the fraction of the blood sample used for gene expression analysis. CD45+ and lymphocyte fractions were isolated from the same tube of blood from patient 45, but EpCAM expression was much higher in the lymphocyte fraction. EpCAM expression in patients 87, 73, and 90 was also different between CD45+ and lymphocyte fractions. This may be due to our method of isolation, which uses a double gradient that separates the buffy coat by cell density. It is possible that CTCs were isolated with the lymphocyte or monocyte fractions, thereby altering overall EpCAM expression in these samples. We also observed variability within the same fraction over time. Two monocyte samples from patient 45 that were collected approximately one week apart had differential EpCAM expression. Despite controlling for run to run variability, an additional 4 genes (*CLDN12, TPBG, TNFRSF21, TSPAN5*) also had variable expression between these two samples. We observed similar differences in EpCAM gene expression using monocyte layers isolated from the same original tube of blood from patient 74. The cause for these differences between samples could be attributed to sample storage conditions in liquid nitrogen. It has been shown that expression profiles from frozen buffy coat samples differs from samples not frozen,

and the length of time in storage affects RNA stability (Debey-Pascher, Hofmann et al. 2011). Future isolation of patient samples should utilize a method proven to provide better RNA stability and quality. Another reason we have seen these differences may be attributed to a change in potential CTCs or cfRNAs in patient blood samples. Enumeration of CTCs is currently utilized to monitor patients and CTC counts have been shown to change through the course of cancer progression and treatment. It is possible that we have captured this in our samples, but we cannot test this hypothesis. Further studies should include determining how many CTCs are captured through isolation, if cfRNAs are present, and using proper storage techniques.

Analysis of patient blood samples has allowed us to identify two possible biomarkers for metastatic PDAC. Both *GJB3* and *MFI2* showed significant differences in survival between low and high gene expression patient groups. *GJB3*, also known as connexin31, showed a trend toward higher expression in the metastatic patient group compared to benign and localized PDAC groups. Although we saw this trend in *GJB3*, patient 75 benign control showed strong expression of this gene, making our results somewhat difficult to interpret. *GJB3* gene expression analysis of additional benign controls will help us determine if this gene was abnormally expressed in patient 75 alone or consistently has variable expression across benign samples. We also observed no significant differences in expression of *GJB3* among patients within the benign, localized and advanced PDAC status groups, indicating that *GJB3* would not be a useful biomarker for early or advanced PDAC. We also analyzed *GJB3* expression in multiple samples from the same patients, which may have biased our results. It is possible that expression of *GJB3* in our samples may be unrelated to cancer, since there is no known cancer function of *GJB3* (Ikeya, Urano et al. 2013). However, greatly reduced or absent expression of other connexins has been found in various cancers, such as lung cancer, suggesting that these connexins, like *GJB3*, play important roles as tumor suppressors by maintaining cell differentiation and preventing transformation (Cronier, Crespin et al. 2009, Naus and Laird 2010). The re-expression of connexins in both non-

metastatic and metastatic tumor cell lines decreases cell proliferation and tumorigenesis, and promotes favorable mesenchymal to epithelial transitions (McLachlan, Shao et al. 2006, Liu, Zhang et al. 2014). Another connexin, *GJB5*, was identified in our screen but did not show significant differences between patient groups. In one study, overexpression of *GJB5* increased the expression of epithelial markers, such as cytokeratin 18, and decreased expression of mesenchymal markers, such as vimentin, indicating *GJB5* mediated a shift from a mesenchymal towards an epithelial phenotype (Zhang, Chen et al. 2012). Conversely, higher expression of connexin26 was associated with poor differentiation and venous invasion, as well as shorter disease free survival and lung metastasis free survival in colorectal cancer patients (Ezumi, Yamamoto et al. 2008). The association of cancer and *GJB3* needs further investigation. Our preliminary data suggests a connection with metastatic disease limited to the pancreas and analysis of other cancers may provide more support for the role of *GJB3* in cancer. Our data indicates that higher expression of *GJB3* may indicate worse prognosis, shorter survival, and advanced disease. We also note that expression of *GJB3* in a control sample may indicate other functions which should be explored. Current cancer research on connexins presents conflicting ideas. Some connexins are correlated with metastasis, worse survival, increased cell proliferation, motility and invasiveness. Yet closer analysis has revealed that connexins promote mesenchymal to epithelial transition, therefore high expression of connexins at later stages of cancer confer metastasis and in early stage cancer, tumor suppression. Our data supports the role of *GJB3* in metastatic disease, but further investigation into the role of connexins in tumor progression is needed.

Also identified in our screen was *MFI2*, a cell-surface glycoprotein. Overexpression of *MFI2* has been observed in metastatic melanoma tissue and is thought to play a role in angiogenesis specifically during metastasis (Sala, Jefferies et al. 2002). However, the specific effects of *MFI*2 in angiogenesis are still under investigation (Neitzel, Neitzel et al. 1999). *MFI2* has also been shown to be involved in migration and cell proliferation (Dunn, Sekyere et al. 2006, Suryo Rahmanto, Dunn et al. 2007). Our data

suggests that high expression of *MFI2* indicates worse prognosis, shorter survival, and advanced disease. High expression of *MFI2* was seen in all pancreatic cancer cell lines, which supports the hypothesis that *MFI2* promotes tumorigenesis. We found significantly improved survival in primary PDAC tumors with low expression of *MFI2*. As previously discussed, multiple samples from the same patient as well as the limited number of samples from different patients may have introduced bias of *MFI2* expression. Analyzing more samples in all patient groups will provide stronger evidence for differential *MFI2* gene expression across different stages of disease. Current research on *MFI2* is aiming to identify the mechanisms by which *MFI2* may promote proliferation, migration, and angiogenesis. Further investigation into the role of *MFI2* in tumorigenesis will provide insight into its potential use as a biomarker in PDAC. It is clear that *MFI2* plays a role in melanoma tumorigenesis through increased proliferation and angiogenesis, but it's involvement in PDAC cancers has yet to be determined.

Additional gene expression analysis using isolated CTCs or cfRNAs may provide us with more information on the roles of *GJB3* and *MFI2* in PDAC. Improving upon the methods used in this study may help us to identify other potential biomarkers from our screen to further investigate.

REFERENCES

Anand, P., A. B. Kunnumakkara, C. Sundaram, K. B. Harikumar, S. T. Tharakan, O. S. Lai, B. Sung and B. B. Aggarwal (2008). "Cancer is a preventable disease that requires major lifestyle changes." Pharm Res **25**(9): 2097-2116.

Ballehaninna, U. K. and R. S. Chamberlain (2012). "The clinical utility of serum CA 19-9 in the diagnosis, prognosis and management of pancreatic adenocarcinoma: An evidence based appraisal." J Gastrointest Oncol **3**(2): 105-119.

Biankin, A. V., N. Waddell, K. S. Kassahn, M. C. Gingras, L. B. Muthuswamy, A. L. Johns, D. K. Miller, P. J. Wilson, A. M. Patch, J. Wu, D. K. Chang, M. J. Cowley, B. B. Gardiner, S. Song, I. Harliwong, S. Idrisoglu, C. Nourse, E. Nourbakhsh, S. Manning, S. Wani, M. Gongora, M. Pajic, C. J. Scarlett, A. J. Gill, A. V. Pinho, I. Rooman, M. Anderson, O. Holmes, C. Leonard, D. Taylor, S. Wood, Q. Xu, K. Nones, J. L. Fink, A. Christ, T. Bruxner, N. Cloonan, G. Kolle, F. Newell, M. Pinese, R. S. Mead, J. L. Humphris, W. Kaplan, M. D. Jones, E. K. Colvin, A. M. Nagrial, E. S. Humphrey, A. Chou, V. T. Chin, L. A. Chantrill, A. Mawson, J. S. Samra, J. G. Kench, J. A. Lovell, R. J. Daly, N. D. Merrett, C. Toon, K. Epari, N. Q. Nguyen, A. Barbour, N. Zeps, I. Australian Pancreatic Cancer Genome, N. Kakkar, F. Zhao, Y. Q. Wu, M. Wang, D. M. Muzny, W. E. Fisher, F. C. Brunicardi, S. E. Hodges, J. G. Reid, J. Drummond, K. Chang, Y. Han, L. R. Lewis, H. Dinh, C. J. Buhay, T. Beck, L. Timms, M. Sam, K. Begley, A. Brown, D. Pai, A. Panchal, N. Buchner, R. De Borja, R. E. Denroche, C. K. Yung, S. Serra, N. Onetto, D. Mukhopadhyay, M. S. Tsao, P. A. Shaw, G. M. Petersen, S. Gallinger, R. H. Hruban, A. Maitra, C. A. Iacobuzio-Donahue, R. D. Schulick, C. L. Wolfgang, R. A. Morgan, R. T. Lawlor, P. Capelli, V. Corbo, M. Scardoni, G. Tortora, M. A. Tempero, K. M. Mann, N. A. Jenkins, P. A. Perez-Mancera, D. J. Adams, D. A. Largaespada, L. F. Wessels, A. G. Rust, L. D. Stein, D. A. Tuveson, N. G. Copeland, E. A. Musgrove, A. Scarpa, J. R. Eshleman, T. J. Hudson, R. L. Sutherland, D. A. Wheeler, J. V. Pearson, J. D. McPherson, R. A. Gibbs and S. M. Grimmond (2012). "Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes." Nature **491**(7424): 399-405.

Blackford, A., G. Parmigiani, T. W. Kensler, C. Wolfgang, S. Jones, X. Zhang, D. W. Parsons, J. C. Lin, R. J. Leary, J. R. Eshleman, M. Goggins, E. M. Jaffee, C. A. Iacobuzio-Donahue, A. Maitra, A. Klein, J. L. Cameron, K. Olino, R. Schulick, J. Winter, B. Vogelstein, V. E. Velculescu, K. W. Kinzler and R. H. Hruban (2009). "Genetic mutations associated with cigarette smoking in pancreatic cancer." Cancer Res **69**(8): 3681-3688.

Bosetti, C., E. Lucenteforte, D. T. Silverman, G. Petersen, P. M. Bracci, B. T. Ji, E. Negri, D. Li, H. A. Risch, S. H. Olson, S. Gallinger, A. B. Miller, H. B. Bueno-de-Mesquita, R. Talamini, J. Polesel, P. Ghadirian, P. A. Baghurst, W. Zatonski, E. Fontham, W. R. Bamlet, E. A. Holly, P. Bertuccio, Y. T. Gao, M. Hassan, H. Yu, R. C. Kurtz, M. Cotterchio, J. Su, P. Maisonneuve, E. J. Duell, P. Boffetta and C. La Vecchia (2012). "Cigarette smoking and pancreatic cancer: an analysis from the International Pancreatic Cancer Case-Control Consortium (Panc4)." Ann Oncol **23**(7): 1880-1888.

Cen, P., X. Ni, J. Yang, D. Y. Graham and M. Li (2012). "Circulating tumor cells in the diagnosis and management of pancreatic cancer." Biochim Biophys Acta **1826**(2): 350-356.

Chaika, N. V., F. Yu, V. Purohit, K. Mehla, A. J. Lazenby, D. DiMaio, J. M. Anderson, J. J. Yeh, K. R. Johnson, M. A. Hollingsworth and P. K. Singh (2012). "Differential expression of metabolic genes in tumor and stromal components of primary and metastatic loci in pancreatic adenocarcinoma." PLoS One **7**(3): e32996.

Chaudhry, Z. W., E. Hall, R. R. Kalyani, D. P. Cosgrove and H. C. Yeh (2013). "Diabetes and pancreatic cancer." Curr Probl Cancer **37**(5): 287-292.

Cronier, L., S. Crespin, P. O. Strale, N. Defamie and M. Mesnil (2009). "Gap junctions and cancer: new functions for an old story." Antioxid Redox Signal **11**(2): 323-338.

Dabritz, J., R. Preston, J. Hanfler and H. Oettle (2009). "Follow-up study of K-ras mutations in the plasma of patients with pancreatic cancer: correlation with clinical features and carbohydrate antigen 19-9." Pancreas **38**(5): 534-541.

Debey-Pascher, S., A. Hofmann, F. Kreusch, G. Schuler, B. Schuler-Thurner, J. L. Schultze and A. Staratschek-Jox (2011). "RNA-stabilized whole blood samples but not peripheral blood mononuclear cells can be stored for prolonged time periods prior to transcriptome analysis." J Mol Diagn **13**(4): 452-460.

DeSantis, C. E., C. C. Lin, A. B. Mariotto, R. L. Siegel, K. D. Stein, J. L. Kramer, R. Alteri, A. S. Robbins and A. Jemal (2014). "Cancer treatment and survivorship statistics, 2014." CA: A Cancer Journal for Clinicians **64**(4): 252-271.

Duffy, M. J., C. Sturgeon, R. Lamerz, C. Haglund, V. L. Holubec, R. Klapdor, A. Nicolini, O. Topolcan and V. Heinemann (2010). "Tumor markers in pancreatic cancer: a European Group on Tumor Markers (EGTM) status report." Ann Oncol **21**(3): 441-447.

Dunn, L. L., E. O. Sekyere, Y. Suryo Rahmanto and D. R. Richardson (2006). "The function of melanotransferrin: a role in melanoma cell proliferation and tumorigenesis." Carcinogenesis **27**(11): 2157-2169.

Ellis, W. J., J. Pfitzenmaier, J. Colli, E. Arfman, P. H. Lange and R. L. Vessella (2003). "Detection and isolation of prostate cancer cells from peripheral blood and bone marrow." Urology **61**(2): 277-281.

Ezumi, K., H. Yamamoto, K. Murata, M. Higashiyama, B. Damdinsuren, Y. Nakamura, N. Kyo, J. Okami, C. Y. Ngan, I. Takemasa, M. Ikeda, M. Sekimoto, N. Matsuura, H. Nojima and M. Monden (2008). "Aberrant expression of connexin 26 is associated with lung metastasis of colorectal cancer." Clin Cancer Res **14**(3): 677-684.

Goggins, M. (2005). "Molecular markers of early pancreatic cancer." J Clin Oncol **23**(20): 4524-4531.
Gold, D., G. Newsome, D. Liu and D. Goldenberg (2013). "Mapping PAM4 (clivatuzumab), a monoclonal antibody in clinical trials for early detection and therapy of pancreatic ductal adenocarcinoma, to MUC5AC mucin." Molecular Cancer **12**(1): 143.

Gold, D. V., J. Gaedcke, B. M. Ghadimi, M. Goggins, R. H. Hruban, M. Liu, G. Newsome and D. M. Goldenberg (2013). "PAM4 enzyme immunoassay alone and in combination with CA 19-9 for the detection of pancreatic adenocarcinoma." Cancer **119**(3): 522-528.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Harouaka, R., Z. Kang, S. Y. Zheng and L. Cao (2014). "Circulating tumor cells: advances in isolation and analysis, and challenges for clinical applications." Pharmacol Ther **141**(2): 209-221.

Harris, L., H. Fritsche, R. Mennel, L. Norton, P. Ravdin, S. Taube, M. R. Somerfield, D. F. Hayes and R. C. Bast, Jr. (2007). "American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer." J Clin Oncol **25**(33): 5287-5312.

Herreros-Villanueva, M., M. Gironella, A. Castells and L. Bujanda (2013). "Molecular markers in pancreatic cancer diagnosis." Clin Chim Acta **418**: 22-29.

Hong, S. M., J. Y. Park, R. H. Hruban and M. Goggins (2011). "Molecular signatures of pancreatic cancer." Arch Pathol Lab Med **135**(6): 716-727.

Iacobuzio-Donahue, C. A., B. Fu, S. Yachida, M. Luo, H. Abe, C. M. Henderson, F. Vilardell, Z. Wang, J. W. Keller, P. Banerjee, J. M. Herman, J. L. Cameron, C. J. Yeo, M. K. Halushka, J. R. Eshleman, M. Raben, A. P. Klein, R. H. Hruban, M. Hidalgo and D. Laheru (2009). "DPC4 gene status of the primary carcinoma correlates with patterns of failure in patients with pancreatic cancer." J Clin Oncol **27**(11): 1806-1813.

Ikeya, S., S. Urano, J. Sakabe, T. Ito and Y. Tokura (2013). "Erythrokeratodermia variabilis: first Japanese case documenting GJB3 mutation." J Dermatol **40**(5): 402-403.

Jahr, S., H. Hentze, S. Englisch, D. Hardt, F. O. Fackelmayer, R. D. Hesch and R. Knippers (2001). "DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells." Cancer Res **61**(4): 1659-1665.

Jazieh, K. A., M. B. Foote and L. A. Diaz, Jr. (2014). "The clinical utility of biomarkers in the management of pancreatic adenocarcinoma." Semin Radiat Oncol **24**(2): 67-76.

Kopreski, M. S., F. A. Benko, L. W. Kwak and C. D. Gocke (1999). "Detection of tumor messenger RNA in the serum of patients with malignant melanoma." Clin Cancer Res **5**(8): 1961-1965.

La Torre, G., A. Sferrazza, M. R. Gualano, C. de Waure, G. Clemente, A. M. De Rose, N. Nicolotti, G. Nuzzo, R. Siliquini, A. Boccia and W. Ricciardi (2014). "Investigating the synergistic interaction of diabetes, tobacco smoking, alcohol consumption, and hypercholesterolemia on the risk of pancreatic cancer: a case-control study in Italy." Biomed Res Int **2014**: 481019.

Liu, H., X. Zhang, J. Li, B. Sun, H. Qian and Z. Yin (2014). "The biological and clinical importance of epithelial-mesenchymal transition in circulating tumor cells." J Cancer Res Clin Oncol.

Lucas, A. L., L. E. Frado, C. Hwang, S. Kumar, L. G. Khanna, E. J. Levinson, J. A. Chabot, W. K. Chung and H. Frucht (2014). "BRCA1 and BRCA2 germline mutations are frequently demonstrated in both high-risk pancreatic cancer screening and pancreatic cancer cohorts." Cancer **120**(13): 1960-1967.

McLachlan, E., Q. Shao, H. L. Wang, S. Langlois and D. W. Laird (2006). "Connexins act as tumor suppressors in three-dimensional mammary cell organoids by regulating differentiation and angiogenesis." Cancer Res **66**(20): 9886-9894.

Naus, C. C. and D. W. Laird (2010). "Implications and challenges of connexin connections to cancer." Nat Rev Cancer **10**(6): 435-441.

Neitzel, L. T., C. D. Neitzel, K. L. Magee and M. P. Malafa (1999). "Angiogenesis correlates with metastasis in melanoma." Ann Surg Oncol **6**(1): 70-74.

Oshima, M., K. Okano, S. Muraki, R. Haba, T. Maeba, Y. Suzuki and S. Yachida (2013). "Immunohistochemically detected expression of 3 major genes (CDKN2A/p16, TP53, and SMAD4/DPC4) strongly predicts survival in patients with resectable pancreatic cancer." Ann Surg **258**(2): 336-346.

Pericleous, M., R. E. Rossi, D. Mandair, T. Whyand and M. E. Caplin (2014). "Nutrition and pancreatic cancer." Anticancer Res **34**(1): 9-21.

Pucciarelli, S., E. Rampazzo, M. Briarava, I. Maretto, M. Agostini, M. Digito, S. Keppel, M. L. Friso, S. Lonardi, A. De Paoli, C. Mescoli, D. Nitti and A. De Rossi (2012). "Telomere-specific reverse transcriptase (hTERT) and cell-free RNA in plasma as predictors of pathologic tumor response in rectal cancer patients receiving neoadjuvant chemoradiotherapy." Ann Surg Oncol **19**(9): 3089-3096.

Rhim, A. D., E. T. Mirek, N. M. Aiello, A. Maitra, J. M. Bailey, F. McAllister, M. Reichert, G. L. Beatty, A. K. Rustgi, R. H. Vonderheide, S. D. Leach and B. Z. Stanger (2012). "EMT and dissemination precede pancreatic tumor formation." Cell **148**(1-2): 349-361.

Sala, R., W. A. Jefferies, B. Walker, J. Yang, J. Tiong, S. K. Law, M. F. Carlevaro, E. Di Marco, A. Vacca, R. Cancedda, F. D. Cancedda and D. Ribatti (2002). "The human melanoma associated protein melanotransferrin promotes endothelial cell migration and angiogenesis in vivo." Eur J Cell Biol **81**(11): 599-607.

Schwarzenbach, H., D. S. Hoon and K. Pantel (2011). "Cell-free nucleic acids as biomarkers in cancer patients." Nat Rev Cancer **11**(6): 426-437.

Shinozaki, M., S. J. O'Day, M. Kitago, F. Amersi, C. Kuo, J. Kim, H. J. Wang and D. S. Hoon (2007). "Utility of circulating B-RAF DNA mutation in serum for monitoring melanoma patients receiving biochemotherapy." Clin Cancer Res **13**(7): 2068-2074.

Siegel, R., D. Naishadham and A. Jemal (2013). "Cancer statistics, 2013." CA Cancer J Clin **63**(1): 11-30.
Singh, P., R. Srinivasan and J. D. Wig (2012). "SMAD4 genetic alterations predict a worse prognosis in patients with pancreatic ductal adenocarcinoma." Pancreas **41**(4): 541-546.

Slater, E. P., K. Strauch, S. Rospleszcz, A. Ramaswamy, I. Esposito, G. Kloppel, E. Matthai, K. Heeger, V. Fendrich, P. Langer and D. K. Bartsch (2014). "MicroRNA-196a and -196b as Potential Biomarkers for the Early Detection of Familial Pancreatic Cancer." Transl Oncol.

Stroun, M., P. Maurice, V. Vasioukhin, J. Lyautey, C. Lederrey, F. Lefort, A. Rossier, X. Q. Chen and P. Anker (2000). "The origin and mechanism of circulating DNA." Ann N Y Acad Sci **906**: 161-168.

Suryo Rahmanto, Y., L. L. Dunn and D. R. Richardson (2007). "Identification of distinct changes in gene expression after modulation of melanoma tumor antigen p97 (melanotransferrin) in multiple models in vitro and in vivo." Carcinogenesis **28**(10): 2172-2183.

Verma, M. and U. Manne (2006). "Genetic and epigenetic biomarkers in cancer diagnosis and identifying high risk populations." Crit Rev Oncol Hematol **60**(1): 9-18.

Yachida, S. and C. A. Iacobuzio-Donahue (2013). "Evolution and dynamics of pancreatic cancer progression." Oncogene **32**(45): 5253-5260.

Yachida, S., C. M. White, Y. Naito, Y. Zhong, J. A. Brosnan, A. M. Macgregor-Das, R. A. Morgan, T. Saunders, D. A. Laheru, J. M. Herman, R. H. Hruban, A. P. Klein, S. Jones, V. Velculescu, C. L. Wolfgang and C. A. Iacobuzio-Donahue (2012). "Clinical significance of the genetic landscape of pancreatic cancer and implications for identification of potential long-term survivors." Clin Cancer Res **18**(22): 6339-6347.

Yu, M., A. Bardia, B. S. Wittner, S. L. Stott, M. E. Smas, D. T. Ting, S. J. Isakoff, J. C. Ciciliano, M. N. Wells, A. M. Shah, K. F. Concannon, M. C. Donaldson, L. V. Sequist, E. Brachtel, D. Sgroi, J. Baselga, S. Ramaswamy, M. Toner, D. A. Haber and S. Maheswaran (2013). "Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition." Science **339**(6119): 580-584.

Zhang, D., C. Chen, Y. Li, X. Fu, Y. Xie, Y. Li and Y. Huang (2012). "Cx31.1 acts as a tumour suppressor in non-small cell lung cancer (NSCLC) cell lines through inhibition of cell proliferation and metastasis." J Cell Mol Med **16**(5): 1047-1059.

Zheng, S., H. Lin, J. Q. Liu, M. Balic, R. Datar, R. J. Cote and Y. C. Tai (2007). "Membrane microfilter device for selective capture, electrolysis and genomic analysis of human circulating tumor cells." J Chromatogr A **1162**(2): 154-161.