IDENTIFYING BIOMARKERS OF RESPONSE TO MODIFIED FOLFIRINOX REGIMENS USING PATIENT DERIVED XENOGRAFT MOUSE MODELS OF PANCREATIC DUCTAL ADENOCARCINOMA

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“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 of Science in the Department of Pharmacology in the School of Medicine.

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ABSTRACT

Meagan Marie Davis Eldridge: Identifying biomarkers of response to modified FOLFIRINOX regimens using patient derived xenograft mouse models of pancreatic ductal adenocarcinoma
(Under the direction of Jen Jen Yeh)

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with limited effective therapies. FOLFIRINOX is a chemotherapeutic regimen for patients with metastatic disease that provides an unprecedented median overall survival of 11.1 months. However, significant toxicities necessitate dose reductions and limit the number of patients eligible for FOLFIRINOX treatment.

I used patient derived xenograft (PDX) mouse models of PDAC to assess efficacy of modified regimens and determine if intra-tumoral heterogeneity plays a role in response. I also used RNA sequencing of PDX tumors to identify potential biomarkers of response.

No significant differences in response to standard and modified FOLFIRINOX regimens were observed; however, intra-tumoral heterogeneity affected responses of one PDX tumor line to modified regimens. Biomarkers associated with differential responses could not be identified by RNA sequencing. RNA sequencing data confirmed previously identified PDAC subtypes, classical and basal-like.
To my husband,
my encourager, best friend, and biggest fan.
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Finally, I thank God, my rock and salvation, for his great love, steadfastness, and provision. “But God, being rich in mercy, because of the great love with which he loved us, even when we were dead in our trespasses, made us alive together with Christ—by grace you have been saved” (Ephesians 2:4–5).
# TABLE OF CONTENTS

**LIST OF TABLES** ........................................................................................................... vii

**LIST OF FIGURES** ................................................................................................. viii

**LIST OF ABBREVIATIONS** .................................................................................... ix

**CHAPTER I** .................................................................................................................. 1

  **INTRODUCTION** ........................................................................................................ 1

  **METHODS** ................................................................................................................ 4

    PDX Tumor Expansions ............................................................................................. 4

    FOLFIRINOX Dosing ................................................................................................. 5

    RNA Sequencing ...................................................................................................... 6

  **RESULTS** .................................................................................................................... 7

    Response to Modified FOLFIRINOX Regimens ...................................................... 7

    Effect of Intra-tumoral Heterogeneity on Response to Modified Regimens ............. 7

    Differentiating PDAC Subtypes using Principal Component Analysis .................... 8

    Identifying Differential Gene Expression Among PancT6 Cohorts ......................... 9

    Evaluating Growth Rate Among PancT6 Cohorts .................................................... 11

    Assessing Differences Between Classical and Basal-like Subtypes ....................... 12

  **DISCUSSION** ............................................................................................................. 13

  **REFERENCES** ........................................................................................................... 19
LIST OF TABLES

Table 1 – Drug dosages for each of the 4 treatment regimens

Table 2 – Differentially expressed genes associated with protein biosynthesis
LIST OF FIGURES

Figure 1 – Study design ........................................................................................................4

Figure 2 – Modified FOLFIRINOX regimens are as effective as the standard regimen ..............................................................6

Figure 3 – Intra-tumoral heterogeneity may affect response to modified FOLFIRINOX regimens ..................................................................................8

Figure 4 – Principal Component Analysis differentiates PDAC subtypes .................................................9

Figure 5 – Pre-treatment growth rates for PancT6 mice assigned to the Low IRI regimen are not significantly different .........................................................11

Figure 6 - PancT6 (Basal-like) tumors are more heterogeneous than P505 (Classical) tumors ..................................................................................13
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FOLFIRINOX</td>
<td>Chemotherapeutic regimen composed of folinic acid, fluorouracil, irinotecan, and oxaliplatin</td>
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<tr>
<td>GATHER</td>
<td>Gene annotation tool to help explain relationships</td>
</tr>
<tr>
<td>IRI</td>
<td>Irinotecan</td>
</tr>
<tr>
<td>OX</td>
<td>Oxaliplatin</td>
</tr>
<tr>
<td>OX/IRI</td>
<td>Irinotecan and oxaliplatin</td>
</tr>
<tr>
<td>PC</td>
<td>Principal component</td>
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<td>PCA</td>
<td>Principal component analysis</td>
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<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
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<tr>
<td>PDX</td>
<td>Patient derived xenograft</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RSEM</td>
<td>Tool used to determine gene expression levels</td>
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INTRODUCTION

Pancreatic cancer is the tenth most commonly diagnosed cancer but is the fourth leading cause of cancer-related death in the United States (1). The overall 5-year survival rate is approximately 5%, with roughly 44,000 newly diagnosed cases of pancreatic cancer in the United States each year (2). Ninety percent of pancreatic cancers are pancreatic ductal adenocarcinomas (PDAC), which are malignancies of the exocrine compartment of the pancreas. The vast majority of patients are diagnosed when the tumor has already involved nearby vessels or has metastasized to distant organs, making them ineligible for potentially curative resection (3). Additionally, the incidence of pancreatic cancer is expected to increase by 55% in the next twenty years, making it the second leading cause of cancer-related death in the United States (2). The rising mortality associated with PDAC is not only due to increased prevalence but also a lack of effective long-term treatments (2).

The nucleoside analog, gemcitabine, is the most commonly prescribed chemotherapeutic regimen for PDAC and has been the standard of care since 1997 when it was directly evaluated against fluorouracil in patients with advanced PDAC. Gemcitabine, however, is only marginally effective, offering a 10% response rate and 7-month median survival (4). Several drugs have been used in combination with gemcitabine in an effort to increase effectiveness, but there have been few significant increases in survival or quality of life reported for these combination regimens (4).

FOLFIRINOX is a novel, promising treatment and has been shown to significantly benefit patients suffering from PDAC. FOLFIRINOX is named for the four drugs of which it is composed: folinic acid (FOL), fluorouracil (F), irinotecan (IRIN), and oxaliplatin (OX) (5). The 3 cytotoxic
components of the regimen, fluorouracil, irinotecan, and oxaliplatin, have different mechanisms of action and non-overlapping toxicities (5). Fluorouracil inhibits the enzyme thymidylate synthase, which is required for DNA synthesis. Folinic acid potentiates the action of fluorouracil by stabilizing the binding of fluorouracil to thymidylate synthase (6). Irinotecan inhibits topoisomerase, the enzyme that unwinds DNA for synthesis, and also prolongs the effect of oxaliplatin, which induces DNA cross-links (7).

In 2011, the efficacy and safety of FOLFIRINOX for patients with metastatic PDAC was assessed against gemcitabine in a phase III randomized controlled clinical trial (5). Previous phase I and II clinical trials showed significant toxicities associated with the FOLFIRINOX regimen (8, 9). Therefore, only patients with good performance status, measured as a 0 or 1 on the Eastern Cooperative Oncology Group performance status scale, were enrolled in the phase III clinical trial (5). Patients with a performance status of 0 or 1 are “fully active, able to carry on all pre-disease performance without restriction” or “restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature,” respectively (10).

Results of this study indicated a significantly better objective response rate for patients that were treated with FOLFIRINOX compared to patients that received gemcitabine (31.6%, 95% CI: 24.7–39.1 versus 9.4%, 95% CI: 5.4–14.7; p<0.001), a greater median overall survival (11.1 months versus 6.8 months, hazard ratio: 0.57; 95% CI: 0.45–0.73; p<0.001), and longer median progression-free survival (6.4 months versus 3.3 months, hazard ratio: 0.47; 95% CI: 0.37–0.59; p<0.001) (5). Patients in the FOLFIRINOX arm of the study also reported longer time until definitive deterioration compared to patients in the gemcitabine arm. Although FOLFIRINOX was more effective than gemcitabine, toxicity occurred much more frequently in patients treated with FOLFIRINOX compared to patients in the gemcitabine arm. FOLFIRINOX-treated patients experienced significantly more neutropenia (p<0.001), febrile neutropenia (p=0.03), thrombocytopenia (p=0.04), diarrhea (p<0.001), and sensory neuropathy (p<0.001). Doses also had to be decreased in many patients. Median relative dose intensities for oxaliplatin, irinotecan, and fluorouracil were 78, 81, and 82% of their full doses, respectively (5).
Although significant toxicities were associated with FOLFIRINOX treatment, this phase III clinical trial demonstrated that FOLFIRINOX is a superior chemotherapy regimen compared to gemcitabine for patients with metastatic pancreatic cancer and good performance status. Despite these findings, the question still remains: how many patients with metastatic pancreatic cancer have good performance status and are likely to benefit from FOLFIRINOX?

Currently, 50% of patients with pancreatic cancer present with metastatic disease, but poor performance status limits the number of patients eligible for FOLFIRINOX treatment (11). However, of the patients with metastatic disease and good performance status, roughly 31% respond to treatment (5), a huge increase in response rate compared to other treatments. It is imperative that we utilize this effective regimen by making it available to a broader patient population. In order to expand the number of patients eligible to receive FOLFIRINOX treatment, we must minimize the toxicity of the regimen while maintaining its efficacy.

Not only must we limit the toxicity of FOLFIRINOX, but we must also identify patients that are most likely to respond to modified regimens. Potential biomarkers of response to therapy can be assessed by interrogating the transcriptome of tumors (12). Subtypes have been identified for several cancers, such as breast, clear cell renal, and bladder cancers, using RNA sequencing and microarrays. Importantly, specific therapeutic targets have been identified for different subtypes, and various subtypes have been shown to respond differently to therapy (13-16). Use of molecular subtyping tools, such as RNA sequencing, is critical to more effectively treat patients by implementing personalized medicine practices.

For this study, I aimed to determine if modified FOLFIRINOX regimens, containing 20% dose reductions in oxaliplatin, irinotecan, or both, are as effective as the human equivalent standard regimen in patient-derived xenograft (PDX) mouse models of PDAC. PDX mice are established by subcutaneously implanting human PDAC tissue that is obtained during a patient’s surgical resection into the flank of an immunodeficient mouse (17-19). I chose to utilize this mouse model because PDX mice accurately recapitulate human response rates and tumor heterogeneity. Furthermore, these models provide biological replicates of the same tumor yielding an invaluable tool for investigating pancreatic cancer therapies (20, 21).
I also sought to determine if intra-tumoral heterogeneity affects response to FOLFIRINOX regimens by using PDX mice that were implanted with tumors that arose from a single original parent donor but were passaged into 3 different cohort donor mice prior to finally being passaged into the experimental PDX mice. This study design provided us with 3 cohorts of experimental PDX mice that were implanted with tumors from 3 different regions of the original parent tumor. I also investigated potential biomarkers of response to FOLFIRINOX regimens using RNA sequencing of the original parent and 3 cohort donor tumors. I repeated this study in 2 different PDX tumor lines, corresponding to tumors originally obtained from 2 individual patients. Our findings suggest that modified FOLFIRINOX regimens are as effective as the Standard FOLFIRINOX regimen; however, I observed different responses to FOLFIRINOX regimens between PDX mice from different cohorts within the same tumor line. RNA sequencing of PDX tumors confirmed PDAC subtypes that were previously identified in our lab using patient and PDX microarray data. The continuation of this work may provide a greater understanding of subtype differences and intra-tumoral heterogeneity that affects response to FOLFIRINOX regimens, thereby enabling the identification of PDAC patients that are most likely to benefit from this promising treatment.

METHODS

PDX Tumor Expansions

The original parent PDX donor tumors (Figure 1a) from lines P505 and PancT6 were harvested and implanted subcutaneously into 3 naïve immunodeficient mice each to establish the cohort donor tumors (Figure 1b). A piece from each original parent tumor was also snap frozen. Cohort donor

![Figure 1: Study design](image-url)

*Figure 1: Study design.* The tumor of an original parent PDX mouse (a) was harvested. A piece of tumor from mouse a was implanted subcutaneously into each of 3 mice (b). Upon reaching max size, the tumors from these mice were harvested and subcutaneously implanted into 30 mice each (c). Approximately 5 mice from each cohort were assigned to one of the 5 treatment groups.
tumors were allowed to grow to approximately 2.0 cm in maximal dimension. Upon reaching maximum size, each cohort donor tumor was removed and cut into 31 pieces. Each piece of tumor was then implanted subcutaneously into a single nude mouse, for a total of 30 experimental PDX mice per cohort donor tumor (90 experimental mice per PDX tumor line (Figure 1c). An additional piece from each cohort donor tumor was also snap frozen.

**FOFIRINOX Dosing**

Because PDX tumors from different lines grow at different rates, I utilized growth data for each tumor line to determine the appropriate tumor size at which to begin treatment. For the P505 tumor line, mice were enrolled in the study when their tumors reached a median volume of 239 mm$^3$ (range, 196–385 mm$^3$). For the PancT6 tumor line, mice were enrolled in the study when their tumors reached a median volume of 196 mm$^3$ (range, 144–486 mm$^3$). Tumor sizes were determined using caliper measurements.

Upon reaching appropriate tumor volume, mice were randomly assigned to one of 4 treatment groups or the placebo group. Treatment regimens were titled as follows: Standard, Low OX (oxaliplatin), Low IRI (irinotecan), Low OX/IRI (oxaliplatin and irinotecan) (Table 1). 0.9% NaCl was used as the placebo control. Drug dosages for the Standard regimen were identified as the maximum tolerated dosages in nude mice, resulting in less than 20% weight loss during a 28-day treatment course. FOLFIRINOX was administered intravenously by tail vein injection on a weekly basis for 28 days. Folinic acid and oxaliplatin were administered first as a combination, mice were rested for 30 minutes, then fluorouracil and irinotecan were administered as a second combination. Tumor size and mouse weight were recorded twice per week. Tumors were harvested 4 hours after the final treatment and snap frozen for storage.

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Drug Dosage (mg/kg)</th>
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<tbody>
<tr>
<td></td>
<td>Folinic Acid</td>
</tr>
<tr>
<td>Standard</td>
<td>100</td>
</tr>
<tr>
<td>Low OX</td>
<td>100</td>
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<tr>
<td>Low IRI</td>
<td>100</td>
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<tr>
<td>Low OX/IRI</td>
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RNA Sequencing

RNA was isolated from snap frozen tumors using the AllPrep DNA/RNA Mini Kit (Qiagen®, Hilden, Germany) and sent to BGI in Hong Kong for library preparation and sequencing. Libraries for RNA sequencing were constructed using oligo (dT) selection methods and consisted of 200 base pair fragments. RNA sequencing was performed using the Illumina HiSeq sequencing platform. Four samples were run per lane, producing approximately 60 million, 50 base pair paired-end reads per sample (BGI Americas, Cambridge, MA).

RNA sequencing data was aligned to human and mouse genomes, hg19 and mm10, respectively, using MapSplice. Reads were classified as human, mouse, or of ambiguous origin using the Xenome algorithm (22). Gene expression levels were determined using the RSEM annotation (23).

Principal component analysis (PCA) was performed to determine sources of gene expression variability across the 3 cohort donor tumors and original parent tumors for the P505 and PancT6 lines. Genes that were not expressed in any of our PDX tumors were eliminated prior to analysis.
RESULTS

Response to Modified FOLFIRINOX Regimens

The doses of FOLFIRINOX that were administered were not toxic based on mouse weight (Figure 2A). Less than 4% of mice died during the course of treatment.

To determine if modified FOLFIRINOX regimens were as effective as the Standard regimen, I calculated the fold change in tumor volume at day 28 for each mouse. Mice from each experimental cohort that received the same regimen were combined into one group for statistical purposes. To account for any variability that may exist among different cohorts, I performed a 2-way ANOVA to compare the response of mice treated with modified FOLFIRINOX regimens to mice treated with the Standard regimen. Because mice from only two of the P505 cohorts were assigned to the Low OX/IRI regimen due to a number of unexpected deaths in cohort 3 prior to study enrollment, a 2-way ANOVA comparing only mice from cohorts 1 and 2 was used to evaluate response to this regimen (Figure 2B). Similarly, mice from only one PancT6 cohort were assigned to the Low OX/IRI regimen, so a t-test was used to assess differences in response of mice from this single cohort donor to the Standard and Low OX/IRI regimens (Figure 2C). For both the P505 and PancT6 PDX tumor lines, modified regimens were as effective as the Standard regimen. Although overall tumor regression did not occur, nearly all FOLFIRINOX regimens provided significant tumor growth inhibition compared to placebo for both tumor lines (Figure 2B-C). Mice in PancT6 cohort 1 that were treated with the Low OX/IRI regimen did demonstrate statistically significant growth inhibition compared to placebo (t-test, p=0.11); however, this is thought to be due to limited sample size (Figure 2C).

Effect of Intra-tumoral Heterogeneity on Response to Modified Regimens

To determine the role of intra-tumoral heterogeneity in response to modified FOLFIRINOX regimens, I evaluated whether mice from different cohorts that were treated with the same regimen demonstrate the same response to therapy. For the P505 tumor line, I did not observe statistically significant differences in response in mice from different cohorts, although
the data suggest that mice in cohorts 2 and 3 demonstrate better overall response to standard FOLFIRINOX than mice in cohort 1 (Figure 3A).

There were statistically significant differences in response to the Low IRI regimen between PancT6 cohorts. Mice in cohorts 1 and 3, denoted by circles and triangles, demonstrated greater growth inhibition compared to mice in cohort 2, denoted by squares, when treated with the Low IRI regimen. While not significant, mice in cohort 3 also demonstrated better response than mice in cohort 2 to the Low OX regimen (p=0.06) (Figure 3B).

Figure 3: Intra-tumoral heterogeneity may affect response to modified FOLFIRINOX regimens. A) P505; B) PancT6. Five to six mice from each cohort were assigned to one of the five treatment groups. Response to treatment was evaluated by tumor volume log₂ fold change at day 28 compared to day 0. Circles, squares, and triangles represent cohorts 1, 2, and 3 for each tumor line, respectively. Line represents median tumor volume log₂ fold change for each group. For most regimens, statistical differences in response between mice from different cohorts were assessed by 1-way ANOVA. # indicates p-value was calculated by unpaired t-Test. * indicates p-value < 0.05.

Differentiating PDAC Subtypes using Principal Component Analysis

I used RNA sequencing to assess gene expression differences among the 3 cohorts for each tumor line that may contribute to variability in response to FOLFIRINOX. Relative expression values for approximately 13,000 genes were obtained after removing genes that were not expressed in any of the cohort or parent donor samples.

I performed PCA to identify differential gene expression that may indicate response to FOLFIRINOX regimens. Principal component (PC) 1 comprised genes that differentiated the 2 PDX tumor lines that were used for our study, P505 and PancT6. Genes with high gene weights in PC1 were positively correlated with PancT6 samples, meaning these genes were relatively
highly expressed in PancT6 tumors. P505 samples were negatively associated with PC1, meaning that genes with low gene weights in PC1 were highly expressed by P505 samples.

Previous work in our lab using microarray data of both patient and PDX tumor samples identified two main subtypes of PDAC, classical and basal-like (24). Out of the 100 most highly weighted genes in PC1, 19 of them were also present in the basal-like gene list, and only 5 genes were also present in the classical gene list. Alternatively, out of the 100 most lowly weighted genes in PC1, 47 of them were also present in the classical gene list, and only 2 were present in the basal-like gene list. The basal-like and classical reference gene lists were composed of a total of 229 and 367 genes, respectively. I determined that the overlap in these gene lists was highly significant (Fisher’s exact test, p-value < 0.0001), indicating that PancT6 and P505 samples are of the basal-like and classical subtypes, respectively. These data corroborate previous findings in our lab demonstrating unique PDAC subtypes (Figure 4).

Identifying Differential Gene Expression Among PancT6 Cohorts

Based on the PCA scatter plot of the first 2 PCs, tumors from PancT6 cohort donors 1 (PancT6.b1) and 3 (PancT6.b3), represented by circles and triangles in Figure 3B, were grouped together and were spatially distinct from cohort donor 2 (PancT6.b2), represented by squares in Figure 3B (Figure 4). The parent tumor (PancT6.a) also grouped...
with cohort donor 2. Because I observed better response to the Low IRI regimen in mice
expanded from cohort donors 1 and 3, I termed this group the “Low IRI responders.” I termed the
cohort donor 2 and the parent tumor group the “Parent + Low IRI non-responder.” These two
groups fell on opposite sides of PC2, so I hypothesized that PC2 may describe differential gene
expression that impacts response to the Low IRI regimen. I used GATHER (Gene Annotation
Tool to Help Explain Relationships), a software tool that returns relevant gene ontologies when
provided a list of genes, to attempt to identify biological functions that are associated with genes
ranked in PC2 (25). Genes to be included in the query were determined by first ranking genes by
PC2 gene weight then identifying genes with the highest relative weights (relative gene weight >
0.0200). I then filtered this gene list to only include genes with positive gene weights in PC1,
making these genes important for the basal-like subtype (PancT6). This gene list was comprised
of 65 genes that were relatively highly expressed by only PancT6 samples and also relatively
highly expressed in the “Parent + Low IRI non-responder” group. Top gene ontologies returned
by GATHER included epidermis development, ectoderm development, and histogenesis. Bayes
factors, which quantify the significance of the association of the input gene list with gene
ontologies, were 9, 8, and 6, respectively. We used a Bayes factor of 6 as a cutoff for significance
as suggested by the makers of GATHER. All three top gene ontologies were based on the same
5 genes from my input list, KRT16, KRT17, S100A7, SPRR1A, and SPRR1B. However, none of
these genes have been previously associated with response to irinotecan or any of the drugs in
the FOLFIRINOX regimen. Although this finding was significant, the biological and clinical
significance of these genes in relation to irinotecan response and pancreatic cancer is unclear.

I also interrogated gene ontologies associated with lowly weighted genes in PC2. Genes
with relatively low gene weights in PC2 were identified (relative gene weight < -0.0200) and then
filtered to include only genes with positive gene weights in PC1. This gene list was composed of
78 genes that were relatively highly expressed by only PancT6 samples and also relatively highly
expressed by the “Low IRI responder” group. GATHER returned no significantly associated gene
ontologies for this input list.
I further interrogated gene expression differences that may contribute to variable PancT6 cohort responses to the Low IRI regimen using manual filtering methods of the full gene expression dataset. Because the PancT6 original parent tumor (PancT6.a) also grouped with cohort donor 2 (PancT6.b2) tumor by PCA, I continued to utilize this grouping for downstream analysis. To filter the data set, I performed unpaired, two-tailed t-tests for each gene between our “Parent + Non-responder” and “Responders” groups and chose genes with a t-test p-value < 0.05 (n=1839). Next, I identified the subset of genes with an average expression difference of 50 RSEM (expression values) or greater between the two groups (n=101). I imported this list of 101 genes into GATHER. Results of this query determined protein biosynthesis to be the most strongly associated gene ontology, involving 18 genes from my input list (Bayes factor, 15). These 18 genes involved in protein biosynthesis were more highly expressed in the “Parent + Low IRI non-responder” compared to the “Low IRI responders.” These genes are listed in Table 2.

Table 2: Differentially expressed genes associated with protein biosynthesis

<table>
<thead>
<tr>
<th>EER1D</th>
<th>EIF4EBP1</th>
<th>EIF5A</th>
<th>HSPB1</th>
<th>ITG44BP</th>
<th>RPL13</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPL18</td>
<td>RPL18A</td>
<td>RPL27</td>
<td>RPL36</td>
<td>RPL5</td>
<td>RPS10</td>
</tr>
<tr>
<td>RPS15</td>
<td>RPS26</td>
<td>RPS28</td>
<td>RPS5</td>
<td>RPS8</td>
<td>RPS9</td>
</tr>
</tbody>
</table>

Evaluating Growth Rate Among PancT6 Cohorts

Because protein synthesis was highly associated with our final gene list based on manual filtering methods, I hypothesized that mice expanded from PancT6 experimental cohort donors 1 and 3 (Low IRI responders) may exhibit different growth rates than mice expanded from experimental cohort donor 2 (Low IRI non-responder). I calculated the amount of time in weeks that it took for the tumors of the mice that were later randomly assigned to the Low IRI regimen to reach 150 mm$^3$. I then used a one-way ANOVA to compare growth rates between PancT6 cohorts. No statistically significant differences were observed between cohorts.
significant differences in growth rate were observed between PancT6 cohorts, indicating that although there seems to be greater expression of genes involved in protein synthesis in PancT6 mice in cohort 2 (Low IRI non-responder) compared to mice in cohorts 1 and 3 (Low IRI responders), enhanced expression of genes involved in protein synthesis does not appear to affect tumor growth rate (Figure 5).

Assessing Differences Between Classical and Basal-like Subtypes

I used a two-way ANOVAs to assess whether classical (P505) and basal-like (PancT6) subtypes have different overall responses to FOLFIRINOX regimens. No differences in overall response to any FOLFIRINOX regimen were observed between subtypes.

To determine whether a greater degree of heterogeneity in response to FOLFIRINOX regimens exists among the classical (P505) or basal-like (PancT6) subtype, I performed an F-test to assess subtype differences in the variances of the log2 tumor volume fold change for mice from different cohort donors that were treated with the same regimen. Mice from no single cohort donor had a statistically significantly greater variance in log2 tumor volume fold change than mice from other cohort donors that were treated with the same regimen. No statistically significant differences in variances of responses were observed between the two subtypes for the Standard, Low OX, Low IRI, or Placebo regimens.

I also investigated whether genes were more heterogeneously expressed among classical cohorts or basal-like cohorts. To quantify potential differences, I first deleted all genes with RSEM values less than 10 for all cohorts to eliminate genes that were very lowly expressed in all of our samples. I then performed an F-test to compare the variances of PancT6 and P505 cohorts. 247 genes were identified as being statistically more variable in the PancT6 cohorts compared to the P505 cohorts. 382 genes were identified as being statistically more variable in P505 cohorts compared to PancT6 cohorts. These data indicate that P505 tumors are more heterogeneous at the gene expression level compared to PancT6 tumors (p<0.0001, binomial test).
I also assessed differences in microscopic heterogeneity within a single PDX tumor line. As expected, I observed obvious differences in histopathology between the two PDX tumor lines, with P505 (classical) cohort donor tumors displaying much more abundant and larger mucin-producing glands than PancT6 (basal-like). P505 cohort donor tumors appeared to be overall very similar in microscopic features; however, PancT6 cohort donor tumors demonstrated visible differences in the abundance of mucin-producing glands. PancT6 cohort donors 1 and 3 (Low IRI responders) had more abundant mucin-producing glands than cohort donor 2 (Low IRI non-responder). Heterogeneity among cohort donor tumors suggests intra-tumoral heterogeneity within the original parent tumor which may influence response to FOLFIRINOX regimens (Figure 6).

**Figure 6:** PancT6 (Basal-like) tumors are more heterogeneous than P505 (Classical) tumors. A piece of each cohort donor tumor from both the P505 and PancT6 tumor lines was fixed in paraffin, sectioned, and stained with hematoxylin and eosin. All 3 P505 cohort donor tumors demonstrate large and abundant mucin-producing glands. PancT6 cohort donors 1 and 3 have more abundant mucin-producing glands (indicated by black-circles) than cohort donor 2, suggesting greater intra-tumoral heterogeneity of PancT6 tumors compared to P505 tumors. All images shown at 4x magnification.

**DISCUSSION**

FOLFIRINOX is the most effective treatment for patients with metastatic PDAC and provides approximately a 4-month increase in median overall survival compared to the previous
standard of care, gemcitabine. However, the number of patients that are eligible to take FOLFIRINOX is severely limited by the toxicities of this regimen (5). In accordance with the necessitated dose reductions in the phase III clinical trial, I assessed the efficacy of FOLFIRINOX regimens with 20% dose reductions of two of the most toxic drugs in the regimen, oxaliplatin and irinotecan. I determined that regimens containing 20% dose reductions in one or both of these drugs were as effective as the Standard regimen, suggesting that the standard dose in patients may provoke more drug-induced toxicities with no definitive benefit in the degree of tumor growth inhibition.

Although the drugs that compose the FOLFIRINOX regimen have different mechanisms of action, this does not indicate that the drugs act completely independently of each other. Therefore, potential synergistic or antagonistic effects of the combination regimen cannot be excluded. It is imperative that we continue to evaluate this regimen in order to identify the optimal dosages at which to treat patients so that therapeutic effects can be maximized while minimizing toxicities. Future studies should assess the efficacies of FOLFIRINOX regimens containing even greater dose reductions in oxaliplatin and irinotecan in addition to determining the effects of lowering the dose of fluorouracil.

Response to chemotherapy may be impacted by the molecular heterogeneity of pancreatic tumors. To address this, I evaluated the efficacy of FOLFIRINOX regimens in PDX mice implanted with tumors from distinct regions of an original parent tumor. PancT6 mice implanted with tumors from different cohort donors responded differently to the Low IRI regimen. Mice from different P505 cohorts showed no statistically significant differences in response to any of the regimens administered; however, mice in cohorts 2 and 3 tended to respond better to the Standard regimen than mice in cohort 1.

The number of mice that were available for enrollment may have limited our ability to identify differential responses between cohorts of the same tumor line. This is a difficult issue to overcome because cohort donor tumors must be harvested upon reaching maximum size and can only be passaged into a fixed number of mice based on tumor size. The process of passaging PDX tumors is also time-sensitive since the tumor is harvested and maintained outside
the body prior to being implanted into more mice. Furthermore, not all tumors implanted into mice grow successfully. Approximately, 20% of tumors passaged into mice do not grow, and/or mice get sick and must be harvested. Out of the 30 mice that were passaged per cohort donor, only 20 to 25 mice were enrolled in our study due to unexpected deaths or lack of tumor growth. These deaths can be attributed to causes unassociated with FOLFIRINOX toxicity. One of the four mice that died during the course of treatment was from PancT6 cohort 2. Without factoring in the death of this mouse, this PancT6 cohort had an unusual number of pre-treatment deaths with 30% of mice from this cohort requiring pre-treatment harvest. These data suggest that the health of many mice in this cohort was compromised prior to treatment. Another mouse that died after beginning treatment had actually been losing weight prior to the start of treatment, and, retrospectively, should have been excluded from our study. The other two deaths that occurred during treatment are also likely due to underlying causes that were unrelated to toxicity as we discovered post-completion of this study that the maximum tolerated dose for fluorouracil in this regimen is 100 mg/kg rather than 50 mg/kg in nude mice. Unexpected deaths also limited our ability to adequately assess the efficacy of the Low OX/IRI regimen. Increasing the number of cohort donors or adding an additional passage prior to establishing the experimental cohorts (i.e. passage each cohort donor into a second round of 3 cohort donors) are methods to address this issue.

I used PCA to assess overall similarities and differences in gene expression in the parent and cohort donor tumors. The results of PCA independently validated previous findings in our lab that suggest two PDAC subtypes, basal-like and classical, based on microarray data of patient and PDX tumor samples. Overall responses to FOLFIRINOX regimens and variances of responses of cohort donor tumors were not statistically significantly different between classical (P505) and basal-like (PancT6) tumors. Several factors may have influenced these negative results. In addition to the limited number of mice enrolled in each cohort for each tumor line, these data account for only one basal-like and one classical tumor line. Because our data consists of an n of 2, the statistical power of our study is lacking. As more basal-like and classical PDX tumor lines are evaluated in the continuation of this study, the differences in FOLFIRINOX response,
variability in response, and heterogeneity in gene expression will be elucidated. The seemingly homogenous responses between classical and basal-like subtypes to FOLFIRINOX may also be due to using a 4-drug combination therapy. Basal-like and classical subtypes may respond differently to one or more drugs in the FOLFIRINOX regimen when used as a single agent. Simultaneous treatment using all four drugs may have abrogated differential responses that may exist for single drugs. Before repeating this study using other PDX tumor lines, each line should be thoroughly evaluated for response to single agent cytotoxics (i.e. fluorouracil, oxaliplatin, and irinotecan) at various concentrations using a similar study design that incorporates potential differences in response due to molecular heterogeneity of the tumor.

Alternatively, homogenous responses by the basal-like and classical subtypes in this study may be due to insufficient dosing of fluorouracil. Following completion of this study, the maximum tolerated dose of FOLFIRINOX was re-evaluated. The phase III clinical trial reported a 31.6% objective response rate, defined by patients who demonstrated either complete or partial responses, in the FOLFIRINOX arm. Additionally, 38.6% of patients achieved stable disease with FOLFIRINOX treatment (5). Although my study design only included two tumor lines, the response data does not appear to reflect the results of the clinical trial, considering that nearly 70% of patients in the trial had a stable, partial, or complete response. Overall, neither PancT6 nor P505 tumors responded to the Standard regimen with a stable, partial, or complete response. It is possible that I did not observe a similar frequency in tumor response to the clinical trial because both of these tumor lines may be resistant to this therapy. However, our data suggest that the Standard regimen may be insufficient to yield stable disease or tumor regression. I re-assessed the maximum tolerated dose of FOLFIRINOX in nude mice and determined that the Standard regimen should contain 100 mg/kg fluorouracil instead of the 50 mg/kg that was used for the current study. For subsequent studies, the Standard regimen will consist of the higher dose of fluorouracil with the original doses of folinic acid, oxaliplatin, and irinotecan.

In addition to validating the PDAC subtypes, PCA also split our PancT6 samples into 2 groups based on PC2. These two groups were largely consistent with the responses that we observed to the Low IRI regimen. Genes and biological functions that may be responsible for the
differentiation between the two groups were interrogated using various statistical methods and tools; however, consistent and significantly associated gene ontologies that may influence differential responses to Low IRI could not be identified. The number of samples with available RNA sequencing data was likely limiting since this data was only available for the parent and cohort donor tumors (n=4). Because one versus one or one versus two comparisons are statistically underpowered, I attempted to reconcile gene expression differences by also including the parent donor in the analysis since it grouped closely with cohort donor 2. This two versus two comparison allowed me to filter my gene list down to 101 genes that were most likely responsible for gene expression and response differences between PancT6 cohorts. However, the most notable gene ontology associated with this list was protein biosynthesis, which mostly involved genes important for ribosome production. Considering that expression of genes in protein synthesis may contribute to growth and proliferation, I compared the amount of time it took mice from each cohort that were later assigned to the Low IRI regimen to reach a pre-treatment tumor volume of 150 mm$^3$. No statistically significant difference in growth rate was observed, nullifying the hypothesis that protein biosynthesis corresponded to enhanced tumor growth.

As previously discussed, future studies should use a greater sample size to better parse subtle differences in gene expression due to intra-tumoral heterogeneity. This could be achieved by including an additional passage of the cohort donor tumors into 3 mice each prior to establishing the experimental cohorts. With this new study design, the parent donor, 3 cohort donors, and the 9 pre-experimental cohort donors can be sequenced and provide a total of 270 experimental mice (9 cohorts of 30 mice each). This larger study design will yield a greater sample size for the experimental cohorts and may also provide greater depth to answer the question of whether intra-tumoral heterogeneity affects response to FOLFIRINOX regimens.

Currently, whole-exome data for each cohort donor and parent tumor is being analyzed and may potentially reveal single nucleotide polymorphisms that might indicate response to FOLFIRINOX regimens. As previously mentioned, future studies will use the re-determined doses for the Standard regimen, containing an increased dosage of fluorouracil. Increasing the dose of fluorouracil in the Standard regimen may be more clinically applicable and potentially better
represent patient response data. Future studies will also assess efficacy and identify potential biomarkers of regimens with even greater dose reductions in the cytotoxic components of the regimen. These studies may set a precedent for the clinical use of equally effective modified FOLFIRINOX regimens that contain substantially decreased doses of one or more of the drugs in the regimen. These modified regimens will likely limit drug-induced toxicity and, therefore, may be available as a treatment option to a broader population of pancreatic cancer patients.
REFERENCES


