Integration of Metabolomics and Transcriptomics Revealed a Fatty Acid Network Exerting Growth Inhibitory Effects in Human Pancreatic Cancer

Geng Zhang1, Peijun He1, Hanson Tan1, Anuradha Budhu1, Jochen Gaedcke6, B. Michael Ghadimi6, Thomas Ried2, Harris G. Yantis3, Dong H. Lee3, Anirban Maitra4, Nader Hanna5, H. Richard Alexander5, and S. Perwez Hussain1

Abstract

Purpose: To identify metabolic pathways that are perturbed in pancreatic ductal adenocarcinoma (PDAC), we investigated gene-metabolite networks with integration of metabolomics and transcriptomics.

Experimental Design: We conducted global metabolite profiling analysis on two independent cohorts of resected PDAC cases to identify critical metabolite alteration that may contribute to the progression of pancreatic cancer. We then searched for gene surrogates that were significantly correlated with the key metabolites, by integrating metabolite and gene expression profiles.

Results: Fifty-five metabolites were consistently altered in tumors as compared with adjacent nontumor tissues in a test cohort (N = 33) and an independent validation cohort (N = 31). Weighted network analysis revealed a unique set of free fatty acids (FFA) that were highly coregulated and decreased in PDAC. Pathway analysis of 157 differentially expressed gene surrogates revealed a significantly altered lipid metabolism network, including key lipolytic enzymes PNLIP, CLPS, PNLIPRP1, and PNLIPRP2. Gene expressions of these lipases were significantly decreased in pancreatic tumors as compared with nontumor tissues, leading to reduced FFAs. More importantly, a lower gene expression of PNLIP in tumors was associated with poorer survival in two independent cohorts. We further showed that two saturated FFAs, palmitate and stearate, significantly induced TRAIL expression, triggered apoptosis, and inhibited proliferation in pancreatic cancer cells.

Conclusions: Our results suggest that impairment in a lipolytic pathway involving lipases, and a unique set of FFAs, may play an important role in the development and progression of pancreatic cancer and provide potential targets for therapeutic intervention.

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States with an estimated 44,920 new cases and 37,390 deaths in 2013 (1). Pancreatic cancer cases and deaths have been on the rise since 1998. The median survival of all pancreatic ductal adenocarcinoma (PDAC) cases is less than 6 months, and only 6% of patients survive 5 years after diagnosis. The mortality rate has not improved significantly, due to late diagnosis and resistance to available chemotherapy. Therefore, a better understanding of molecular mechanisms of disease progression and discovery of novel therapeutic targets are desperately needed to improve outcomes in patients with PDAC.

Genetic alterations have been extensively characterized in pancreatic cancer. We and others have previously identified gene markers of PDAC which have prognostic and therapeutic significance (2, 3). However, the impact of those gene alterations on metabolism and gene-metabolite networks in PDAC has not been clearly defined. Several oncogenes and tumor suppressors such as c-Myc and P53 control the activity of different metabolic pathways to support the metabolic transformation of a cancer cell (4). A growing body of evidence showed a strong connection between cancer and metabolism, including the discovery that some key metabolic enzymes such as succinate dehydrogenase, fumarate hydratase, isocitrate dehydrogenase, and phosphoglycerate dehydrogenase, if mutated, could
Zhang et al.

**Translational Relevance**

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies worldwide. Altered metabolism is considered as one of the hallmarks of cancer. Therefore, a better understanding of metabolic dysregulation in pancreatic cancer could lead to the discovery of novel therapeutic targets. In the present study, we investigated metabolic dysregulation and gene-metabolite networks in pancreatic cancer, with integration of metabolomics and transcriptomics. Our results suggest that the impaired lipolytic network involving four lipases and a unique set of fatty acids may play an important role in the development and progression of pancreatic cancer. Furthermore, our study showed that integration of omics data provide a systems level perspective of pancreatic cancer that could facilitate the development of novel treatments for this disease.

Metabolic profiling of PDAC

Metabolic profiling of PDAC samples (tumor and adjacent nontumor tissues) was carried out at Metabolon Inc. using the general protocol as outlined earlier (refs. 12, 13; see more details in the Supplementary File). Metabolon analytic platform incorporates two separate ultra-high-performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) injections and one gas chromatography/mass spectrometry injection per sample. The UHPLC injections are optimized for basic species and acidic species. This integrated platform enabled the high-throughput collection and relative quantitative analysis of analytic data and identified a large number and broad spectrum of molecules with a high degree of confidence (12). A total of 469 known metabolites were measured.

Weighted coexpression network analysis

Weighted coexpression network analysis (WGCNA) has been implemented in R, a free and open source statistical programming language (14). We followed the protocols of WGCNA to create metabolite networks. Briefly, for each metabolite profiling dataset, Pearson correlation coefficients were calculated for all pairwise comparisons of metabolites across all tumor samples. The resulting Pearson correlation matrix was transformed into an adjacency matrix using a power function, which resulted in a weighted network (14). WGCNA defines modules as a group of densely interconnected molecules with high topological overlap in weighted network analysis. For each dataset, we used average linkage hierarchical clustering with a dynamic tree-cutting algorithm to identify modules on the basis of the topological overlap dissimilarity measure (15).

Ingenuity pathways analysis

Canonical pathway analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant to the dataset. The association between the dataset and the canonical pathway was measured in 2 ways: (i) The statistical significance: Fischer exact test was used to calculate a P value, determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone; (ii) a ratio of the number of genes from the dataset that map to the given pathway divided by the total number of genes in that canonical pathway (16).

lead to different forms of cancer (5). The development of pancreatic cancer has also been linked to abnormal glucose metabolism, which is induced by long-term type-II diabetes, a known risk factor for pancreatic cancer (6). Metabolites are central in intermediary metabolism, and they provide substrates for biologic processes, and have an active role in regulating cell cycle, proliferation, and apoptosis (7). Recently, there has been an increased interest in global analysis of metabolites for cancer biomarker discovery and identification of potential novel therapeutic targets. New technologies applying chromatography-mass spectrometry (MS) provide sensitive and reproducible detection of hundreds to thousands of metabolites in a single biofluid or tissue sample, and allows nontargeted high-throughput metabolic profiling analysis (8). Furthermore, integration of comprehensive gene expression profile with metabolic profiling has been shown to be an innovative way to reveal the complex regulatory networks involving genes and metabolic pathways in cancers (9, 10).

In the present study, we conducted global metabolite profiling analysis in two independent cohorts of PDAC cases to identify critical metabolite alterations that may contribute to the progression of pancreatic cancer. We then searched for gene surrogates that were significantly correlated with the key metabolites using transcriptomic profiling data of the same samples in the test cohort from our previous study (2). The integrative analysis of metabolomics and transcriptomic data revealed a lipid metabolism network involving four lipases and a unique set of free fatty acids (FFA) that may play an important role in pancreatic tumor progression and could provide potential targets for therapeutic intervention.

Materials and Methods

Tissue collection

Primary pancreatic tumor and adjacent nontumor tissues were collected from patients with PDAC at the University of Maryland (Göttingen, Germany), and from the University of Maryland Medical Center (Baltimore, MD) through the NCI-UMD resource contract. Tissues were flash frozen immediately after surgery. Demographic and clinical information for each tissue donor, including age, sex, clinical staging, resection margin status, survival times from diagnosis, and receipt of adjuvant chemotherapy were collected. Tumor histopathology was classified according to the World Health Organization Classification of Tumor system (11).

Use of these clinical specimens was reviewed and approved by the NCI-Office of the Human Subject Research (OHSR, Exempt # 4678) at the NIH (Bethesda, MD).

**Metabolic profiling of PDAC**

Metabolic profiling of PDAC samples (tumor and adjacent nontumor tissues) was carried out at Metabolon Inc. using the general protocol as outlined earlier (refs. 12, 13; see more details in the Supplementary File). Metabolon analytic platform incorporates two separate ultra-high-performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) injections and one gas chromatography/mass spectrometry injection per sample. The UHPLC injections are optimized for basic species and acidic species. This integrated platform enabled the high-throughput collection and relative quantitative analysis of analytic data and identified a large number and broad spectrum of molecules with a high degree of confidence (12). A total of 469 known metabolites were measured.

**Weighted coexpression network analysis**

Weighted coexpression network analysis (WGCNA) has been implemented in R, a free and open source statistical programming language (14). We followed the protocols of WGCNA to create metabolite networks. Briefly, for each metabolite profiling dataset, Pearson correlation coefficients were calculated for all pairwise comparisons of metabolites across all tumor samples. The resulting Pearson correlation matrix was transformed into an adjacency matrix using a power function, which resulted in a weighted network (14). WGCNA defines modules as a group of densely interconnected molecules with high topological overlap in weighted network analysis. For each dataset, we used average linkage hierarchical clustering with a dynamic tree-cutting algorithm to identify modules on the basis of the topological overlap dissimilarity measure (15).

**Ingenuity pathways analysis**

Canonical pathway analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant to the dataset. The association between the dataset and the canonical pathway was measured in 2 ways: (i) The statistical significance: Fischer exact test was used to calculate a P value, determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone; (ii) a ratio of the number of genes from the dataset that map to the given pathway divided by the total number of genes in that canonical pathway (16).
RNA isolation and transcriptome profiling
RNA from frozen tissue samples was extracted using standard TRIzol (Invitrogen) protocol. RNA quality was confirmed with the Agilent 2100 Bioanalyzer (Agilent Technologies) before the microarray gene expression profiling. Tumors and paired nontumor tissues from the Germany cohort were profiled using the Affymetrix GeneChip Human 1.0 ST arrays, according to the manufacturer’s protocol at the LMT microarray core facility at National Cancer Institute (Frederick, MD). All arrays were RNA normalized and gene expression summaries were created for each gene by averaging all probe sets for each gene using Partek Genomics Suite 6.5. All data analysis was conducted on gene-summarized data. The microarray gene expression data has been deposited in the National Center for Biotechnology Information’s (NCBI) Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) with accession number GSE28735.

Quantitative RT-PCR
Total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative Reverse Transcription PCR (qRT-PCR) reactions in 384-well plates were carried out using TaqMan Gene Expression Assays on an ABI Prism 7900HT Sequence Detection instrument from Applied Biosystems. Expression levels of GAPDH were used as the endogenous controls. All assays were conducted in triplicate. For quality control, any sample with a gene cycle value more than 36 was considered of poor quality and removed. All the primers for qRT-PCR in the present study were purchased from Applied Biosystems (Supplementary Table S1).

Cell lines and culture conditions
Human pancreatic carcinoma cell lines hTERT-HPNE (CRL-4023), MIApaca2 (CRL-1420) and Panc1 (CRL-1469) were obtained from American Type Culture Collection. Cells were maintained in Gibco RPMI Media 1640 supplemented with GlutaMAX-I (Invitrogen), penicillin–streptomycin (50 IU/mL and 50 mg/mL, respectively), and 10% (v/v) fetal calf serum (FCS). Fatty acids were purchased from Sigma. The stock solutions of fatty acids bound to bovine serum albumin (BSA) were prepared as described earlier (17). A 5% FFA-free BSA solution was prepared in H2O and dissolved for 30 minutes at 55°C in a water bath. The appropriate amount of 50 mmol/L FFA stock solution was added to BSA solution and incubated for 8 hours at 37°C under nitrogen atmosphere to prevent oxidation. The FFA/BSA stock solution was then cooled to 25°C, filter sterilized, and stored at −20°C.

MTT assay
Cells were seeded in 96-well plates (3,000–5000 cells/well) and incubated for 2 to 10 days. Then, the MTT solution was added and incubated for 4 hours. The solution was aspirated and 100 μL DMSO was added to each well. The absorbance was measured at 570 and 650 nm.

Aptosis assay
Panc1 and MIApaca2 cells were seeded and incubated for 24 hours in standard medium. After 12 hours of serum starvation, cells were incubated in serum-free medium with BSA-bound fatty acids or BSA control for 12 hours and 24 hours. Caspase activity was measured by Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) and potency of caspase activation was calculated, compared with control cells.

Quantification of TRAIL protein level by ELISA
TRAIL protein level was directly quantified in pancreatic cancer cells treated with 0.1 μmol/L FFA or BSA for 24 hours. Cells were lysed (5 × 10⁵ cells/100 μL lysis buffer) and TRAIL production in cell lysates was measured using Quantikine Human TRAIL/TNFSF10 ELISA KIT (R&D Systems) according to the manufacturer’s instructions. Optical density of each well was then determined using a microplate reader set to 450 nm. TRAIL concentrations were calculated using a standard curve and linear regression analysis.

Statistical analysis
A student t test was used to compare metabolite or gene expression between tumors and nontumor tissues using GraphPad Prism 5.0 (GraphPad Software Inc). Correlation analysis and Kaplan–Meier analysis were conducted with GraphPad Prism 5.0 (GraphPad Software Inc). Fisher exact test, correlation analysis, and Cox Proportional-hazards regression analysis were conducted using Stata 11 (StataCorp LP). Univariate Cox regression was conducted on genes and clinical covariates to examine the influence of each on patient survival. For these analyses, resection margin status was dichotomized as positive (R1) versus negative (R0); TNM staging was dichotomized on the basis of non-metastatic (I-IIA) versus metastatic (IIB-IV) disease; histologic grade was dichotomized on the basis of well and moderately differentiated (G1 and 2) versus poorly differentiated (G3 and 4). All Cox regression models were tested for proportional hazards assumptions based on Schoenfeld residuals, and no model violated these assumptions. The statistical significance was defined as P < 0.05. All P values reported were two-sided.

Results
Metabolomics of PDAC
The characteristics of the patients with PDAC in a test cohort (N = 33) and a validation cohort (N = 31) are shown in Supplementary Table S1. The two cohorts were similar in TNM staging, resection margin status, grade, and cancer-specific mortality (P = 0.76; Kaplan–Meier log rank) with 1-year survival rate of 50.8% for the test cohort and 51% for the validation cohort.

Metabolite profiling was conducted using liquid and gas chromatography coupled with mass spectrometry to identify and statistically compare the relative metabolite expression levels between tumor and nontumor tissues from PDAC cases. We identified 55 metabolites that were differentially expressed in tumors as compared with nontumor
tissues \( (P < 0.01) \) in both test and validation cohorts (Supplementary Table S2).

**Weighted network analysis identified a unique set of fatty acids that are coregulated in PDAC**

We constructed coexpression networks using the 55 metabolite profiling data in two independent cohorts. According to recently described methodology (14), the connectivity \( (k) \) was determined for all metabolites in the network by taking the sum of their connection strengths (coexpression similarity) with all other nodes in the network. To identify modules of highly coregulated metabolites, we used average linkage hierarchical clustering to group metabolites based on the topologic overlap of their connectivity (see Materials and Methods for details). WGCNA identified three modules of highly connected metabolites. Each module was assigned a unique color identifier (Fig. 1A), with the remaining, poorly connected

![Figure 1](image-url)

**Figure 1.** Integration of metabolomics and transcriptomics revealed altered lipid metabolism pathways in PDAC. A, weighted network analysis showed that the turquoise-colored module was highly conserved in both test and validation cohort. 8 fatty acids (indicated by stars) with high connectivity \( \text{IMconn} > 6 \), Table 1) in both cohorts represent the main hubs in the metabolite network that were chosen for integration analysis. B, canonical pathways analysis identified 10 pathways from the Ingenuity Pathways Analysis (IPA) library of canonical pathways that were most significant \( (P < 0.05) \) to the data set of 157 surrogate genes for the unique set of 8 fatty acids in PDAC. Glycerolipid metabolism was the most significantly enriched pathway. C, IPA analysis revealed a lipid metabolism network involving 4 lipases and fatty acids highlighted here, that may potentially regulate and interact with apoptosis signaling pathways. Lipolytic genes \( \text{PNLIP}, \text{CLPS}, \text{PNLIPR1}, \) and \( \text{PNLIPR2} \) from the surrogate gene dataset, play central roles in lipolysis.
metabolites colored gray. In a topological overlap matrix (TOM) plot, the increasing color intensity indicates higher connectivity among metabolites in the network (Fig. 1A). Metabolites with the greatest connectivity index represent network "hubs" and are localized in the center of individual modules. Highly connected and correlated hub metabolites are often sharing common pathways and tightly coregulated within the same metabolism networks (18).

Because of the indicated importance of hubs in the network, we ranked metabolites within turquoise module (Supplementary Table S3), based on their intramodular connectivity to identify module hubs (Table 1). Eight metabolites with high connectivity (IMconn > 6) in the turquoise module represented the main hubs in both test and validation cohorts. Interestingly, this set of highly coregulated hub metabolites are all free fatty acids, suggesting that fatty acid metabolism may be significantly altered in PDAC.

Integration of metabolomics and transcriptomics revealed altered lipid metabolism pathway in PDAC

To define genetic alterations and the molecular pathways associated with 8 fatty acids identified from WGCNA, we first searched for gene surrogates that were significantly correlated with these 8 fatty acids within the same 33 samples in test cohort. Using transcriptomic profiling data from our previous study (2), we conducted Pearson correlation analysis between gene expression and metabolite profile data of 8 fatty acids. These analyses identified 157 gene surrogates that were highly correlated with the set of 8 fatty acids (Pearson correlation $P < 0.01$) and differentially expressed between tumors and nontumors (t-test, FDR-corrected $P < 0.01$, |fold change$|>1.5$, Supplementary Table S4).

Furthermore, Ingenuity Pathways Analysis showed that 157 gene surrogates of 8 fatty acids were highly enriched for glycerolipid metabolism, axonal guidance signaling, starch and sucrose metabolism, intrinsic prothrombin activation pathway, and other pathways. The glycerolipid metabolism pathway is the top hit with Fisher exact test $P$ value of 0.001, representing the most significant pathway that is associated with our dataset (Fig. 1B). Significant enrichments were also observed for surrogate genes associated with apoptosis and Wnt signaling, which are known to play critical roles in tumorigenesis. IPA's network analysis further identified interacting modules involved in lipid metabolism and apoptosis signaling networks, which include fatty acids and their surrogate genes PNLIP (pancreatic lipase), CLPS (collase, pancreatic), PNLIPRP1 (pancreatic lipase-related protein 1), and PNLIPRP2 (pancreatic lipase-related protein 2; Fig. 1C).

Lypolytic enzymes PNLIP, CLPS, PNLIPRP1, and PNLIPRP2 are significantly decreased in PDAC

It should be noted that surrogate genes PNLIP, CLPS, PNLIPRP1, and PNLIPRP2 which encode key lypolytic enzymes playing central roles in glycerolipid metabolism, were decreased in tumors as compared with adjacent nontumor tissues in the test cohort (Fig. 2A), which is consistent with the downregulation of FFAs in pancreatic tumors (Table 1).

To validate the association of these lipases with the set of 8 fatty acids identified in metabolic profiling, we then used the validation cohort of PDAC cases ($N = 31$) to examine the gene expression of PNLIP, CLPS, PNLIPRP1, and PNLIPRP2 by qRT-PCR. Our data confirmed that the expression of all 4 lipases were decreased in tumors, as compared with surrounding nontumor tissues in two independent cohorts (Fig. 2), and consistently all 8 fatty acids were also decreased in tumors from both cohorts (Table 2). Particularly, qRT-PCR data in the validation cohort showed approximately 100- to 1,000-fold decrease in the gene expression of these lipases in pancreatic tumors. Our data also showed that gene expression of PNLIP, CLPS, PNLIPRP1, and PNLIPRP2 are lower in PDAC cell lines Panc1 and MIapaca2, as compared with nontumorigenic hTERT-HPNE cells (Supplementary Fig. S1). These data are consistent with other publicly available transcriptional profiling data in Oncomine database (Supplementary Table S5), suggesting that the gene expression of these lipases are potential diagnostic markers for PDAC.

Pancreatic lipases directly hydrolyse triglycerolipids into fatty acid and glycerol, therefore regulating fatty acid turnover and signaling (19). In both test and validation cohorts, gene expressions of these lipases are significantly correlated with the set of 8 FFAs (Supplementary Table S6), indicating that markedly decreased expression of PNLIP, CLPS, PNLIPRP1, and PNLIPRP2 may have a role in reduced fatty acid level in pancreatic tumors.

PNLIP is a predictor of cancer-specific mortality in PDAC

There is no significant association between 8 fatty acids and cancer-specific mortality in both cohorts of PDAC cases (data not shown). We then tested whether the 4 lipolytic genes were associated with patient outcome. The association of gene expression with cancer-specific mortality was evaluated in both test and validation cohort using Cox regression analysis, in which we dichotomized high and low gene expression as values above and below the median. In our study, univariate Cox regression analysis (Table 2) for all cases showed that only PNLIP was associated with prognosis in both test cohort $[HR 0.36; 95\%\text{ confidence interval (CI), }0.15\text{--}0.88; P = 0.023]$ and validation cohort (HR 0.36; 95\% CI, 0.15--0.87; $P = 0.02$). Therefore, surrogate gene PNLIP maybe a prognostic marker for pancreatic cancer.

Palmitate and stearate inhibits cell growth in vitro

IPA Canonical Pathway analysis identified an enriched set of interactions between the lipid metabolism and the apoptosis signaling pathway (Fig. 1B and C), leading to the hypothesis that fatty acids may regulate apoptosis and cell growth of pancreatic tumors. Therefore, we studied the effects of this unique set of FFAs on the proliferation of human pancreatic cancer cell lines Panc1 and MIApaca2. Palmitate, stearate, linoleate, and oleate
were chosen because they are the most abundant fatty acids in animals (17). MTT cell proliferation assays showed that palmitate and stearate significantly inhibit Panc1 and MIApaca2 cell growth in a dose-dependent manner. In contrast, linoleate and oleate had little effect on cell proliferation in both cells (Fig. 3A). Cell counting and bromodeoxyuridine assays, in addition to MTT assay, also showed significant growth inhibitory effect of palmitate and stearate on Panc1 and MIApaca2 cells (Supplementary Fig. S2). To better assess the growth-inhibitory effect of palmitate and stearate, cell growth curve was generated by incubating Panc1 and MIApaca2 cells in the media containing either BSA alone (as a control) or 0.25 mmol/L FFAs over a 8-day period (Fig. 3B). Our data showed that palmitate and stearate substantially inhibit the growth of pancreatic cancer cells.

**Palmitate and stearate induce TRAIL expression and promote apoptosis in pancreatic cancer cells**

To determine whether palmitate and stearate could affect apoptosis, we examined the caspase-3 activity in Panc1 and MIApaca2 cells following treatment of FFAs for 24 hours. Our data showed that palmitate and stearate (0.25 mmol/L) significantly increased caspase-3/7 activity by 2- to 3- fold as compared with controls in both cell lines \( (P < 0.01, \text{Fig. 4A}) \). To elucidate the potential underlying mechanisms of apoptosis regulation by palmitate and stearate, we then analyzed the gene expression of apoptosis related genes in response to FFA treatment using qRT-PCR and found that palmitate and stearate significantly induced the expression of the pro-apoptotic gene TRAIL by 2- to 3- fold in pancreatic cancer cell lines as compared with controls \( (P < 0.01, \text{Fig. 4B}) \). Consistent with the gene expression, the protein level of TRAIL was also increased by about 3- to 4- fold following 24-hour incubation with 0.25 mmol/L palmitate or stearate. Taken together, these results show that palmitate and stearate induce TRAIL expression and trigger apoptosis in pancreatic cancer cells.

**Discussion**

Altered metabolism is considered as one of the hallmarks of cancer (20). Genetic alterations enable cancer cells to
reprogram metabolism to meet increased energy demands for cell proliferation and to survive in hypoxic and nutrient-deprived tumor microenvironment (21). In this regard, a better understanding of metabolic dysregulation in pancreatic cancer could lead to the discovery of novel therapeutic targets (22). Integrative postgenomic studies and systems biology approaches have emerged with the aim of developing a more comprehensive understanding of cellular physiology and metabolism (23, 24). To the best of our knowledge, here, we report for the first time, the implementation of a systems biology approach to investigate gene-metabolite networks and metabolic dysregulation in pancreatic cancer, with integration of metabolomics and transcriptomics.

Metabolomics allow for global assessment of a cellular state within the context of the immediate environment, taking into account genetic regulation, altered kinetic activity of enzymes, and changes in metabolic reactions (25). Thus, compared with transcriptomics or proteomics, metabolomics reflects changes in phenotype and therefore cellular function (26). Metabolomics strategies have been applied to tissues, serum, and other body fluid, to develop novel early diagnostic biomarkers in human cancers (27–30).

In this study, we identified 55 differentially expressed metabolites using metabolic profiling in two independent cohorts of PDAC cases. Next, we applied WGCNA to analyze metabolic networks in PDAC. WGCNA is a systems biology-based network analysis that has been shown to be an important alternative and a more meaningful tool for discovery of molecular interaction networks and candidate biomarkers (31–35). Using WGCNA, we have identified 8 highly connected fatty acid hubs in a conserved lipid module, which are decreased in tumors as compared with adjacent non-tumor tissues in two independent cohorts of PDAC. Further data mining revealed significant involvement of these fatty acids in cell proliferation and tumorigenesis (36). FFAs play an important role in numerous biologic functions. They serve as a source of energy and as precursors of many signaling and cellular components. The effect of different types of FFAs on cell proliferation and apoptotic activity in pancreatic cancer remains unclear.

### Table 1. A set of coregulated fatty acids are identified using WGCNA on two independent cohorts

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Test cohort</th>
<th>Validation cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P (^a)</td>
<td>Ratio (^b)</td>
</tr>
<tr>
<td>linoleate (18:3n3 or 6)</td>
<td>2.3E-04</td>
<td>0.53</td>
</tr>
<tr>
<td>palmitate (16:0)</td>
<td>4.2E-04</td>
<td>0.53</td>
</tr>
<tr>
<td>margarate (17:0)</td>
<td>1.0E-03</td>
<td>0.56</td>
</tr>
<tr>
<td>stearate (18:0)</td>
<td>1.5E-03</td>
<td>0.58</td>
</tr>
<tr>
<td>linoleate (18:2n6)</td>
<td>1.9E-03</td>
<td>0.58</td>
</tr>
<tr>
<td>oleate (18:1n9)</td>
<td>3.1E-03</td>
<td>0.59</td>
</tr>
<tr>
<td>eicosenoate (20:1n9 or 11)</td>
<td>3.9E-03</td>
<td>0.59</td>
</tr>
<tr>
<td>10-nonadecenoate (19:1n9)</td>
<td>4.1E-03</td>
<td>0.59</td>
</tr>
</tbody>
</table>

\(^a\)P-value calculated using t test in each cohort.

\(^b\)Ratio of tumor vs. non-tumor.

\(^c\)Intramodular connectivity represents the strength of co-expression for each metabolite in network analysis.

### Table 2. Univariate Cox regression analysis on test and validation cohorts

<table>
<thead>
<tr>
<th>Variables (comparison/referent)</th>
<th>Test cohort</th>
<th>Validation cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>PNLIP (high/low)/C3</td>
<td>0.36 (0.15–0.88)</td>
<td>0.023</td>
</tr>
<tr>
<td>CLPS (high/low)</td>
<td>0.34 (0.14–0.84)</td>
<td>0.027</td>
</tr>
<tr>
<td>PNLIPRP1 (high/low) (^a)</td>
<td>0.37 (0.15–0.89)</td>
<td>0.028</td>
</tr>
<tr>
<td>PNLIPRP2 (high/low) (^a)</td>
<td>0.35 (0.14–0.85)</td>
<td>0.020</td>
</tr>
<tr>
<td>Grading (G3&amp;4/1&amp;2)</td>
<td>1.94 (0.89–4.26)</td>
<td>0.097</td>
</tr>
<tr>
<td>Resection Margin (R1/R0)</td>
<td>1.18 (0.54–2.58)</td>
<td>0.677</td>
</tr>
<tr>
<td>Tumor stage (IIIB-IV/I-IIA)</td>
<td>1.07 (0.54–2.14)</td>
<td>0.844</td>
</tr>
</tbody>
</table>

\(^a\)Gene expression value was dichotomized into high and low groups using median. P-value was calculated using univariate cox regression analysis.
this study, we showed for the first time that two major saturated FFAs, palmitate and stearate, exert a strong growth inhibitory effect in pancreatic cancer cells. Our data also showed that palmitate and stearate significantly induce apoptosis in pancreatic cancer cells. These findings are consistent with previous reports on induction of apoptosis by palmitate in other cell types, including breast cancer cells (37), hematopoietic cells (38), pancreatic β-cells (39), and cardiomyocytes (40). In contrast, many studies reported contradictory findings with respect to the role of unsaturated fatty acids in tumor growth, particularly for oleic acid, in breast cancer cells and tumor xenograft models (41). Our functional investigation in different pancreatic cancer cell lines showed that unsaturated FFAs, linoleate and oleate, have no significant effect on the proliferation of pancreatic cancer cells.

It has been proposed that excess palmitate could induce cell death through increased intracellular concentration of ceramide (38, 39), a metabolite exclusively produced from saturated FFAs. However, other studies suggested that apoptosis induced by palmitate could occur through the generation of reactive oxygen species (ROS) rather than ceramide synthesis (40). FFA-induced production of mitochondrial ROS is linked to the activation of protein kinase C (PKC) and the redox-sensitive transcription factor NF-κB (42), which might be involved in the regulation of apoptosis (43). However, the biochemical pathways by which fatty acids influence pancreatic cancer cell growth and death have not been adequately defined. Our data show that palmitate and stearate can upregulate TRAIL expression in pancreatic cancer cells, which may contribute to the apoptosis induced by these two fatty acids.

To further define the genetic alterations and molecular pathways associated with this unique set of fatty acids identified by metabolic profiling, we integrated transcriptomics and metabolomics, and identified 157 gene surrogates for the fatty acid set that is associated with PDAC. Pathway and network analysis revealed that the expected lipid metabolism, particularly in lipolytic pathway involving gene surrogates PNLIP, CLPS, PNLIPRP1, and PNLIPRP2, is significantly altered in PDAC (Fig. 1B and C). Pancreatic lipase, also known as pancreatic triacylglycerol lipase, is encoded by PNLIP, and secreted by the pancreas, and is the primary lipase that hydrolyzes lipids, converting triglyceride substrates to monoglycerides and FFAs (44). Unlike some pancreatic enzymes that are activated by proteolytic cleavage, pancreatic lipase is secreted in its final form. Colipase, encoded by the CLPS gene, is a protein coenzyme required for optimal enzyme activity of pancreatic lipase (45). PNLIPRP1 and PNLIPRP2 code for two novel human pancreatic lipase-related proteins in pancreatic juice, referred to as PNLIP-related proteins 1 and 2, each showing an amino acid sequence identity of 68% to PNLIP. PNLIPRP2 shows a lipolytic activity that is only marginally dependent on the presence of colipase, whereas the function of PNLIPRP1 remains unclear (44). Overall, these functionally related lipases play key roles in direct regulation of fatty acid turnover and signaling. Therefore, decreased expressions of lipases eventually lead to reduced levels of FFAs. Consistent with the function of lipases, our data showed positive correlations between FFA levels and gene expression of these lipases in tumor samples, indicating that a profound dysregulation of the lipolytic network exists in PDAC and may play an important role in tumor growth (Fig. 1C).
Excessive production of pancreatic lipase may indicate the presence of certain disorders, most notably inflammation of the pancreas or pancreatitis (46). Elevated levels of pancreatic lipases also occur in bowel obstruction or kidney disease (47, 48). On the other hand, individuals with Crohn disease, cystic fibrosis, and celiac disease suffer from lipase deficiency, in which the cells of the pancreas responsible for producing this enzyme may be irreversibly damaged (49). The most common symptoms associated with lipase deficiency are muscle spasms, acne, arthritis, gallbladder stress and formation of gallstones, bladder problems, and cystitis. Therefore, pancreatic lipase supplements are used to treat PNLIP deficiency diseases (49). However, the role of PNLIP in pancreatic cancer remains unknown. In this study, we have shown striking decreases (>100-fold) in the gene expression of all four lipases including PNLIP in pancreatic tumors, and consistently, immunohistochemical staining on paraffin sections also showed that PNLIP protein level is lower in PDACs as compared with normal ducts (Supplementary Fig. S3). In addition, our study also provided the first evidence that a lower expression of PNLIP is associated with poor outcome in PDAC.

In summary, we have used a systems biology approach and identified an altered lipolytic network involving lipase genes and a unique set of fatty acids that are associated with pancreatic cancer. This approach moves beyond single gene investigation to provide a systems-level perspective on the potential relationships among members of a biologic network in pancreatic cancer. Our results suggest that the impaired lipolytic pathway may contribute to the development and progression of PDAC. In conclusion, our study showed significant decrease in fatty acids and their surrogate lipase genes in PDAC and showed tumor inhibitory roles of palmitate and stearate in pancreatic cancer. Furthermore, to the best of our knowledge, this is the first report indicating the potential prognostic significance of PNLIP in pancreatic cancer. Further studies are needed to determine the mechanism underlying the altered expression of these lipase genes, and to explore their potential clinical application in PDAC. These studies will lead to a better understanding of the aggressiveness of pancreatic cancer and may facilitate therapeutic target discovery.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: G. Zhang, A. Maitra, S.P. Hussain
Development of methodology: G. Zhang, P. He, T. Ried
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.:) G. Zhang, H. Tan, J. Gaedcke, B.M. Ghadimi, H.G. Yfantis, N. Hanna, H.R. Alexander
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): G. Zhang, P. He, A. Budhu, J. Gaedcke, B.M. Ghadimi, T. Ried, S. P. Hussain

Figure 4. Palmitate and stearate induce TRAIL expression and promote apoptosis. A, there are significant increases in caspase-3/7 activity in Panc1 and MIapaca2 cells with palmitate and stearate treatment. Relative Caspase-3/7 activity represents the effect of FFAs on apoptosis compared with control cells after 24-hour incubation. B, palmitate and stearate upregulate TRAIL expression. Cell lysates were collected after 24-hour of incubation with 0.25 mmol/L FFAs or BSA control. Real-time PCR was conducted to determine TRAIL mRNA levels. TRAIL protein level in cell lysates was measured using an ELISA kit (R&D Systems) according to the manufacturer's instructions. Data represent means ± S.D. from 3 independent experiments. *, t test P < 0.01; ANOVA P < 0.01.
References


Integration of Metabolomics and Transcriptomics Revealed a Fatty Acid Network Exerting Growth Inhibitory Effects in Human Pancreatic Cancer

Geng Zhang, Peijun He, Hanson Tan, et al.

*Clin Cancer Res* Published OnlineFirst August 5, 2013.

**Updated version**

Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-0209

**Supplementary Material**

Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2013/08/02/1078-0432.CCR-13-0209.DC1

**E-mail alerts**

Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**

To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/early/2013/08/15/1078-0432.CCR-13-0209. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.