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A Novel Assessment of Metabolic Pathways in Peritoneal Metastases from Low Grade Appendiceal Mucinous Neoplasms

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Abstract

Background—There is a paucity of targeted therapies for patients with pseudomyxoma peritonei (PMP) secondary to low grade appendiceal mucinous neoplasms (LAMNs). Dysregulated metabolism has emerged as a hallmark of cancer, and the relationship of metabolomics and cancer is an area of active scientific exploration. We sought to characterize phenotypic differences found in PM derived from LAMN versus adenocarcinoma.

Methods—Tumors were washed with PBS, microdissected, then dissociated in ice cold methanol dried and reconstituted in pyridine. Samples were derivatized in TBDMS and subjected to gas chromatography-coupled mass spectrometry. Metabolites were assessed based on a standard library. RNA sequencing was performed, with pathway and network analyses on differentially expressed genes.

Results—Eight peritoneal tumor samples were obtained and analyzed: LAMN (4), and moderate to poorly differentiated adenocarcinoma (colon [1], appendix [3]). Decreases in pyroglutamate, fumarate, and cysteine in PM from LAMN were found compared to adenocarcinoma. Analyses showed the differential gene expression was dominated by the prevalence of metabolic pathways, particularly lipid metabolism. The gene retinol saturase (*RETSAT*), downregulated by LAMN, was involved in the multiple metabolic pathways that involve lipids. Using network mapping, we found IL1B signaling to be a potential top level modulation candidate.

Conclusions—Distinct metabolic signatures may exist for PM from LAMN versus adenocarcinoma. A multitude of genes are differentially regulated, many of which are involved

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in metabolic pathways. Additional research is needed to identify the significance and applicability of targeting metabolic pathways in the potential development of novel therapeutics for these challenging tumors.

Introduction

Peritoneal metastases (PM) are a common site of metastasis from gastrointestinal tumors, particularly of appendiceal and colorectal origin, and remain a major contributor to poor outcomes in these disease processes[1,2]. However, utilization of cytoreductive surgery (CRS) with or without hyperthermic intraperitoneal chemotherapy (HIPEC) has been associated with improved survival across multiple histologies[3–5]. In particular, patients with pseudomyxoma peritonei (PMP) secondary to low grade appendiceal mucinous neoplasms (LAMNs) can experience durable, long-term survival in the setting of an optimal cytoreduction[5]. Unfortunately at times, due to disease burden and location, an optimal cytoreduction is not possible, and systemic cytotoxic therapies for the management of recurrent and/or unresectable disease in this setting are not effective in improving survival[5–7]. Therefore, there is a need for continued advancements in the developments of novel therapeutics to potentially better control these tumors.

The peritoneal cavity is a metabolic microenvironment unlike any other in the body. In the absence of consistent blood supply, these tissues must use the carbon sources available to them in order to expand and grow. Thus, metabolic adaptations that tumors in this body cavity employ could prove to be a unique opportunity for therapies. Dysregulated metabolism has emerged as a hallmark of cancer[8,9]. As a result, metabolic pathways have enormous potential in the clinic, whether via targeting signaling pathways[10,11] or nutrient supplementation[12]. Moreover, it is becoming clear that tumors, particularly metastatic tumors, acquire major metabolic adaptations in order to survive harsh nutrient depleted environments as the tumor expands away from the blood supply[13]. The relationship of metabolomics and cancer is an area of active scientific exploration; however, literature on metabolomics in PM is relatively sparse. Recent studies have begun to elucidate the metabolic environment in PM from ovarian cancer, but this has not been well-described in PM from LAMN[14–16].

Thus, we sought, first, to investigate a comprehensive comparative metabolic profile for PM from different histological backgrounds using gas chromatography coupled mass spectrometry (GC-MS). We then performed global gene expression using RNA Sequencing and performed pathway analyses in PM from LAMN versus adenocarcinoma. We found tumors from these subtypes exhibit significant differences in carbon metabolism, lipid pathways and lipid content. Insight from these studies provide a first look into the phenotypic differences found in PM derived from LAMN. These results in combination with the ability to access these tumors suggest metabolic interventions may be advantageous to pursue for the treatment of these tumors.

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Methods

Tumor Acquisition

Deidentified peritoneal tumor samples were obtained, frozen, and transferred for processing via the University of California, Irvine Experimental Tissue Shared Resource Facility in accordance with university policy, procedures, and protocols. Tumors were washed with PBS, micro-dissected and frozen at −80C.

Untargeted Metabolomics

50mg of frozen tumors was pulverized in −20C 50%:50% methanol:water using the PerCellys bead blaster. Samples were spun and the top aqueous layer was collected to a new tube. These samples were dried in a speed vac. Samples were reconstituted in pyridine containing 10mg/ml MOX overnight at RT. Samples were derivatized in TBDMS at 70C for 1hr and subjected to gas chromatography-coupled mass spectrometry on the Agilent 7820A GC/5977B GC/MSD[17]. Metabolites were identified by comparing them to a library of purified standards using the MassHunter suite (Agilent).

RNA Sequencing

RNA was extracted from 100mg of tumor using Trizol (Invitrogen). rRNA depletion was performed and the library was constructed using the Illumina TruSeq stranded Total RNA Ribo-Zero Gold kit. Illumina RNA sequencing was performed using the MiSeq System (Illumina), with 20M paired end reads per sample. Reads were trimmed using the Trimmomatic[18] package and observed for quality using Fastqc (Babraham Bioinformatics). Genome alignment to the human genome sequence 43 was performed using the STAR alignment tool[19]. Counts were generated using FeatureCounts[20] and differential expression was performed using the DESeq2 package[21]. Differentially expressed genes were subjected to pathway analysis using the Gene Set Enrichment Analysis (GSEA) and Molecular Signatures Database (MSigDB) software suite[22]. Network analysis was performed on differentially expressed genes using the Cytoscape software suite[23]. All scripts for this project can be found at https://github.com/erichanse/RNASeqCode/. Sequence data will be deposited to the Gene Expression Omnibus.

Results

To investigate potential metabolic differences, a total of eight peritoneal tumor samples were obtained and analyzed. The tumors represented the following primary histologies: low grade appendiceal mucinous neoplasm (4), and moderate to poorly differentiated adenocarcinoma (colon [1], appendix [3]). Detailed information about the patient cohort can be found in Table 1. We extracted metabolites from these samples and subjected them to GC-MS to identify the relative concentrations of some of the most common amino acids and TCA metabolites.

Metabolic differences between PM from LAMN and Adenocarcinoma

A heat map of the identified metabolites PM from LAMN and adenocarcinoma is demonstrated in Figure 1A. Statistically significant decreases in pyroglutamate, fumarate,

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and cysteine in PM from LAMN were found compared to adenocarcinoma (Figure 1B). Fumarate is part of the TCA cycle and may indicate a slowing of the TCA cycle in LAMN, although more studies need to be performed to conclude this. Interestingly, cysteine and pyroglutamate are both metabolites involved in glutathione (GSH) synthesis, and deficiency in GSH enzymes can lead to spikes in pyroglutamate levels[24,25] . Glutathione is a critical modulator of reactive oxygen species in proliferating cells and tumors particularly when metabolism is strained [26–28].

Comparative expression analysis between PM from LAMN and Adenocarcinoma

RNA sequencing of tumors from LAMN and Adenocarcinoma was performed to better understand the phenotypic differences observed in these tumors clinically. We found that a principal component analysis showed LAMN samples cluster close together compared to the adenocarcinoma samples, which showed a much wider variance amongst samples (Figure 2A). These data suggest LAMNs may share a conserved biology and make them a more uniform therapeutic target. The top 25 up- and down-regulated genes in LAMN versus adenocarcinoma PMs are shown in Figure 2B. Many of the genes found to be differentially regulated include unidentified open reading frames. Figure 2C is a heatmap of the top 50 up- and down-regulated genes. The samples also mainly cluster within subtypes. We next employed GSEA pathway analysis on the complete list of differentially expressed genes (Figure 3A). We found this list was dominated by the prevalence of metabolic pathways, particularly lipid metabolism (Figure 3A–3B). To gain some insight into the regulation of these pathways, we next looked at leading edge analysis to identify which genes overlapped the most in these pathways (Figure 3C). We found that the gene retinol saturase (*RETSAT*), downregulated by LAMN, is involved in the multiple metabolic pathways that involve lipids, but not carbohydrate metabolism. Next, we analyzed the network signaling regulation of these pathways in the hopes of identifying top level signaling contributors to these pathways. Using Cytoscape network mapping, we found IL1B signaling to be a potential top level modulation candidate (Figure 3D). Importantly, IL1B was found to be one of the top down-regulated genes in LAMN versus adenocarcinoma RNASeq (Figure 2B). Together, these results suggest PM from LAMN regulate lipid metabolism differently than PM from adenocarcinoma and represent a potential window of opportunity for new therapeutic avenues.

Discussion

The presence of altered metabolism in cancer cells was first described by Warburg: the term “Warburg Effect” is characterized by increased utilization of glycolysis even in the presence of oxygen, and therefore termed as aerobic glycolysis[29]. The exact role of these metabolic processes in relation to cell proliferation and oncogenesis is an active area of therapeutic pursuit. The tumor’s ability to regulate various biosynthetic pathways during periods of metabolic stress may potentially provide a conduit for to identify novel therapeutic targets. The development, proliferation, and propagation of peritoneal metastases in the abdominal cavity contains a number of ongoing mechanistic explorations regarding the peritoneal tumor microenvironment. For instance, given the heterogeneity of vascular supply to these tumors, recent studies have explored the role of angiogenesis in

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the development and propagation of PM[30]. Other studies have investigated the dynamic immune environment in relation to responsiveness (or lack thereof) to systemic therapies and checkpoint inhibition[31,32]. Yet, research on the metabolic microenvironment of PM and furthermore, the application of metabolomics in peritoneal metastasis research is in its nascent stages.

Pan et al analyzed metabolic components of peritoneal lavage fluid in patients with gastric cancer and found that metabolites such as sulfite, retinol, glutamyl alanine, and 3-methylalanine were potential independent risk factors for peritoneal metastasis in some groups[33]. Interestingly, this study also noted that some of these metabolites were indeed lipid metabolites. In contrast to GI-related PM, the literature on ovarian cancer has explored the mechanistic impact of metabolomics in a more in-depth manner. For example, Mukherjee et al performed unbiased mass-spectrometry-based metabolomics on ovarian cancer cells co-cultured with adipocytes. The authors found that the lipid chaperone protein FABP4 was upregulated in the presence of adipocytes, and resultant knockdown models demonstrated downregulation of gene signatures and reduced metastatic tumors in mice[15]. More recently, Yang et al reported on ovarian cancer progression with alkaline malignant ascites, and patients with more advanced cancer had higher ascitic concentrations of alanine, isoleucine, phenylalanine, and glutamine[16].

One of the main observations from our data is the prevalence of genes involved in lipid metabolism pathways in the setting of untargeted metabolomics; this suggests that targeting and/or manipulating these pathways may indeed be impactful. Another observation is the differential regulation of genes involved in oxidative phosphorylation pathways, which suggests a relative difference in Warburg effect between PM from LAMN versus adenocarcinoma; however, much more work needs to be done to further elucidate any pathways implicated by these data. Interestingly, our data suggests a potential role for glutathione in LAMN. Glutathione generation is extremely important for the protection of the proteome and genome from reactive oxygen species (ROS)[34]. However, tumor cells benefit from the high mutation rate low glutathione levels afford. Thus, glutathione level modulation could be another potential avenue of therapeutic pursuit for these patients. Interestingly, one of the differentially down-regulated genes in PM from LAMN, *RETSAT*, has been shown to be down-regulated in other human cancers[35,36]. However, higher *RETSAT* levels in pancreatic ductal adenocarcinoma were found to be associated with decreased responsiveness to gemcitabine-based chemotherapy and poorer survival[35]. IL1B is an important cytokine that has been described as being a part of the general inflammation of adipose tissue[37]. In the colon, IL-1B signaling is found to be correlated with increased CRC[38]. Moreover, IL1B is part of a signature of cytokines that conveys resistance to EGFR targeted therapies in CRC[39]. This raises an interesting question as to whether IL1B may be playing a high-level role in promoting not only the lipid metabolism, but some of the other phenotypic differences we observe between these subtypes. With the generation of IL1B receptor sponges such as rilonacept and the monoclonal antibody canakinumab, potential usage for PM could be beneficial. Unbiasedly, the most significantly modulated pathways in LAMN versus adenocarcinoma human patients involved some form of lipid metabolism. We hypothesized metabolism pathways would be different between these groups, but we were

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surprised to find an unbiased prevalence of lipid metabolism pathways different between these groups.

Limitations of this study include the deidentified acquisition of peritoneal tumors and therefore inability to correlate with clinical outcomes. The number of tumor samples was low and heterogeneous, and we are actively working on increasing our numbers for future experiments. In addition, while the metabolic microenvironment is dynamic, these tumors each represent a discrete time point. Planned likely next steps include additional mechanistic exploration (perhaps via knockout models) and assessing these findings via the development of organoids, and subsequently the responses of the organoid models to metabolic alterations.

We believe that our study is the first to report on findings highlighting the different metabolic profile between patients with PM secondary to LAMN versus higher-grade adenocarcinoma. This is certainly important as it provides an opportunity to explore the mechanistic implications of the observed differential gene expressions and metabolic pathways for potential future therapeutics.

In conclusion, distinct metabolic signatures may exist for PM from LAMN versus adenocarcinoma. A multitude of genes are differentially regulated, many of which are involved in metabolic pathways. Additional research is needed to identify the significance and applicability of targeting metabolic pathways in the potential development of novel therapeutics for these challenging tumors.

Acknowledgements

EAH, OSE and MK conceived the project. MK and OSE supervised the project. EAH conducted all experiments and analyzed data. EAH and OE wrote the manuscript. TW provided technical assistance with metabolomics experiments and technical assistance with RNASeq data. DT provided tumor samples. MS and ACK provided valuable clinical advice. This work was supported by NIH R01GM132142 and R01CA244360 (MK).

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SYNOPSIS

Distinct metabolic signatures may exist for PM from LAMN versus adenocarcinoma. A multitude of genes are differentially regulated, many of which are involved in metabolic pathways.

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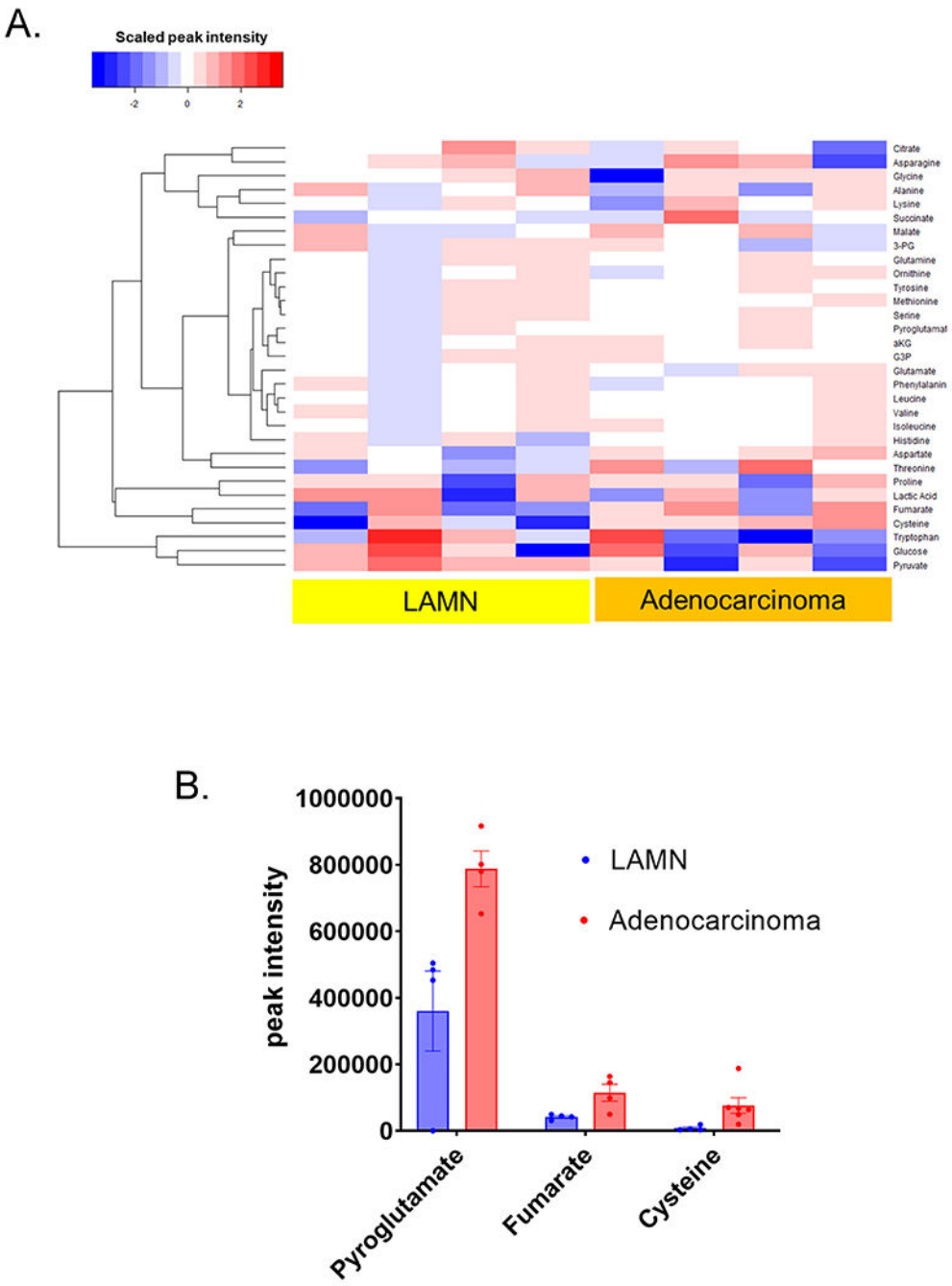

Figure 1. Metabolomic Fingerprint of PM LAMN and Adenocarcinoma.

(A) Heatmap showing the scaled peak intensity for identified metabolites.

(B) Shown are the significantly different metabolites between LAMN and Adenocarcinoma after averaging spectral counts and comparing between groups for each metabolite using a Student's T-test. Results are representative of at least two separate GC/MS runs.

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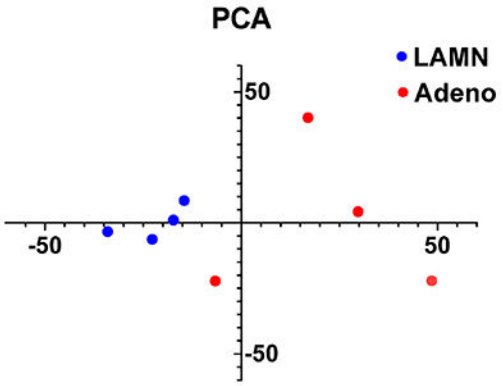
A.



B.

Gene Name	log2FoldChange	pvalue	Gene Name	log2FoldChange	pvalue
ENSG00000203335	5.85	1.8E-07	PLK1	-4.17	7.2E-08
ENSG00000286848	5.36	6.1E-04	FMN1	-4.17	3.1E-04
ENSG00000278456	4.09	2.1E-09	TSHZ2	-4.23	2.3E-05
SMARCB1	4.06	1.5E-04	GTF2A2	-4.30	3.2E-06
GNGT1	3.97	4.5E-04	ENSG00000269495	-4.32	7.5E-07
ETV7	3.72	5.0E-05	HOXB-AS3	-4.49	3.6E-12
LINC01940	3.58	7.9E-05	RNF38	-4.61	1.1E-05
ENSG00000205037	3.48	7.8E-05	C9orf50	-4.66	1.3E-07
PXT1	3.40	8.2E-04	MFSD14B	-4.68	3.3E-07
ABCA8	3.16	2.1E-04	IL1B	-4.75	4.1E-06
ENSG00000290062	3.15	1.4E-05	IL1A	-4.80	6.7E-06
EVC	3.15	2.2E-04	CROCC2	-4.97	4.8E-06
KCTD19	3.14	1.4E-04	BAHCC1	-5.16	1.7E-05
ENSG00000290010	3.06	1.5E-04	HOXB-AS4	-5.20	4.0E-07
ENSG00000264843	3.06	2.2E-03	GDE1	-5.23	1.8E-06
LINC00702	3.05	1.9E-04	IDI1	-5.57	2.1E-06
ENSG00000290038	2.93	1.0E-04	C12orf43	-5.63	1.8E-12
STK32A	2.92	2.3E-03	MYO16	-5.69	1.4E-05
ALDH1A3-AS1	2.86	4.4E-04	GRTP1-AS1	-5.80	2.6E-13
TMEM52B	2.84	2.4E-03	USP37	-5.82	5.6E-20
GNA12	2.84	1.3E-04	LINC02997	-6.33	2.3E-06
TSTD2	2.82	2.6E-03	PNPO	-6.45	6.9E-10
ENSG00000253420	2.80	3.2E-06	LINC02042	-6.70	4.1E-08
ENSG00000254192	2.73	4.5E-05	PKD2L2-DT	-6.99	1.5E-07

C.

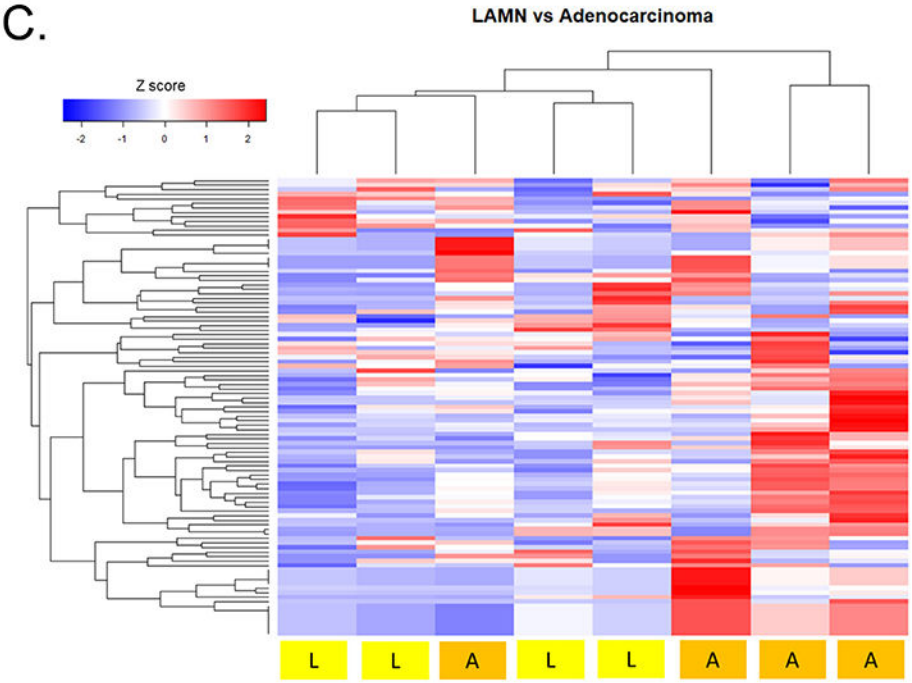


Figure 2. Expression profiling between PM LAMN and Adenocarcinoma.

(A) Principal component analysis demonstrating the variance between samples. Notice the LAMN samples mostly cluster together indicating a more uniform expression profile among these samples. Adenocarcinoma samples were unrelated to LAMN and less related to each other.

(B) Top up- and down- regulated genes found by RNASeq.

(C) Heatmap and hierarchal clustering of the gene sets and samples showing LAMN cluster together, while adenocarcinomas show more variance.

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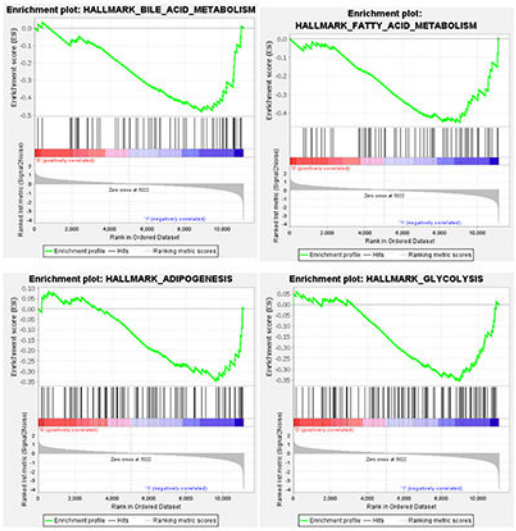
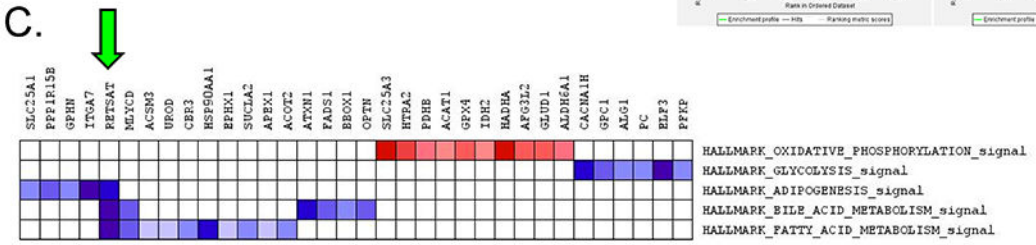
A.

	GSEA Pathway	NOM p-val	FDR q-val	FWER p-val
1	HALLMARK_BILE_ACID_METABOLISM	0.04	0.327	0.204
2	HALLMARK_FATTY_ACID_METABOLISM	0	0.237	0.279
3	HALLMARK_ADIPOGENESIS	0	0.185	0.315
4	HALLMARK_PEROXISOME	0.089	0.143	0.315
5	HALLMARK_E2F_TARGETS	0	0.28	0.647
6	HALLMARK_CHOLESTEROL_HOMEOSTASIS	0	0.267	0.713
7	HALLMARK_GLYCOLYSIS	0.033	0.252	0.754
8	HALLMARK_MITOTIC_SPINDLE	0.056	0.299	0.802
9	HALLMARK_MYC_TARGETS_V1	0.111	0.287	0.802
10	HALLMARK_ESTROGEN_RESPONSE_EARLY	0.08	0.316	0.861
11	HALLMARK_MYOGENESIS	0.145	0.394	0.956
12	HALLMARK_G2M_CHECKPOINT	0.176	0.369	0.956
13	HALLMARK_DNA_REPAIR	0.183	0.357	0.973
14	HALLMARK_SPERMATOGENESIS	0.116	0.465	0.993
15	HALLMARK_IL6_JAK_STAT3_SIGNALING	0.262	0.442	0.993
16	HALLMARK_HEME_METABOLISM	0.155	0.425	0.993
17	HALLMARK_ESTROGEN_RESPONSE_LATE	0.319	0.526	0.993
18	HALLMARK_XENOBIOTIC_METABOLISM	0.241	0.505	0.993
19	HALLMARK_COMPLEMENT	0.19	0.5	0.993
20	HALLMARK_APICAL_JUNCTION	0.232	0.488	0.993

B.



C.



D.

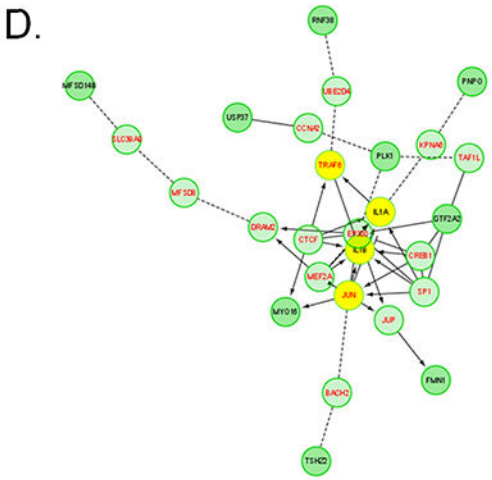


Figure 3. Pathway Analysis of gene expression profiling between PM LAMN and Adenocarcinoma.

(A) Top unbiased GSEA/MSigDB Hallmark pathways identified as being affected by the gene set shown in Figure 2.

(B) Enrichment Plots for lipid and carbohydrate metabolism pathways identified in (A).

(C) Leading Edge Analysis identifies RETSAT as overlapping in all of the down-regulated (in LAMN) lipid metabolism pathways.

(D) Gene network analysis using Cytoscape, identifies IL1B as potentially playing a role in orchestrating some of the gene network regulation observed.

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Table 1.

Patient demographics.

Age	Sex	Race/Ethnicity	Prior Systemic Therapy	Diagnosis
54	M	White	No	Low grade appendiceal mucinous neoplasm
42	M	White	No	Poorly differentiated appendiceal adenocarcinoma
56	F	Other/Mixed	No	Low grade appendiceal mucinous neoplasm
67	M	White	FOLFOX/Avastin, Lonsurf	Poorly differentiated appendiceal adenocarcinoma
63	F	White	XELOX, FOLFIRI	Moderately differentiated colon adenocarcinoma
71	M	White	No	Low grade appendiceal mucinous neoplasm
51	M	Other/Mixed	FOLFOX	Moderately differentiated appendiceal adenocarcinoma
63	F	White	No	Low grade appendiceal mucinous neoplasm