Brief Communication
Insights into autotaxin- and lysophosphatidate-mediated signaling in the pancreatic ductal adenocarcinoma tumor microenvironment: a survey of pathway gene expression

Matthew GK Benesch[1]*, Rongrong Wu[1,2]*, Colin J Rog[1], David N Brindley[3], Takashi Ishikawa[2], Kazuaki Takabe[1,2,4,5,6,7]

[1]Department of Surgical Oncology, Roswell Park Comprehensive Cancer Center, Buffalo, New York 14263, USA; [2]Department of Breast Surgery and Oncology, Tokyo Medical University, Tokyo 160-8402, Japan; [3]Cancer Research Institute of Northern Alberta, Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2S7, Canada; [4]Department of Gastroenterological Surgery, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan; [5]Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata 951-8520, Japan; [6]Department of Breast Surgery, Fukushima Medical University School of Medicine, Fukushima 960-1295, Japan; [7]Department of Surgery, University at Buffalo Jacobs School of Medicine and Biomedical Sciences, State University of New York, Buffalo, New York 14263, USA. *Equal contributors.

Abstract: Lysophosphatidate (LPA)-mediated signaling is a vital component of physiological wound healing, but the pathway is subverted to mediate chronic inflammatory signaling in many pathologies, including cancers. LPA, as an extracellular signaling molecule, is produced by the enzyme autotaxin (ATX, gene name *ENPP2*) and signals through six LPA receptors (LPARs). Its signaling is terminated by turnover via the ecto-activity of three lipid phosphate phosphatases (LPPs, gene names *PLPP1-3*). Many pharmacological developments against the LPA-signaling axis are underway, primarily against ATX. An ATX inhibitor against pancreatic ductal adenocarcinoma (PDAC), a very aggressive disease with limited systemic therapeutic options, is currently in clinical trials, and represents the first in-class drug against LPA signaling in cancers. In the present study, we surveyed the expression of ATX, LPARs, and LPPs in human PDACs and their clinical outcomes in two large independent cohorts, the Cancer Genome Atlas (TCGA) and GSE21501. Correlation among gene expressions, biological function and the cell composition of the tumor microenvironment were analysed using gene set enrichment analysis and cell cyber-sorting with xCell. *ENPP2, LPAR1, LPAR4, LPAR5, LPAR6, PLPP1*, and *PLPP2* were significantly elevated in PDACs compared to normal pancreatic tissue, whereas *LPAR2, LPAR3,* and *PLPP3* where downregulated (all P≤0.003). Only *ENPP2* demonstrated survival differences, with overall survival favoring *ENPP2*-high patients (hazard ration 0.5-0.9). *ENPP2* was also the only gene with enriched gene patterns for inflammatory and tissue repair gene sets. Epithelial (cancer) cells had increased *LPAR2, LPAR5* and *PLPP2* expression, and decreased *ENPP2, LPAR1, PLPP1*, and *PLPP3* gene expression (all P<0.02). Tumor fibroblasts had increased *ENPP2, LPAR2, LPAR4, PLPP1*, and *PLPP3* expression and decreased *LPAR2, LPAR5*, and *PLPP2* expression in both cohorts (all P≤0.01). Immune cell populations were not well correlated to gene expression in PDACs, but across both cohorts, cytolytic scores were increased in high-expressing *ENPP2, LPAR1, LPAR6, PLPP1*, and *PLPP3* tumors (P<0.01). Overall, in PDACs, *ENPP2* may switch from an anti-to-pro tumor promoting gene with disease progression. *LPAR2* and *PLPP2* inhibition are also predicted to have potential therapeutic utility. Future multi-omics investigations are necessarily to validate which LPA signaling components are high-value candidates for pharmacological manipulation in PDAC treatment.

Keywords: Bioinformatics, drug candidates, lysophosphatidic acid, prognostic biomarkers, pancreatic cancer, stroma
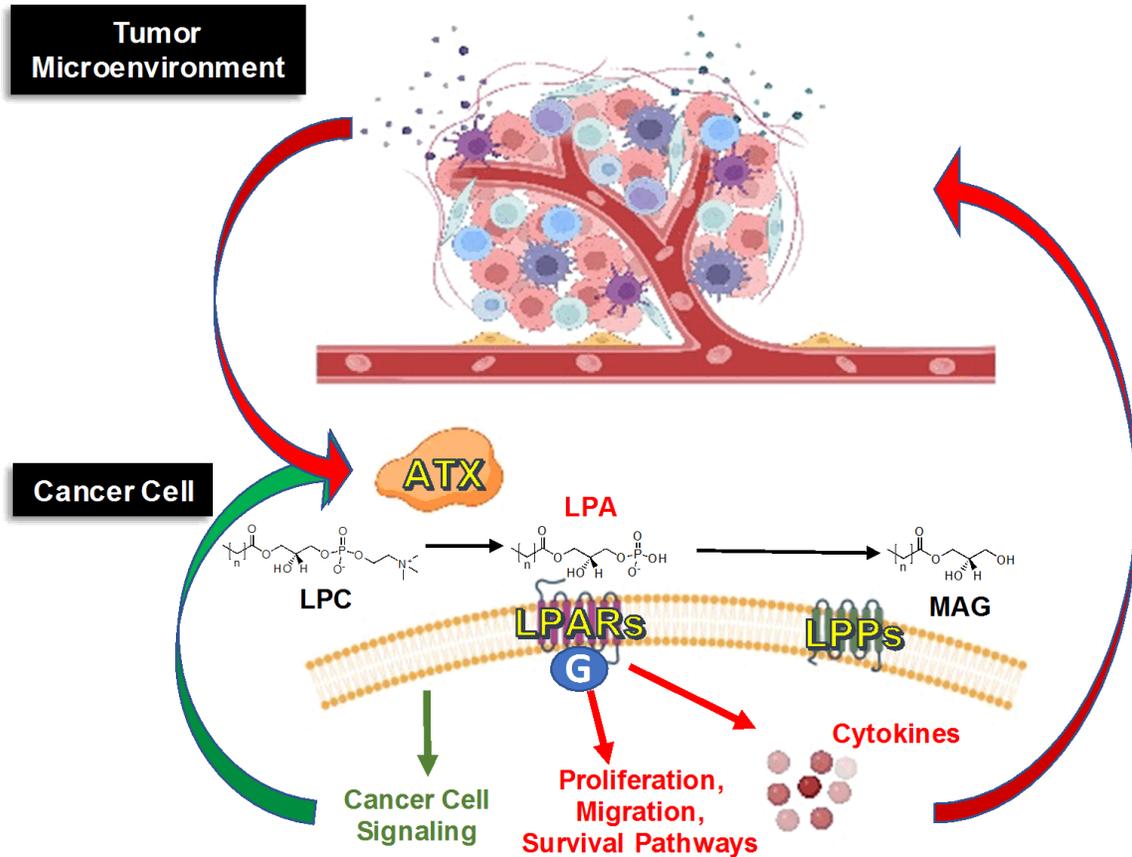
Figure 1. Overview of lysophosphatidate (LPA) signalling in the cancer and the surrounding tumor microenvironment. Extracellular LPA is produced by the lysophosphatidate D activity of the extracellular enzyme autotaxin (ATX) from plasma lysophosphatidylcholine (LPC). LPA signals through six G-protein receptors (LPARs) to activate many signaling pathways involved in cancer pathogenesis. LPA signaling is terminated by its breakdown into monoacylglycerol (MAG) via the ecto-activity of the lipid phosphate phosphatases (LPPs). ATX can be produced by cancer cells in an autocrine fashion by upregulated carcinogenic pathways, including LPA induced cytokine-mediated signaling, establishing a positive feedback loop. Cytokines within the surrounding tumor microenvironment can also induce ATX upregulation in tumor stroma for paracrine-mediated LPA signaling in cancer cells.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer that has not benefited well from continued advances in multimodal therapeutic options, with 5-year overall survival rates essentially plateaued at about 11% [1]. Although it is the tenth most diagnosed cancer, it is third in annual cancer deaths, and is projected to overtake colorectal cancer for second place before the end of the decade [1, 2]. Extensive research efforts are ongoing to overcome this aggressive tumor biology defined by its dense desmoplastic tissue and low tumor mutational burden, which both support a pro-survival milieu and tumor microenvironment [3-5].

Lysophosphatidate (LPA) functions as an extracellular bioactive lipid with numerous physiological roles central to proper embryogenesis and wound healing [6, 7]. However, these mechanisms are readily hijacked in malignancies to fuel pathways of chronic inflammation to both promote cancer progression and metastasis, and loss of efficacy for both chemotherapy and radiotherapy regimens [8]. Extracellular LPA is produced primarily from albumin-bound lysophosphatidylcholine (LPC) by the lysophospholipase D activity of autotaxin (ATX, gene name *ENPP2*) [9, 10] (**Figure 1**). ATX, primarily produced within the local cellular microenvironment, interacts with extracellular-membrane integrins to concentrate LPA production within the vicinity of the targeted cells [11]. LPA then

signals through six known G-protein coupled receptors (LPARs, gene names *LPAR1-6*) to elicit its mechanistic effects [12] (**Figure 1**). Receptor affinity to differing G-protein combinations result in synergetic, redundant, and antagonistic intracellular responses that ultimately define the nature of the LPA-signaling transduction cascade [6, 13]. Extracellular LPA-mediated signaling is terminated by its degradation into monoacylglycerols (MAGs) and inorganic phosphates by the ecto-activities of three unique lipid phosphate phosphatases (LPPs, gene names *PLPP1-3*), particularly LPP1 [14] (**Figure 1**).

In general, the LPA signalling cascade is upregulated in aggressive cancers, resulting in a tumor microenvironment that favors disease progression [8, 10, 15]. This occurs via several concurrent mechanisms. First, LPA concentrations are typically increased through increased ATX production [16]. This ATX can either be overexpressed by the cancer cells themselves, as seen in melanoma, thyroid carcinoma, hepatocellular carcinoma, and glioblastoma multiforme. Alternatively, tumor-induced inflammation increases ATX synthesis by cells in the surrounding tumor stroma such as in breast cancer and PDAC [6, 7, 15]. Additionally, ATX is within the top 40-50 most upregulated genes in both locally invasive and metastatic tumors [17, 18]. This additional LPA signals to elicit the mechanisms of cancer progression and therapy resistance through an enriched cancer cell LPAR profile [19]. LPP1 and LPP3 tend to be overall suppressed in most cancers, resulting in decreased ecto-LPP activity and therefore less turnover of LPA [14, 20, 21]. LPP2 functions differently and it is increased in tumors resulting in increased rates of S-phase entry through the cell cycle via upregulation of transcription by c-Myc [20, 22, 23].

The LPA pathway has been the target of much therapeutic development over the past 20 years for both cancer and chronic inflammatory diseases [6, 9]. Inhibitors against ATX and the LPARs have been studied in clinical trials, primary for idiopathic pulmonary fibrosis (IPF) [24]. Zirtaxestat, also known as GLPG1690, was the first ATX inhibitor to enter clinical trials, culminating into two phase III double-blinded and placebo-controlled trails combining zirtaxestat with standard of care therapies for IPF

(ISABELA 1 and 2) [25]. Additionally, there are at least two other ATX inhibitors (cudetaxestat or BLD-0409 and BBT-877) currently in phase II trials for IPF, with results expected in mid-to-late 2024 [26, 27]. Another ATX inhibitor, IOA-289, has been shown to inhibit tumor growth and lung and bone metastases in synergistic immunocompetent orthotopic murine models of breast cancer [28, 29], similar to other ATX inhibitors trialed in pre-clinical settings [30, 31]. IOA-289 has also been shown to reduce gastrointestinal cancer progression in pre-clinical models, including those for PDAC [32]. IOA-289 has since entered a phase 1b, open label, dose-escalation study in combination with gemcitabine/nab-paclitaxel, a standard of care treatment regimen [33], in patients with metastatic PDAC [34]. This clinical trial represents the first LPA pathway inhibitor used in cancer therapy.

Compared to most other common cancer types, the role of LPA signaling in the PDAC tumor microenvironment has not been as well studied. We have previously explored the role of mRNA expression in PDAC using *in silico* research methodologies [35-38]. We have also used these techniques to explore ATX, LPAR, and LPP expression in human breast tumors, thereby allowing for meaningful comparisons to the evolving body of literature in pre-clinical models [39-41]. In this study, we combine our expertise from these investigations to survey the effects of LPA signaling in PDACs by tumor cell populations using large databases of two independent cohorts. We develop novel insights in the role of LPA-mediated signaling in human PDACs, which may facilitate both the interpretation of the upcoming results from the IOA-289 PDAC clinical trial, and identity other high yield targets in the LPA-signaling pathway for future trials.

Methods

Clinical and mRNA expression PDAC data was obtained from two well-resourced databases: The Cancer Genome Atlas Program (TCGA) (n=146) via the cBioPortal (https://www.cbioportal.org), and a validation cohort of 132 patients, GSE21501 via the Gene Expression Omibus (GEO) repository of the United States National Institutes of Health (https://www.ncbi.mln.nih.gov/geo) [42, 43]. The expression data

for TCGA was log-transformed using "data_mrna_seq_v2_rsem", while GSE21501 data was downloaded already normalized and used without any further processing, as previously described [37, 39, 44]. Briefly, after the gene symbols were annotated with the specified Platform (GPL) accession number, the average value was used if the same gene was assigned to multiple probes. Gene expression data from 167 samples of normal pancreatic tissue from Genotype-Tissue Expression (GTEx) was obtained from the University of California Santa Cruz Xena Portal (https://xena.ucsc.edu) [45, 46]. As all data was obtained from deidentified databases in the public domain, ethics approval requirements were waived by the Roswell Park Institutional Review Board.

Functional enrichment analysis of genes examined was performed by gene set enrichment analysis (GSEA) [47] on the Molecular Signatures Database Hallmark collection (http://www.gsea-msigdb.org) [48]. Gene sets with a false discovery rate (FDR) <0.25 specified enriched signaling [47]. High and low gene expression groups were dichotomized by median gene expression. Positive normalized enriched scores (NES) indicate enriched signaling in the high expression group and negative NES indicate enriched signaling in the low expression group.

The xCell algorithm (https://xcell.ucsf.edu) [49] was used to correlate gene expression to the infiltrating fraction of tumor and stromal cells (epithelial cells, endothelial cells, and fibroblasts), and immune cells (CD8+, T helper cell (Th)1 and Th2 cells, T-regulator cells, M1 and M2 macrophages, and dendritic cells) as described [50-53]. The pancreatic cancer mutational landscape (intratumor heterogeneity, homologous recombination defects, fraction genome altered, silent mutation rate, non-silent mutation rate, single-nucleotide neoantigens, and indel mutations) was examined from data derived by Thorsson *et al.* [54]. Immune cytolytic activity (CYT) in the tumor microenvironment was calculated as the geometric mean of the expression of perforin (*PRF1*) and granzyme A (*GZMA*) mRNA expression, which measures the anti-cancer ability of cytotoxic T cells [55].

Statistical analyses and figure production were performed with R-4.2.1 and BioRender (https://www.biorender.com). mRNA levels for individual genes were dichotomized into low and high groups based on the median expression level. All results are plotted as box plots, with the lower and upper bounds representing the maximum and minimum values, the upper and lower ends of box representing the 25th and 75th percentile values and the bolded bar within the box representing the median value. Two group comparisons were performed using the Mann-Whitney U test and multiple group comparisons by the Kruskal-Wallis test. The R survival software package was used to analyze survival based on high or low gene expression via Cox-proportional hazards regression, and Kaplan-Meier survival curves were compared by the log rank test. $P<0.05$ was set for statistical significance.

Results

When comparing expression levels between normal pancreatic tissue to PDAC, *ENPP2, LPAR1, LPAR4, LPAR5, LPAR6, PLPP1*, and *PLPP2* were significantly elevated in the PDAC group, whereas *LPAR2, LPAR3,* and *PLPP3* were downregulated (all P≤0.003) (**Figure 2**). However, within the PDAC tumors, there was no significant correlation between gene expression level and stage of disease (**Figure 3A**). Apart from *PLPP1*, which showed a significant decrease in expression level with grade progression (Grade 1 to 3) (P=0.04), there were no other correlations between tumor grade and gene expression levels (**Figure 3B**). Also, apart from *LPAR5*, which showed higher levels of Ki67 scoring in tumors with high-*LPAR5* expression in both cohorts (all P≤0.005), there were no consistent correlations between Ki67 scoring and gene expression when dichotomized on the median into low- and high-expression groups (**Figure 3C**).

We next examined survival parameters based on median dichotomized gene expression. The only gene with any statistically significant survival differences in either cohort was *ENPP2*. In the TCGA data, progression-free survival, disease-free survival, disease-specific survival, and overall survival favored the *ENPP2*-high expressing group, with hazard ratios (HRs) ranging from 0.25-0.47 (all P<0.001, **Figure 4**). However, in GSE21501 where overall survival was the only recorded metric, there were no
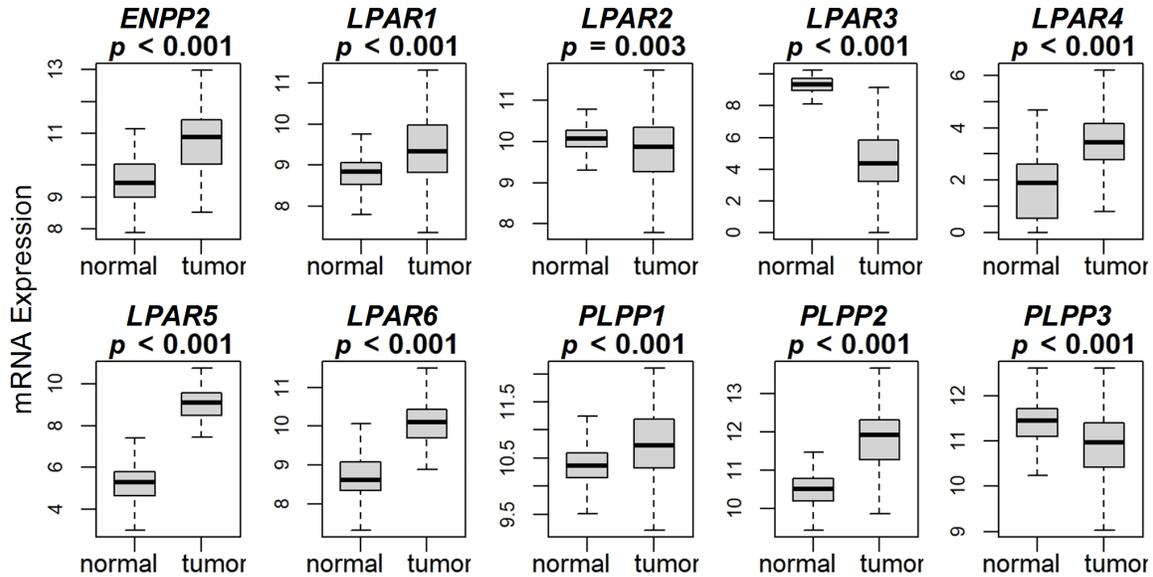
Figure 2. LPA signaling-related gene expression in PDACs compared to normal pancreatic tissues. mRNA expression from 167 normal pancreas in the GTex database is compared to 146 PDACs. Results are plotted as box plots with the bolded center bar within the box plots represents the median, the lower and upper box bounds represent the 25th and 75th percentiles, respectively, and the lower and upper tails represent the minimum and maximum values, respectively.

meaningful differences between the two groups (P=0.8, **Figure 4**). We then performed gene set enrichment analyses (GSEA) using the Hallmark gene sets across all genes in both cohorts. Again, *ENPP2* was the only gene with significant normalized enrichment scores (NES) in both cohorts (allograft rejection, complement, and IL6-JAK-STAT3 signaling) (all NES 1.5-2). *LPAR3, LPAR4, LPAR6, PLPP1*, and *PLPP2* had significantly enriched gene sets in at least one cohort, but were not validated in the other cohort (**Figure 5**). There were no enriched gene sets in *LPAR1, LPAR2, LPAR5,* or *PLPP3* in either cohort. The full GSEA output for all genes by the Hallmark gene set is available in Supplementary Table 1.

Although PDACs tend to have a lower overall tumor mutational burden compared to most cancers, it is a prognostic marker of aggressive tumor biology [56]. We examined common scores of tumor mutational burden by median gene expression. Intratumor heterogeneity scores were not correlated with any of the genes (**Figure 6**). However, high-*ENPP2* expression correlated to lower scores for homologous recombination defects (HRDs), fraction genome altered (FGA), silent mutation rate (SMR), non-silent mutation rate (NSMR), single-nucleotide

variant (SNV) neoantigens, and indel mutations (all P<0.04, **Figure 6**). This same pattern occurred also in high-expressing *LPAR1, LPAR4, PLPP1,* and *PLPP3* tumors (all P< 0.001), except for indel mutations (**Figure 6**). HRD and FGA were correlated to high-expression *LPAR2* tumors (all P<0.04, **Figure 6**). High *LPAR5*-expressing tumors correlated with increased FGA, SMR, NSMR, and indel mutations (all P<0.04, **Figure 6**). High *LPAR6*-expressing correlated with lower FGA, SMR, and NSMR scores (all P≤0.04, **Figure 6**). Finally, high *PLPP2*-expressing tumors correlated to higher scores for HRD, FGA, SMR, NSMR, and SNV neoantigen scores (all P<0.001, **Figure 6**).

We next examined gene expression by cyber-sorted tumor cell populations. Among epithelial cells, representing cancer cells within the PDAC tumor, their levels were decreased in high-expressing *ENPP2, LPAR1, PLPP1,* and *PLPP3* tumors across both cohorts, while levels were increased in high *LPAR2, LPAR5,* and *PLPP2* tumors (all P<0.02, **Figure 7A**). The converse was essentially observed for endothelial cells, where their populations were enriched in high-expressing *ENPP2, LPAR1, LPAR4, LPAR6, PLPP1,* and *PLPP3* tumors, and in low-expressing *LPAR2* and *PLPP2* tumors in both cohorts
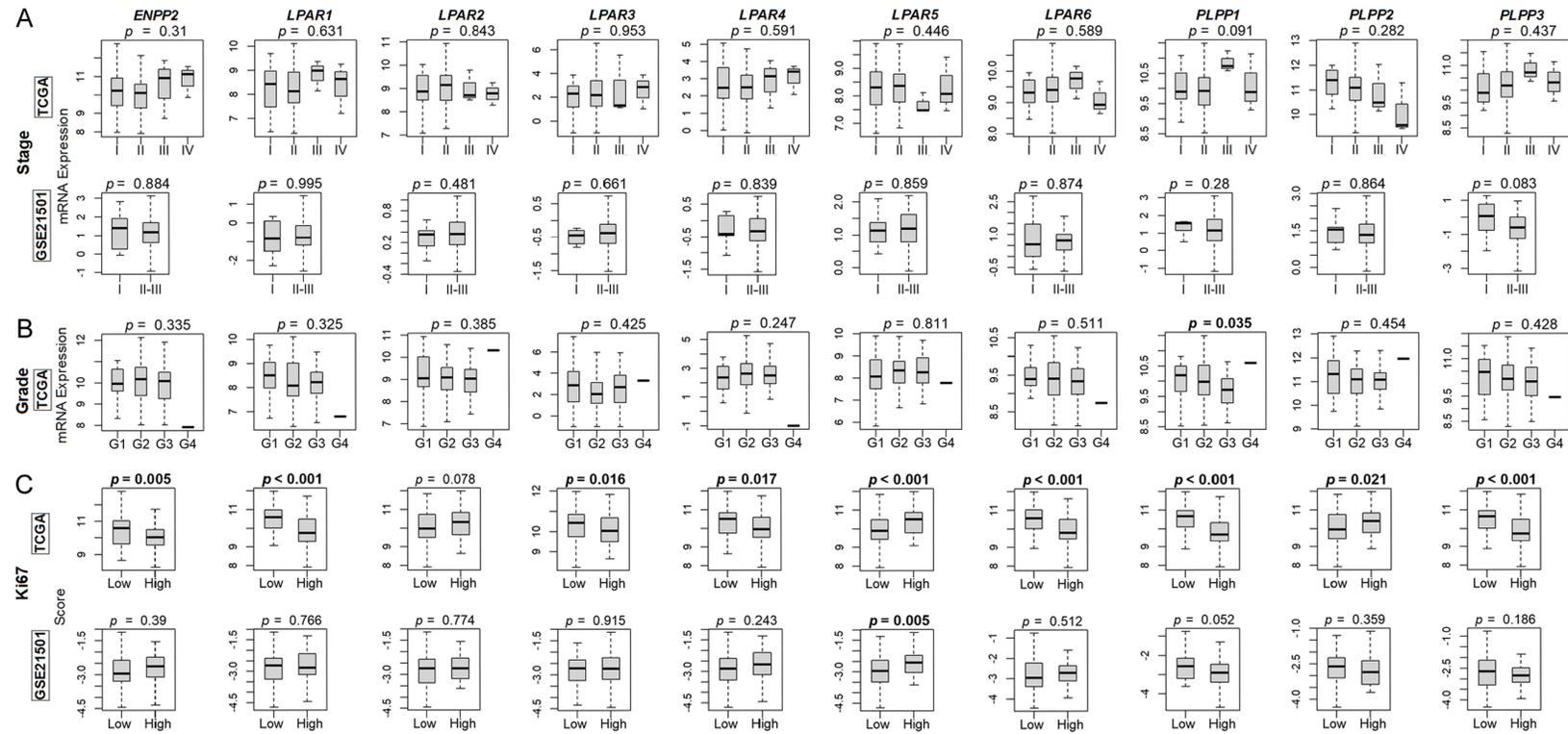
Figure 3. LPA signaling-related gene expression by PDAC characteristics. A. Staging according to the American Joint Committee on Cancer (AJCC). Counts per subgroup. TCGA: I-12, II-127, III-3, IV-3; GSE21501: I-8, II-III-92. B. Grading according AJCC. Grading information not available for GSE21501. Counts per subgroup for TCGA: G1-21, G2-83, G3-41, G4-1. C. Ki67 scoring dichotomized by median gene expression. Results are plotted as box plots with the bolded center bar within the box plots represents the median, the lower and upper box bounds represent the 25th and 75th percentiles, respectively, and the lower and upper tails represent the minimum and maximum values, respectively.
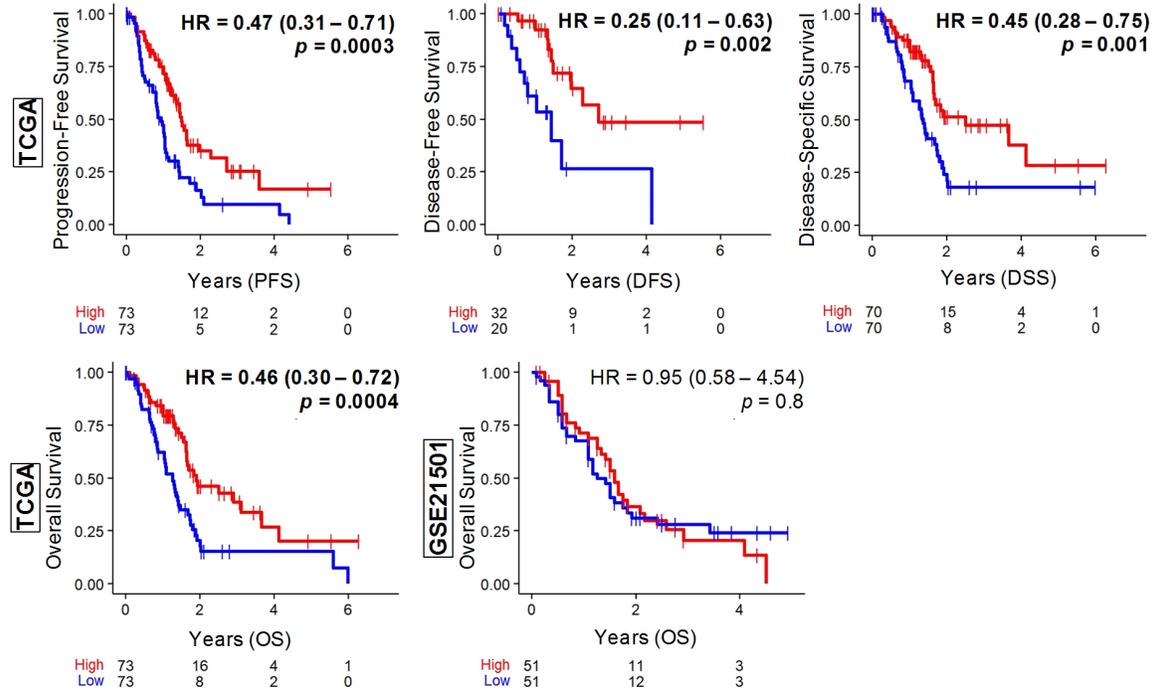
Figure 4. Survival plots for low and high *ENPP2* (ATX) expression in PDACs. First row shows progression-free survival (PFS), disease-free survival (DFS), and disease-specific survival (DSS) for the TCGA cohorts. Second row shows overall survival for the TCGA and GSE21501 cohorts. *ENPP2* expression is dichotomized into low and high groups by the median. The hazard ratio (HR) compares the high group against the low group. *P* values by log rank test. There were no significant findings in survivals for any of the LPAR or LPP genes.

(all P≤0.005, **Figure 7B**). There was no correlation between gene expression and pericyte levels for any of the genes (not shown). Because PDACs have robust stroma and desmoplastic reactions, we also examined stromal marker scores and fibroblast composition. Stromal fraction was not significantly increased for any genes, but TGF-β response, a surrogate of stromal modulation [57], was significantly elevated in high-expressing *ENPP2, LPAR1, LPAR4,* and *PLPP3* tumors, and in low-expressing *LPAR2, LPAR5,* and *PLPP2* tumors (**Figure 8A**, all P<0.05). TGF-β response mirrored fibroblast composition, with fibroblast enrichment in high-expressing *ENPP2, LPAR2, LPAR4, PLPP1,* and *PLPP3* tumors and in low-expressing *LPAR2, LPAR5,* and *PLPP2* tumors across both the TCGA and GSE21501 cohorts (all P≤0.01, **Figure 8B**).

Lastly, we correlated immune cell populations to gene expression levels. On analysis of prototypical anti-cancerous immune cells, particularly among CD8+ T cells, M1 macrophages, and dendritic cell populations, these cell populations were significantly increased in high expressing *ENPP2* and *LPAR1* tumors (all P<0.01, **Figure 9A-D**) across both cohorts. Similar results were also observed in both cohorts of high-expressing *PLPP1* and *PLPP2* tumors for CD8+ T cells and dendritic cells (all P<0.01, **Figure 9A**, **9D**). In breast and melanoma models, increased LPAR5-mediated signaling is associated with suppressed tumor CD8+ cell concentrations [28, 58]. In the TCGA cohort, CD8+ T cells were significantly suppressed in *LPAR5*-high tumors, but there was no correlation in the GSE21501 cohort (**Figure 9A**). We also examined pro-cancerous cell populations in the two cohorts, for which no genes were significantly different by medial dichotomization among the regulatory T cell and Th2 cell populations (**Figure 10A**, **10B**). However, M2 macrophage levels were significantly elevated in high-expressing *ENPP2* and *PLPP3* tumors in both cohorts (all P<0.001, **Figure 10C**). On examination of immune scores by Thorsson et al. [54], leukocyte fractions and lymphocyte infiltration scores were increased in high-expressing *ENPP2, LPAR1, LPAR4, PLPP1,* and *PLPP3* tumors, and decreased in high-expressing *LPAR2, LPAR5,* and *PLPP2* tumors (all

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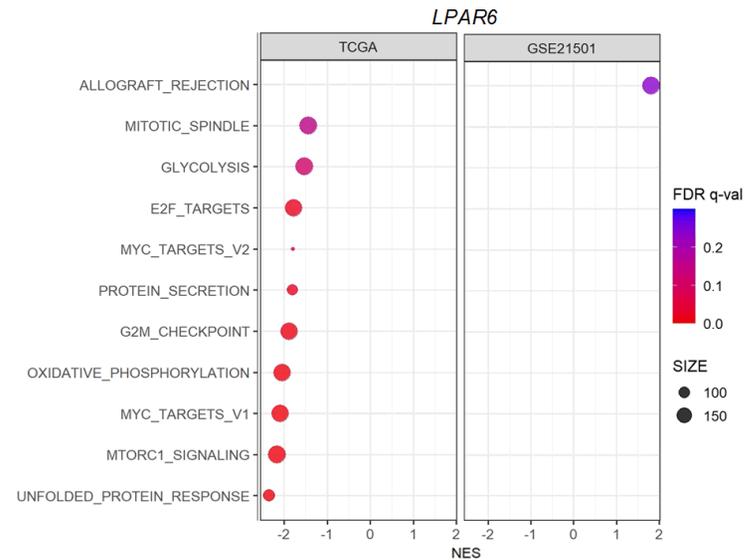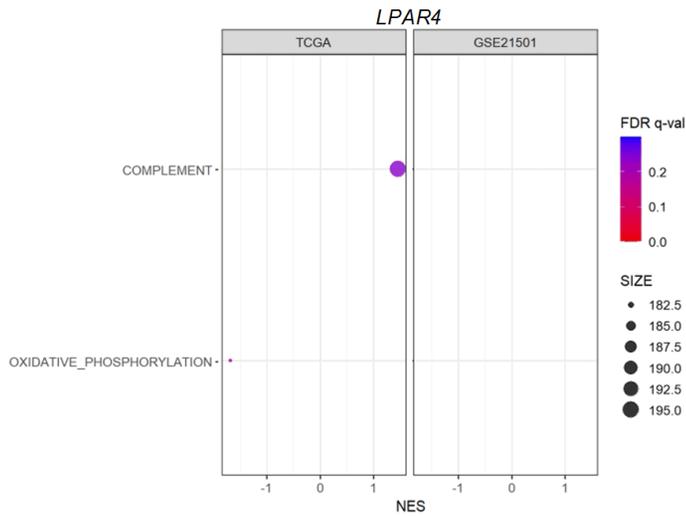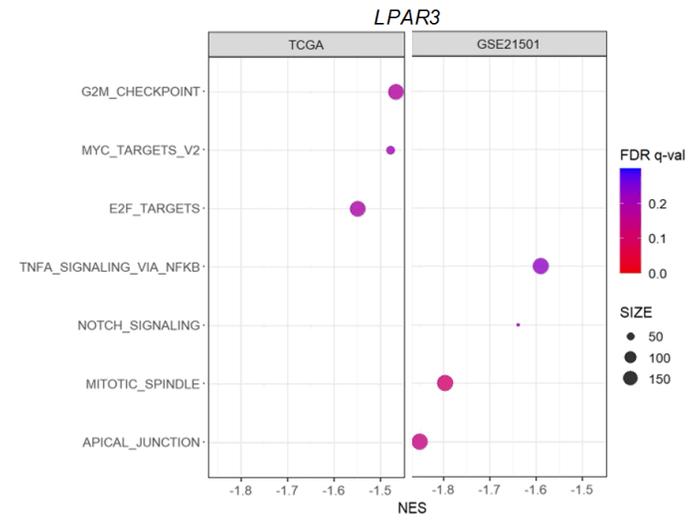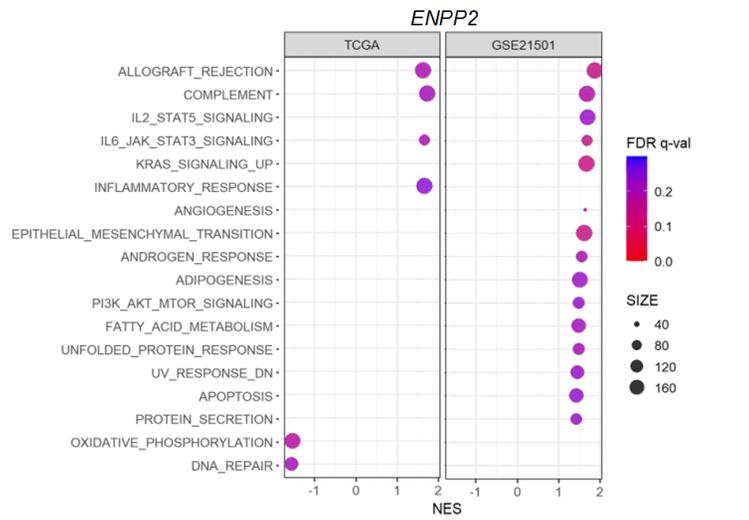
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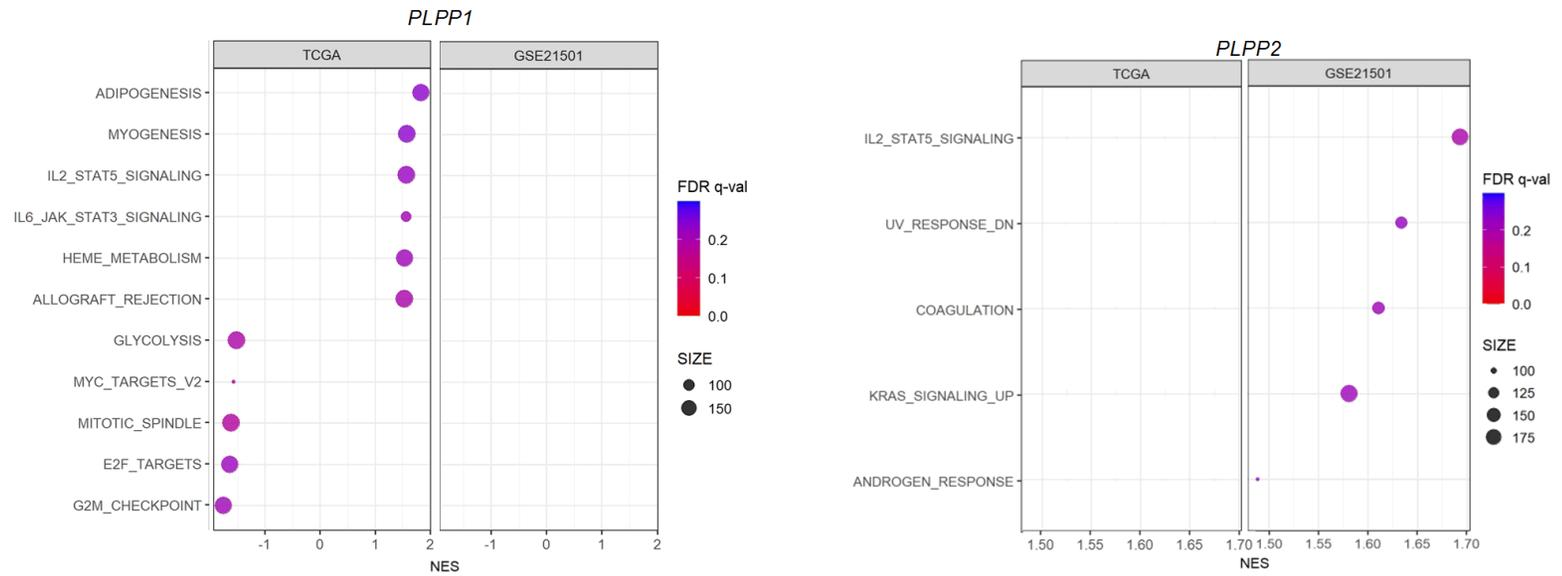
Figure 5. Gene set enrichment analysis (GSEA) for LPA signaling-related expression. GSEA results from the Hallmark gene sets in genes and cohorts that reached significance. A false discovery rate (FDR) of less than 0.25 was considered statistically significant (illustrated by the color of the dot). Dot size represents number of genes in the gene set after filtering out those genes not in the expression dataset. There were no significantly enriched gene sets in either of the cohorts for *LPAR1, LPAR2, LPAR5,* or *PLPP3*.
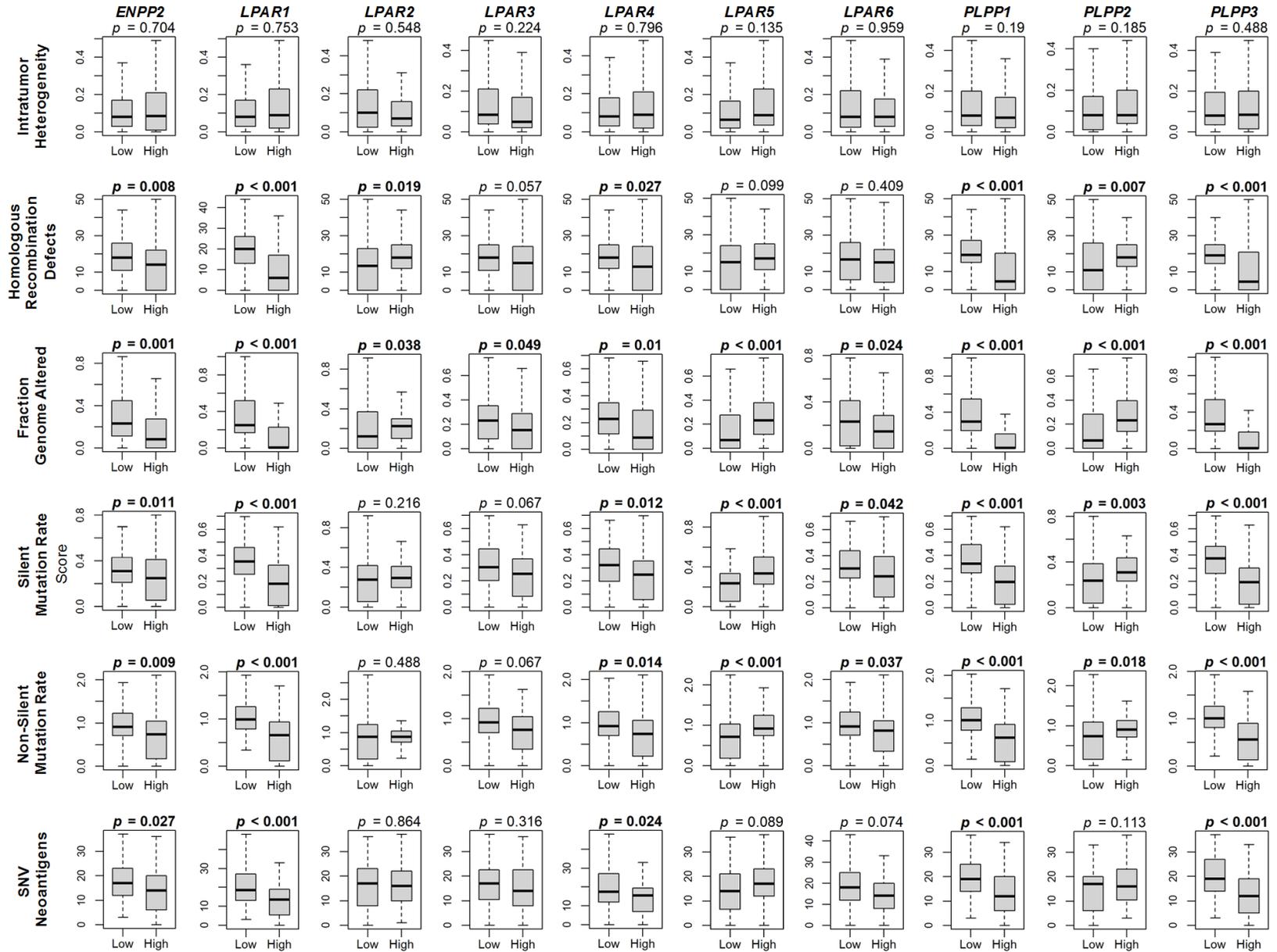
Lysophosphatidic acid signaling in pancreatic ductal adenocarcinoma

Figure 6. LPA signaling-related gene expression association with PDAC mutations. Box plots of intratumor heterogeneity, homologous recombination defects, fraction genome altered, silent mutation rate, non-silent mutation rate, single-nucleotide variant (SNV) neoantigens, and indel mutations. Data is based on the scores by Thorsson, *et al.* [54]. Gene expression is dichotomized by median expression. Results are plotted as box plots with the bolded center bar within the box plots represents the median, the lower and upper box bounds represent the 25th and 75th percentiles, respectively, and the lower and upper tails represent the minimum and maximum values, respectively.
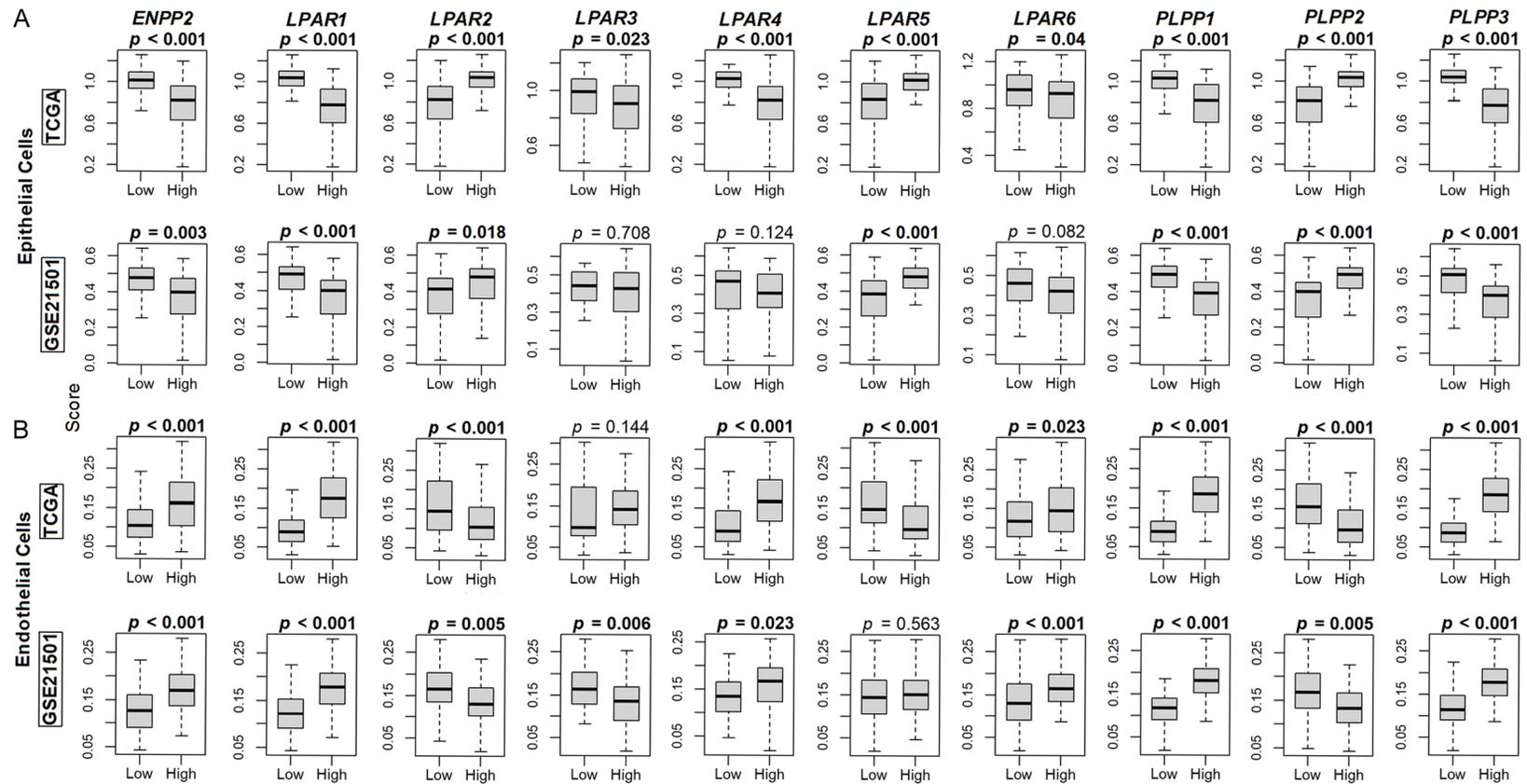
Figure 7. Epithelial and endothelial composition correlation with LPA signaling-related gene expression in PDAC. A. Box plots of epithelial cell composition based on the xCell algorithm for the TCGA and GSE21501 cohorts. B. Box plots of endothelial cell composition based on the xCell algorithm for the TCGA and GSE21501 cohorts. Results are plotted as box plots with the bolded center bar within the box plots represents the median, the lower and upper box bounds represent the 25th and 75th percentiles, respectively, and the lower and upper tails represent the minimum and maximum values, respectively.
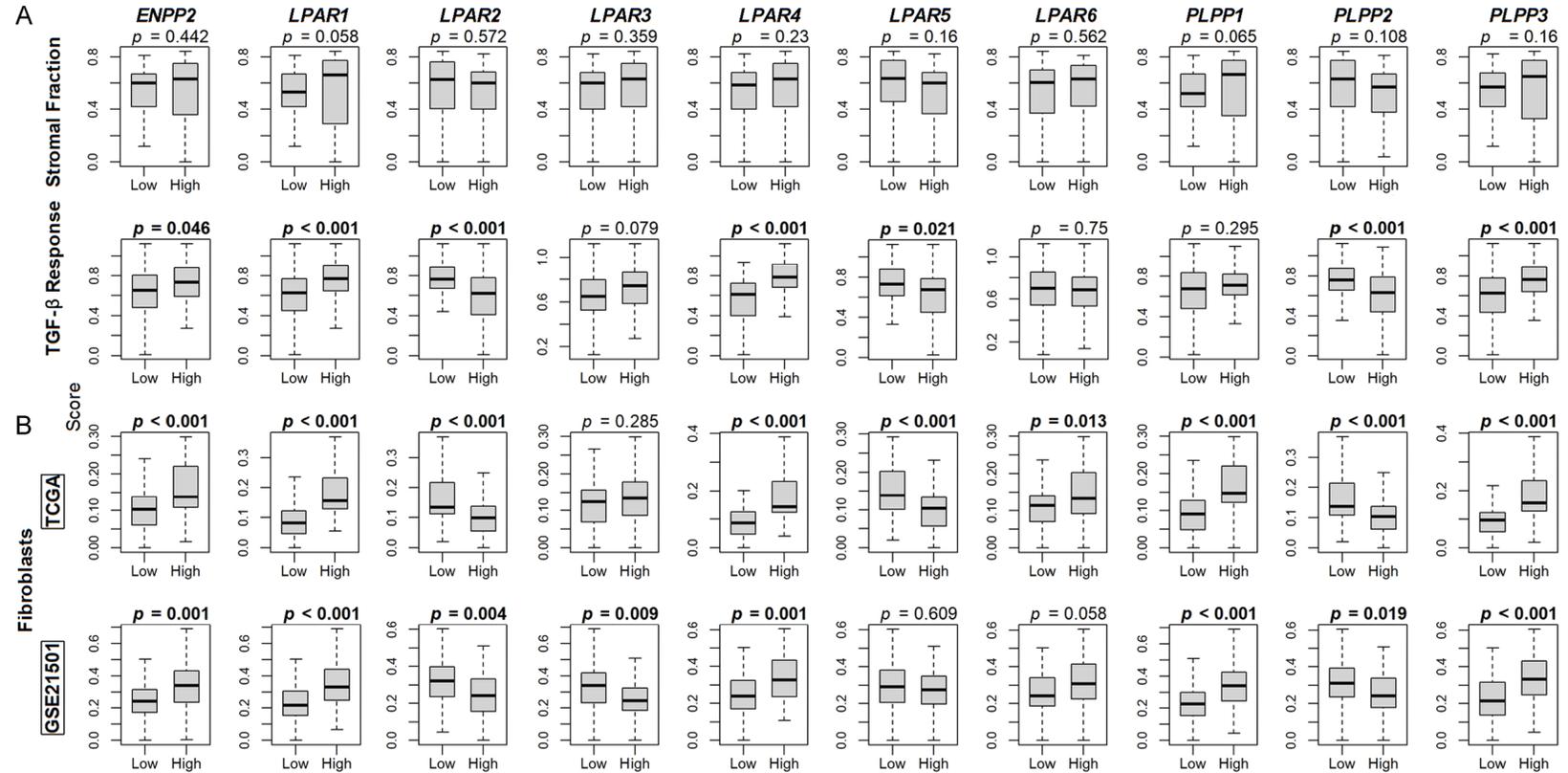
Figure 8. Stromal related scores and fibroblast composition correlation with LPA signaling-related gene expression in PDAC. A. Box plots of calculated scores for stromal fraction and TGF-β response, based on the scores by Thorsson, *et al.* [54]. B. Box plots of fibroblast cell composition based on the xCell algorithm for the TCGA and GSE21501 cohorts. Results are plotted as box plots with the bolded center bar within the box plots represents the median, the lower and upper box bounds represent the 25th and 75th percentiles, respectively, and the lower and upper tails represent the minimum and maximum values, respectively.
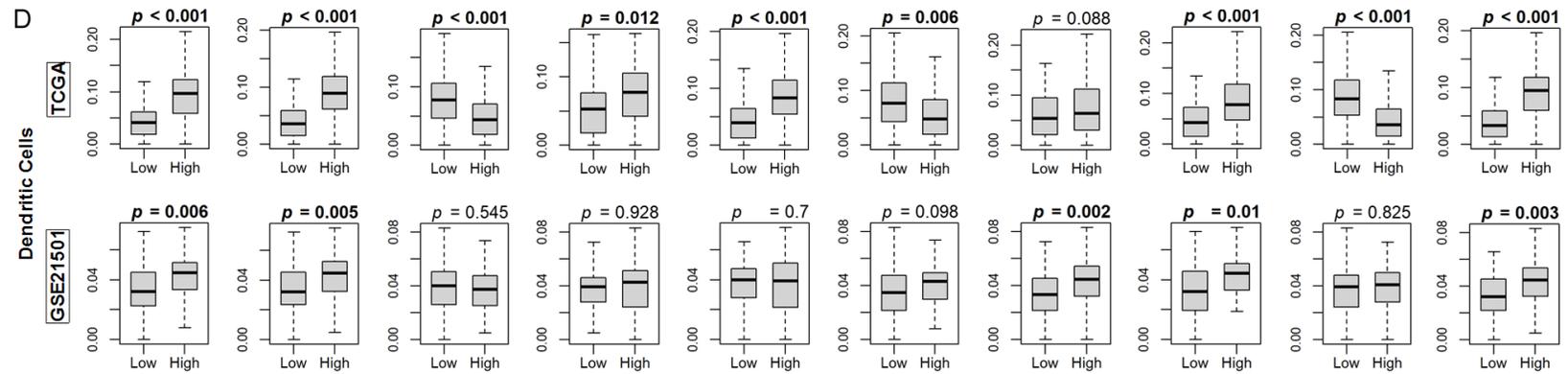
Figure 9. Anti-cancerous immune cell correlation with LPA signaling-related gene expression in PDAC. A. Box plots of CD8+ cell composition based on the xCell algorithm for the TCGA and GSE21501 cohorts. B. Box plots of Th1 cell composition based on the xCell algorithm for the TCGA and GSE21501 cohorts. C. Box plots of M1 macrophage cell composition based on the xCell algorithm for the TCGA and GSE21501 cohorts. D. Box plots of dendritic cell composition based on the xCell algorithm for the TCGA and GSE21501 cohorts. Results are plotted as box plots with the bolded center bar within the box plots represents the median, the lower and upper box bounds represent the 25th and 75th percentiles, respectively, and the lower and upper tails represent the minimum and maximum values, respectively.

Figure 10. Pro-cancerous immune cell correlation with LPA signaling-related gene expression in PDAC. A. Box plots of regulatory T cell composition based on the xCell algorithm for the TCGA and GSE21501 cohorts. B. Box plots of Th2 cell composition based on the xCell algorithm for the TCGA and GSE21501 cohorts. C. Box plots of M2 macrophage cell composition based on the xCell algorithm for the TCGA and GSE21501 cohorts. Results are plotted as box plots with the bolded center bar within the box plots represents the median, the lower and upper box bounds represent the 25th and 75th percentiles, respectively, and the lower and upper tails represent the minimum and maximum values, respectively.

P≤0.01, **Figure 11**). Tumor infiltration lymphocyte (TIL) fraction was increased in high expressing *ENPP2* and *LPAR3* tumors (all P<0.04, **Figure 11**). Macrophage regulation scores were significantly different for all genes: increased in high-expressing *ENPP2, LPAR1, LPAR3, LPAR4, LPAR6, PLPP1*, and *PLPP3* tumors, and decreased in high-expressing *LPAR2, LPAR5*, and *PLPP2* tumors. Wound healing scores, which typically relate to decreased overall survival [54], tended to have an opposite relation compared to the other scores, particularly, decreased in high-expressing *ENPP2, LPAR1, LPAR6, PLPP1*, and *PLPP3* tumors (all P<0.01, **Figure 11**). Across both cohorts, cytolytic (CYT) scores were increased in high-expressing *ENPP2, LPAR1, LPAR6, PLPP1,* and *PLPP3* tumors (all P<0.01, **Figure 12**). CYT scores were decreased in high-expressing *LPAR2* and *PLPP2* tumors in the TCGA cohort (all P<0.001), but did not reach significance in the GSE21501 cohort (**Figure 12**).

Discussion

The LPA pathway has been extensively researched for more than 30 years as a potentially druggable target at multiple levels. The goal of targeting LPA signaling has been primarily to mitigate the development of cancer therapy resistance either through blockade of resistance mechanisms or by potentiating therapeutic synergism with conventional therapies. Multiple agents against the LPA axis players have been developed and tested primarily preclinically against cancer and other diseases of chronic inflammation. However, the ATX inhibitor IOA-289 has become the first agent to enter clinical trials for cancer. It is currently being investigated in a phase 1b, open label, dose-escalation study in combination with gemcitabine/nab-paclitaxel in patients with metastatic PDAC, with preliminary results showing a reduction in CA19-9 of greater than 50%, and durable partial responses beyond those achieved in the control cohorts [59]. Hence, our

motivation for this study was to survey expression patterns of the LPA-related signaling pathway genes within the PDAC TME in order to predict future directions of research for ongoing LPA-pathway targeting therapeutic interventions.

In the study, we showed that *ENPP2, LPAR1, LPAR4, LPAR5, LPAR6*, and *PLPP1* were upregulated in PDAC compared to normal pancreatic tissue. No genes showed a strong correlation to either disease stage or grade. Only *ENPP2* showed consistent upregulation of immune related and inflammatory gene sets in both cohorts. Like human breast cancers, *ENPP2* expression was enriched in tumor stroma cells (fibroblasts and endothelial cells) rather than tumor epithelial cells [39]. *LPAR2, LPAR5*, and *PLPP2* were upregulated in tumor epithelial cells, while *LPAR1, PLPP1*, and *PLPP3* were downregulated. Immune cell infiltration scores and CYT scores were significantly increased in high-*ENPP2* expressing tumors, which are typically markers of decreased tumorgenicity. Only *ENPP2* expression correlated to patient survival outcomes, where high *ENPP2* expression tended to have better survival characteristics, particularly in the TCGA cohort. This finding was unexpected according to the conventional model of ATX expression and tumorgenicity, as high tumor ATX expression is predicted to correlate with a more aggressive phenotype [6, 9]. However, we have observed a similar phenotype in high-expressing *ENPP2* early breast cancer tumors [13, 39]. In the TCGA cohort, 95 percent of PDAC patients had early-stage disease (stage I or II), whereas the GSE21501 cohort tended to have higher-stage disease (>90% stage II or III). This likely explains why overall survival favored the high-*ENPP2* group in TCGA but lost significance in the GSE21501 cohort. Additionally, Ki67 scores, a marker of cellular proliferation, were significantly lower in the high-*ENPP2* group in the TCGA group, but trended non-significantly towards being higher in the GSE21501 cohort. This finding would support our similar conclusion in early breast
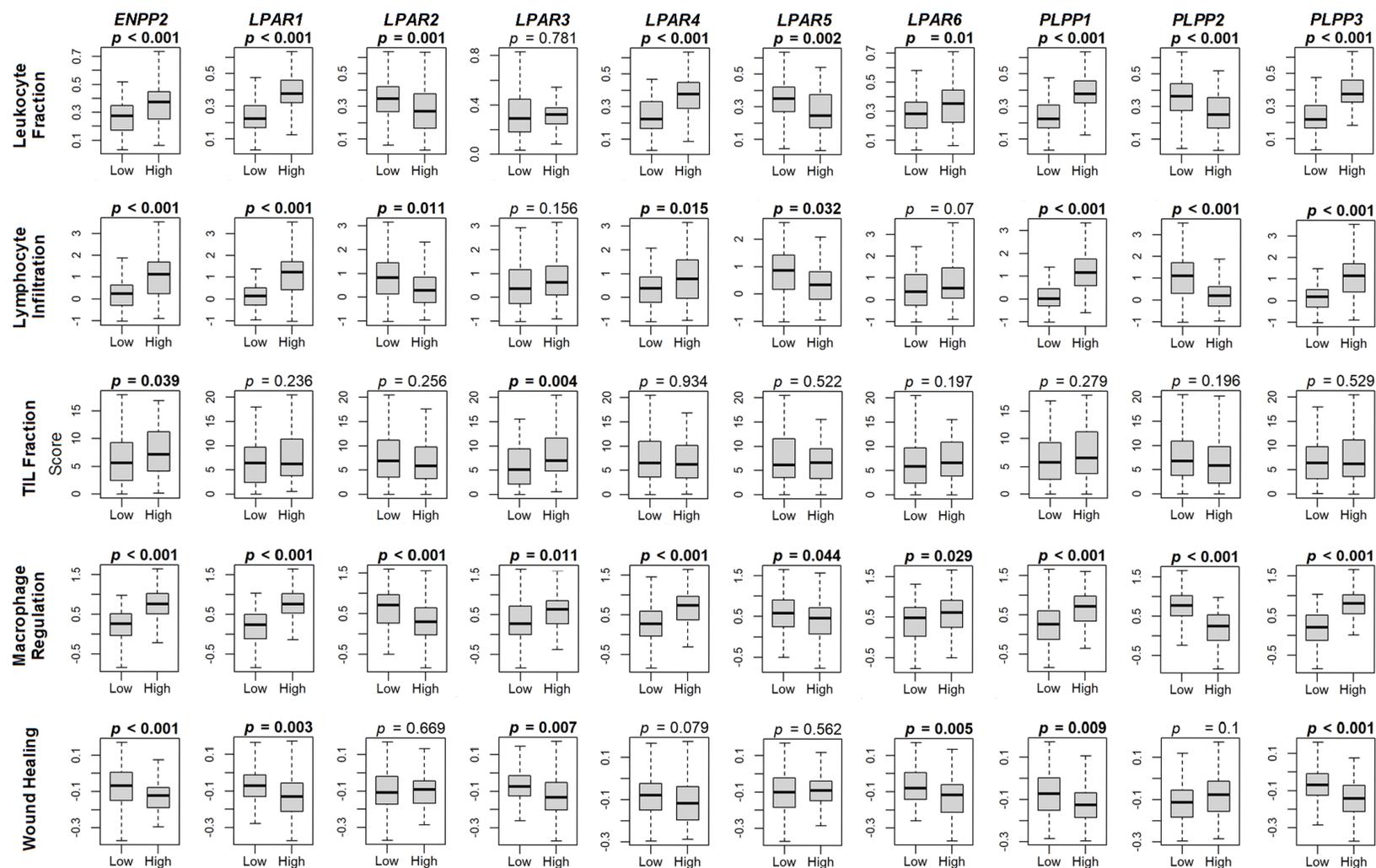
Figure 11. Immune scores for markers of tumor immune cell populations correlation with LPA signaling-related gene expression in PDAC. Box plots of immune scores (leukocyte fraction, lymphocyte infiltration, tumor infiltration leukocyte (TIL) fraction, macrophage regulation, and wound healing) are based on the scores by Thorsson, *et al.* [54]. Results are plotted as box plots with the bolded center bar within the box plots represents the median, the lower and upper box bounds represent the 25th and 75th percentiles, respectively, and the lower and upper tails represent the minimum and maximum values, respectively.
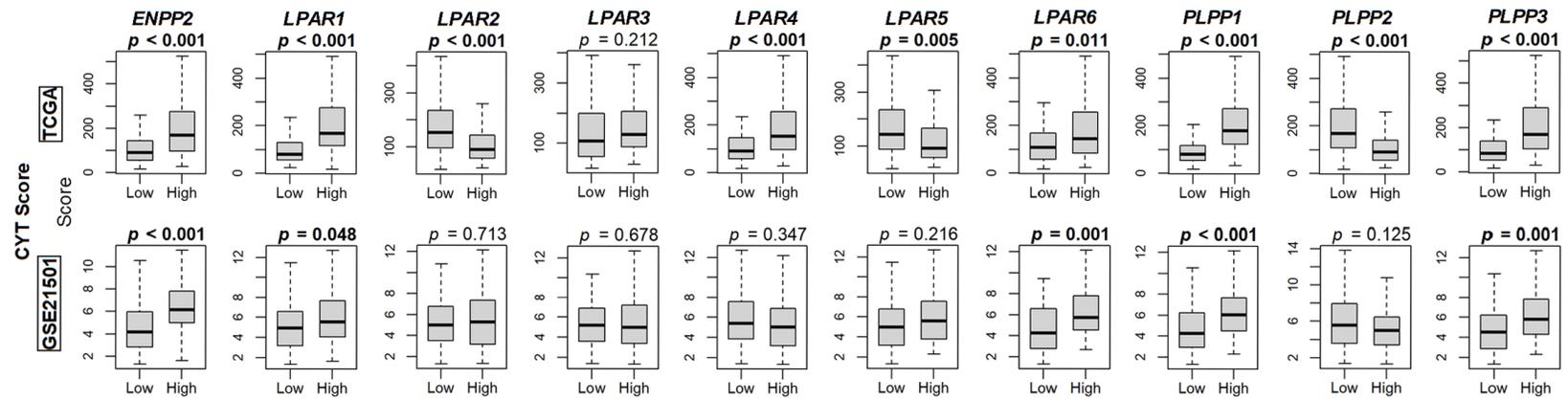
Figure 12. Cytolytic (CYT) score correlation with LPA signaling-related gene expression in PDAC. Box plots of CYT scores based on the xCell algorithm for the TCGA and GSE21501 cohorts. Results are plotted as box plots with the bolded center bar within the box plots represents the median, the lower and upper box bounds represent the 25th and 75th percentiles, respectively, and the lower and upper tails represent the minimum and maximum values, respectively.

cancer where *ENPP2* levels, predominantly expressed in the tumor stroma, may function primarily in a physiological wound healing role to suppress tumor progression [13, 39]. However, at some point, these tumors express their underlying propensity to hijack ATX production and subsequent LPA signaling for progressive tumorigenesis in the context of more advanced or biologically aggressive disease [13, 60].

Recent emerging evidence supports ATX as a promoter of tumor progression in PDAC. Auciello et al. demonstrated that as pancreatic stellate cells transformed into cancer-associated fibroblasts, tumor stroma increased both the LPC concentrations and ATX levels [60]. In both in vitro experiments and murine models, the ATX-LPA axis promoted PDAC cell proliferation, migration, and AKT activation, all of which could be suppressed with potent oral ATX inhibition, resulting in suppressed tumor growth [60]. ATX in the PDAC TME can suppress the infiltration of eosinophils into the TME, thereby shielding the tumor from the immune system, a phenomenon that can be blocked with potent ATX inhibition in murine models [61, 62]. Another group also demonstrated ATX production in inflammatory cancer-associated fibroblasts in PDAC mediates adaptive resistance to TGF-β receptor-mediated inhibition. Treatment with the ATX inhibitor IOA-289 was synergistic with the TGF-β receptor inhibitor galunisertib to improve the efficacy of gemcitabine in PDAC murine models [15]. In breast cancer murine models, IOA-289 treatment both decreased TGF-β1/β2 cytokine signaling and increased anti-tumor CD8α+ T-cell tumor infiltration, resulting in decreased tumor growth [28, 29]. IOA-289 treatment may have similar effects in PDAC.

The majority of ATX in the breast is produced by adipocytes and this is increased by tumor-induced inflammation. Knockout of ATX in adipocytes decreased plasma ATX by ~40%, but this did not affect breast tumor growth [28]. By contrast, treatment with IOA-289 to block total ATX activity decreased tumor growth by ~60%, demonstrating that another source of ATX drives tumor growth [28]. This is likely to come from tumor stromal cells such as fibroblasts, leukocytes or endothelial cells, which express the majority of ATX within mouse [28] and human breast tumors [39]. Similarly, in this study, ATX expression was enriched in tumor

stromal cells compared to pancreatic cancer (epithelial) cells. These observations demonstrate that bulk ATX concentrations are much less important than where the ATX is produced specifically to drive tumor growth and metastasis. This specificity is explained because secreted ATX acts locally by attaching to integrins [11, 63, 64] or syndecan-4 [65] on adjacent cells. Cell-associated ATX acts as a chaperone for LPA by specifically channeling LPA to activate its receptors. The Type IV ATX inhibitors that are in clinical trials are designed to block the binding of LPA to an allosteric tunnel in ATX and diminish this channeling of LPA to its receptors [63]. These inhibitors are particularly effective in decreasing the inflammatory cycle, tumor growth and the accumulation of inflammatory macrophages [9, 66]. The recruitment of CD8+ T-cells is also decreased through activation of LPAR5 [67-69]. Thus, inhibition of ATX with a Type IV inhibitor such as IOA-289, increases the accumulation of CD8+ T-cells in breast tumors, which should increase immune-surveillance [28, 29]. Similar biological effects are predicted to occur in PDACs with IOA-289, though confirmatory investigations are required.

Regarding the LPARs in PDACs, there is a paucity of literature and virtually none concerning the LPPs. In PDAC cell cultures, *LPAR2* and *LPAR3* levels significant increased in response to hypoxic conditions (5% or less oxygen) [70, 71]. Similarly, cultured PDAC cells increased *LPAR2* expression following exposure to X-ray radiation or oxidative stress following exposure to hydrogen peroxide, with cell motility and survival rates increased following treatment with LPAR2 agonists [72]. LPAR1 signaling has been shown to interact with β-catenin signaling pathway mediators to promote invasion in cell culture assays [73]. Short-hairpin RNA knockdown of *LPAR4* and *LPAR5* has been reported to enhance of cell motility and invasion of PDAC cultured cells, whereas knockdown of *LPAR6* inhibited these tumorigenic traits [74]. In this study, we showed that *LPAR2* expression was predominantly enriched in the epithelial cell portion of PDAC tumors. We showed a similar result in human breast tumors, and *LPAR2*-overexpressing breast cancer cells have the most tumorigenic properties of any the LPARs in both in in vitro and pre-clinical animal models [13, 40, 75]. Therefore, selective LPAR2-inhibition, in combination with

potent ATX inhibition [76], is likely to provide the most robust blockage of the LPA signaling axis [13]. Finally, with respect to the LPPs, human PDAC tumors demonstrate the classical low PLPP1/3, high PLPP2 expression profile seen in multiple other types of malignancies. While there are currently no known LPP2 inhibitors, or specific pharmacological inducers of LPP1/LPP3 expression to increase LPA turnover in the TME, developing such compounds would be a novel area of investigation [13].

As a retrospective analysis, our study does have several limitations. Although we use two independent cohorts to validate our key findings, the two cohorts are relatively small, and comprised of heterogenous patient populations and treatments with varied outcomes. Bioinformatics data cannot be used to necessarily imply mechanisms of action, but their utility is to provide comparative analysis to experimental pre-clinical models and insightful perspectives for designing future investigations. The findings of this study should be interpreted as hypothesis generating, and will require multi-omics analysis to validate and delineate the mechanisms of action of the mediators of LPA signaling in the PDAC TME. Critical to future LPA-targeting pharmacological development for PDAC and other malignancies will be determining conditions where the physiological wound healing effects of LPA signaling are subverted into maladaptive effects that promote tumor progression. This is likely a phenomenon that occurs across multiple tumor sites once disease progresses beyond early stages. Under such conditions, inhibitors of ATX-LPA signaling in combination with other treatments might have the largest therapeutic opportunity.

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Disclosure of conflict of interest

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Address correspondence to: Dr. Kazuaki Takabe, Department of Surgical Oncology, Roswell Park Comprehensive Cancer Center, Elm and Carlton Streets, Buffalo, New York 14263, USA. Tel: 1-716-845-5540; E-mail: kazuaki.takabe@roswellpark.org

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