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Investigating the influence of taurochenodeoxycholic acid (TCDCA) on pancreatic cancer cell behavior: An RNA Sequencing Approach

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Abstract

Pancreatic cancer (PC) poses a substantial global health challenge, ranking as the fourth leading cause of cancer-related deaths due to its high mortality rate. Late-stage diagnoses are common due to the absence of specific symptoms. Pancreatic ductal adenocarcinoma (PDAC) accounts for the majority of PC cases. Recent research has suggested a potential link between elevated serum levels of bile acids (BAs) and tumorigenesis of PDAC. This study aims to understand how taurochenodeoxycholic acid (TCDCA), a secondary BA, influences PDAC using RNA sequencing techniques on the Capan-1 cell line. We identified 2,950 differentially expressed genes (DEGs) following TCDCA treatment, with 1,597 upregulated and 1,353 downregulated genes. These DEGs were associated with critical PDAC pathways, including coagulation, angiogenesis, cell migration, and signaling regulation.

Furthermore, we reviewed relevant literature highlighting genes like DKK-1, KRT80, UPLA, and SerpinB2, known for their roles in PDAC tumorigenesis and metastasis. Our study sheds light on the complex relationship between BAs and PDAC, offering insights into potential diagnostic markers and therapeutic targets. Further research is needed to unravel these findings' precise mechanisms and clinical implications, potentially improving PDAC diagnosis and treatment.

Keywords: pancreatic cancer, taurodeoxycholic acid, RNA sequencing, Capan-1 cell line

1. INTRODUCTION

Pancreatic cancer (PC) presents a formidable global health challenge, ranking as the fourth leading cause of cancer-related deaths due to its high mortality rate (Siegel et al., 2022)(Saad et al., 2018). Unfortunately, the absence of specific symptoms often leads to late-stage diagnoses in around 80% of patients, rendering them inoperable (Kanno et al., n.d.)(Bausch and Keck, 2018)(Jeune et al., 2019). Among PC cases, pancreatic ductal adenocarcinoma (PDAC) accounts for approximately 90%, making it the dominant form of this malignancy (Singhi et al., 2024)(Adamska et al., 2017). PDAC usually originates from ductal cells in the pancreas's head, often leading to obstructive jaundice (OJ) due to disrupted bile flow. Recent investigations have suggested that elevated serum levels of bile acids (BAs) might have tumorigenic potential, as observed in gastrointestinal and breast cancer (Li et al., 2022)(Ciaula et al., 2019).

BAs are soluble amphiphilic compounds recognized for their crucial physiological role in aiding the absorption of fat-soluble nutrients (Li and Chiang, 2014). Moreover, emerging research has unveiled their broader impact on human metabolism, brought about by interactions with various receptors and pathways (Martinot et al., 2017). These receptors include nuclear receptors like farnesoid X receptor (FXR), pregnane X receptor and vitamin D receptor, as well as membrane receptors such as G-protein coupled BA receptor-1 (GPBAR-1 or TGR5) and sphingosine-1-phosphate receptor 2), alongside signaling pathways like c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) (Martinot et al., 2017)(Bertolini et al., 2022). The intricate interplay between BAs and these receptors allows for a regulatory role in nutrient metabolism, influencing processes related to energy, glucose, lipid, and lipoproteins (Li and Chiang, 2014)(Zhou and Hylemon, 2014)(Chávez-Talavera et al., 2017)(Vítek and Haluzík, 2016).

Given the central role of BAs in metabolism, researchers have explored their potential connections with cancer. BAs have been implicated in several pathways linked to cancer development, such as oxidative stress, DNA damage, genomic instability, apoptosis, and epigenetic factors (Payne et al., 2008)(Barrasa et al., 2013)(Yang et al., 2015)(Matsuzaki et al., 2013). Furthermore, the interactions of BAs with gut microbiota also have implications for cancer (Malhotra et al., 2023)(Long et al., 2017)(Adolph et al., 2019).

Recent studies have notably hinted at possible links between BAs and PC. The reflux of BAs into the pancreatic duct and their systemic association with obesity, diabetes, and hypertriglyceridemia (well-established risk factors for PC) raises interest in investigating their involvement (Feng and Chen, 2016). Moreover, elevated levels of BAs in the serum and pancreatic juice of PC patients have been observed, hinting at their potential role in the disease's pathogenesis (Joshi et al., 2016)(Gál et al., 2020). Furthermore, the nuclear receptor FXR, affected by BAs, has shown varying effects in different cancer types, being associated with improved clinical outcomes in colon and breast cancer but linked to a poorer prognosis in PC (Alasmael et al., 2016)(Lax et al., 2012)(Hu et al., 2017)(Lee et al., 2011).

This study aims to advance our understanding of how BAs influence PC, building on previous findings that revealed increased proliferation, migration, adhesion, colony formation, and MUC4 expression in PDAC cells exposed to BAs (Gál et al., 2020). To achieve this objective, we employed RNA sequencing techniques to investigate the impact of taurochenodeoxycholic acid (TCDCA) on the Capan-1 cell line, aiming to uncover potential connections between BAs and alterations in cell behavior at the cellular transcriptomic level.

2. MATERIALS AND METHODS

2.1 Cell lines and tissue culture

The Capan-1 human PDAC cell line was obtained from the American Type Culture Collection. Cells were used between 30-35 passage numbers, cultured in RPMI-1640 supplemented with 15% fetal bovine serum, 1% antibiotic-antimycotic, and 1% glutamine. Cells were kept in a humidified incubator at 37°C. The medium was replaced every second day. Cell passage was done using trypsin/ethylenediaminetetraacetic acid; after they reached ~80% confluency, they were seeded to a new flask for BA treatment.

2.2 Bile acid treatment

Capan-1 cells were seeded at 6×10^5 cells/ 75 cm^2 tissue culture flasks and were grown to 70-80% of confluence two days before the BA treatment. Cells were treated with 500 µM TCDCA for 24 hours. After the treatment, RNA isolation was performed for the RNAseq analysis.

2.3 RNA Isolation

For RNA Sequencing, cells were collected, and the total RNA was isolated by the NucleoSpin RNA Kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's protocol.

2.4 RNA-Sequencing

High-throughput mRNA sequencing analysis was performed on the Illumina sequencing platform to obtain global transcriptome data. According to the manufacturer's protocol, total RNA sample quality was checked on Agilent Bioanalyzer using the eukaryotic Total RNA Nano Kit. Samples with an RNA integrity number (RIN) value >7 were accepted for the library preparation. RNA-

Seq libraries were prepared from total RNA using the Ultra II RNA Sample Prep kit (New England BioLabs) according to the manufacturer's protocol. Oligo-dT conjugated magnetic beads captured poly-A RNAs, and then the mRNAs were eluted and fragmented at 94 °C. First-strand cDNA was generated via random priming reverse transcription, and after the second-strand synthesis step, double-stranded cDNA was generated. After repairing ends, A-tailing, and adapter ligation steps, adapter-ligated fragments were amplified in enrichment PCR, and finally, sequencing libraries were generated. The sequence runs were executed on the Illumina NextSeq 500 instrument using single-end 75-cycle sequencing.

2.5 Data Analysis

The complete workflow of the NGS analysis is shown in **Figure 1**. The the quality of raw sequencing data was assessed by FastQC (FastQC: default settings, version 0.11.9). Reads were trimmed using Trimmomatic (settings: ILLUMINACLIP:Poly_A_TruSeq3-SE.fa:2:30:10, SLIDINGWINDOW:4:20, MINLEN:30) and aligned to the human genome (GRCh38) with HISAT2 (default settings, version 2.2.1). Feature Counts (FeatureCounts: flags used: -s 0, subread version 2.0.3) were used to create the count table for the gene expression analysis performed in R (version 4.3.2). As a pre-filtering step, genes with low expression values - rows with only 10 counts across all samples were removed. Principal component analysis (PCA) was used with the R package PCA tools to visualize the association between samples. MultiQC (default settings, version 1.11) was used for aggregate the results. Differential expression analysis was performed using DESeq2 (DESeq2: version 1.44.0). Differentially expressed genes (DEGs) were defined based on adjusted p-value < 0.05 and the basline LFC threshold = 0. Settings were the follows:

```
gene_counts: a txt file generated by FeatureCounts
samples = colnames(gene_counts)
condition = factor(c(rep("ctrl", 3),
        rep("treated", 3)))
coldata = data.frame(row.names=samples, condition)
dds = DESeqDataSetFromMatrix(countData = gene_counts,
                             colData = coldata,
                             design = ~ condition)
keep = rowSums(counts(dds)) > 10
dds = dds[keep,]
dds_norm = DESeq(dds)
res_0 = results(dds_norm, name="condition_treated_vs_ctrl", alpha = 0.05, lfcThreshold = 0)
res = subset(res_0, padj < 0.05)
resLFC = lfcShrink(dds_norm, coef="condition_treated_vs_ctrl", type="ashr", res = res_0)
resLFC_subset = subset(resLFC, padj < 0.05)
```

These results are presented on a heat map and a Volcano plot. Heat map visualization of all the DEGs was performed using the R package Complex Heatmap, where Pearson correlation was used on rows, and z-scores were calculated from count data transformed with DESeq2's variance stabilizing transformation (VST). An enhanced Volcano package was used to make the volcano plots. In our research, we used data from the Human Protein Atlas version 22.0 (accessible at https://www.proteinatlas.org/humanproteome/pathology) to compare our DEGs obtained from TCDCA-treated and control samples against data from the Pancreas and other tumor tissues.

This approach helped us build a solid foundation for our research findings."

2.6 Pathway analysis

Gene-set enrichment was conducted based on DEGs ordered by their log 2-fold changes. The R package cluster Profiler (clusterProfiler: version 4.4.0) was used for the analysis as background Gene Ontology (https://geneontology.org/) and KEGG (https://www.genome.jp/kegg/) databases were used. We additionally analyzed gene sets carefully collected from publications. The normalized counts of the intercept of the DEGs and these pre-selected gene sets are shown on heatmaps. (Normalization was done with DESeq2: normalized count = counts (dds, normalized =TRUE).

```
gsea = gseGO(geneList     = geneList,
             OrgDb        = org.Hs.eg.db,
             keyType      = "ENSEMBL",
             ont          = "BP",
             minGSSize    = 10,
             maxGSSize    = 200,
             pvalueCutoff = 0.05,
             verbose      = TRUE)
gsea_KEGG = gseKEGG(geneList     = geneList_KEGG,
                    organism     = 'hsa',
                    minGSSize    = 5,
                    maxGSSize    = 200,
                    pvalueCutoff = 0.05,
                    verbose      = FALSE)
```

3. RESULTS

We performed a differential gene expression analysis to thoroughly comprehend the alterations in gene expression and their associated pathways in the RNA sequencing data. Our inquiry into signaling pathways took two approaches: an unbiased gene set enrichment analysis (GSEA) utilizing GO and KEGG databases and a targeted examination of pre-selected pathways. After a 24-hour treatment of Capan-1 cells with TCDCA (500 µM), RNA samples were collected and analyzed. The Principal Component Analysis (PCA) plot separated the controls and the treated cluster (**Fig. 2A).** The PCA plot with PC1 explaining 86% and PC2 explaining 7% of the variance effectively distinguishes between the control and TCDCA-treated Capan-1 cell groups. Next, we further analyzed our dataset, and we identified 1597 + 1353 DEG within this set; 1597 genes exhibited upregulation, while 1353 showed negative log fold change (LFC), highlighting the significant impact of TCDCA treatment on gene expression (**Fig. 2B and 2C and Table S1**).

Further downstream analysis identified the specific genes and related pathways driving these differences and provided a deeper understanding of the underlying effects. **Table 1** presents the top 20 genes with the highest expression and the bottom 20 with the lowest expression, as determined by their log2 fold change values. The most highly up-regulated genes have established roles in the tumorigenesis, metastasis and cell migration of PC cells such as Dickkopf-1 (DKK-1), SerpinB2, Keratin80 (KRT80), Follistatin (FST), fibroblast growth factor binding protein 1 (FGFBP1), interleukin-1 receptor-like 1 (IL1RL1), Ankyrin Repeat Domain1 (ANKRD1) and urokinase plasminogen activator (UPLA). (**Table 1).** To explore our findings more deeply, we assessed the alterations within well-established molecular pathways, as illustrated in the GSEA analysis **(Fig. 3).** The input DEGs were ranked based on their log fold changes. Our comprehensive

GSEA revealed that 37 pathways displayed an upregulation, while 2 pathways exhibited downregulation in the GO database (**Fig. 3A and Table S2**).

During our KEGG pathway analysis, we detected a notable upregulation in the ribosome gene set, which stands in contrast to the downregulated expression observed in pathways related to drug metabolism by cytochrome P450, neutrophil extracellular trap formation, tyrosine metabolism, and various other pathways **(Fig. 3B and Table S3)**. These findings offer valuable insights into the intricate mechanisms that underlie the influence of TCDCA on the progression of PDAC. Notably, the enhanced ribosome pathway is a pivotal element in the mechanism by which TCDCA significantly improves the viability and functional characteristics of treated Capan-1 cells. The upregulation in pathways related to blood coagulation regulation, hemostasis, and wound healing intimates that BAs might create an environment conducive to tumor growth. The positive modulation of cellular motility and migration pathways, such as endothelial and epithelial cell migration (Carstens et al., 2021), suggests that BAs may enhance cancer cells' migratory and invasive characteristics. This enhancement could occur through the upregulation of enzymes involved in extracellular matrix (ECM) remodeling, including the activation of matrix metalloproteinases (MMPs) **(Figure 3)**.

Furthermore, the enrichment of cellular movement and locomotion pathways suggests that TCDCA may potentially increase the motility and dissemination of Capan-1 cells. Additionally, stimulating endothelial cell proliferation and angiogenesis-related pathways indicates that BAs could facilitate the development of new blood vessels, supporting tumor growth. Notably, our analysis also brought attention to pathways related to signaling regulation, especially the path associated with the negative regulation of transmembrane receptor protein serine/threonine kinase signaling **(Figure 3)**.

Metastasis, tissue invasion, plays a significant role in tumor progression. The process itself is complex but, in many ways, analogous to tissue regeneration and wound healing. It is also an escape mechanism for the cancer. In the following analysis, we have grouped genes involved in tumor development, lineage differentiation, stem cell-EMT (epithelial-mesenchymal transition) processes, ECM formation, cell outgrowth, and wound healing. The gene expression pattern clearly distinguished treated and untreated cells, suggesting that TCDCA treatment induces changes in Capan-1 cells that may promote the abovementioned processes. After cancer cells migrate and spread, it is essential that the cells can proliferate and locally reduce or inhibit the anti-tumor immune response. In the following sections, we have collected genes involved in the cell cycle, cell viability, cell senescence regulation, DNA repair, and the precursors' metabolism. Genes that regulate the local immune response and the production of cytokines and growth factors that regulate the immune response were investigated **(Figures 4 and 5, Table 2, Suppl. figures S1 and S2).** These alterations were calculated for the pathways outlined in **Suppl. table S2**.

Furthermore, these findings are also visually represented in the Volcano plots **(Suppl. figures S1 and S2).** Notably, the Volcano plots reveal that DKK-1, Coagulation factor III or tissue factor (F3), SerpinB2, FST, FGFBP1, and UPLA are common genes across these pathways. This highlights the importance of increasing the expression of these cancer-related genes in driving the EMT, cell cycle viability, wound healing, and tumor development. Moreover, DKK-1's involvement in enhancing the aggressiveness of PDAC and the migration of tumor cells underscores its critical role in the onset of PC (Igbinigie et al., 2019)(Liu et al., 2017). SerpinB2 and UPLA were previously explored concerning ECM degradation, cancer invasion, and metastasis (Harris et al., 2017). F3 and FGFBP1 are cancer-related genes categorized as unfavorable prognostic markers in PC in the Human Protein Atlas database.

In contrast, the FOS (AP-1 or c-Fos) cancer-related gene showed reduced expression in several pathways **(Suppl. figures S1 and S2)**. Interestingly, high c-Fos levels are associated with improved survival in some cancers but indicate a poor prognosis in PC (Guo et al., 2015). This association is linked to negative factors such as lymph node metastasis and drug resistance (Guo et al., 2015). Notably, c-Fos seems to have a dual role in cancer, both promoting and inhibiting it, as demonstrated in ovarian carcinomas where lower c-Fos expression aligns with disease progression (Mahner et al., 2008). In the analyzed pre-selected gene sets, we illustrate the intersections of DEGs using the UpSet plot **(Figure 6)**. Notably, the most frequent DEGs are found between those associated with immune response and cell cycle viability.

3.1. Cancer-specific and prognostic genes

Aside from the analysis of DEGs derived from the comparison of TCDCA-treated Capan-1 cells against the control group, we also cross-referenced this data with the Human Protein Atlas, examining the distribution of our defining DEGs in various tumors as well as pancreatic tumor tissue and normal pancreas. The results of this comparison can be found in Figure 6. We comprehensively analyzed cancer-specific genes, employing various categories to assess their expression patterns. In **Figure 7A,** we illustrate the distribution of genes across five distinct categories: low cancer-specific, cancer-enhanced, cancer-enriched, group-enriched, and undetected. To provide a more detailed perspective, we computed each cancer type's cumulative counts of cancer-enriched, group-enriched, and cancer-enhanced genes (**Figure 7B**). Furthermore, we investigated prognostic genes, categorizing them into favorable and unfavorable prognostic groups (Figure 6C). These gene sets were determined based on differential expression analysis. Our findings are visually presented in a bar plot, offering insights into the potential prognostic markers for each cancer type. In this comprehensive analysis, we identified potential molecular

players implicated in the development and progression of PC. Through the examination of RNA cancer specificity and functional annotations, we highlighted key genes with altered expression in the treated Capan-1 cells, shedding light on their potential roles in PC. In the cancer-enhanced group, the following genes have positive log fold change: DHRS9 (fragments per kilobase per million mapped reads, FPKM: 16.6) is a key oxidoreductase enzyme involved in lipid and steroid metabolism. It is expressed mainly by squamous epithelial cells, and its increase implies an unfavorable prognostic marker in PC. NMUR2 (FPKM: PC: 1.8; stomach cancer: 2.3) is a G-protein coupled receptor; its heightened protein expression is measured in malignant pancreatic and stomach cancer tissues. The PADI1 (FPKM: Pancreatic cancer: 13.5), a hydrolase, is involved in the late stages of epidermal differentiation and has an unfavorable prognostic marker in renal cancer. The G-protein coupled receptor NTSR1 (FPKM: Pancreatic cancer: 2.9) and the PTPRH (FPKM: Pancreatic cancer: 16.5) protein phosphatase has a role in apoptosis and secretion. The actin-binding protein-coded gene VILL (FPKM: pancreatic cancer: 22.4) also showed positive changes in actin dynamics, indicating its potential contribution to the altered cytoskeletal organization observed in cancer cells. RN2 is involved in apoptosis and transcription regulation and had a negative log fold change. This multifunctional enzyme is identified as cancer-enhanced, particularly associated with colorectal cancer (FPKM: 17.0) and pancreatic cancer (FPKM: 19.5). The CF transmembrane conductance regulator, CFTR2 is particularly related to colorectal cancer (23.9) and PC (28.0) along with ADRA2A a G-protein coupled receptor (FPKM: cancer .17.7). ERN2, CFTR, and ADRA2A show associations with cancer, particularly colorectal and pancreatic cancers, and CFTR and ADRA2A are identified as FDA (Food and Drug Administration, USA) approved drug targets. **(Table S4).** Several other genes showed negative fold change in the group-enriched set. PINK4, a serine protease inhibitor (FPKM: colorectal cancer (237.5), pancreatic

cancer (269.7), and stomach cancer (111.0), is a favorable prognostic marker in colorectal cancer. ONECUT2 (FPKM: pancreatic cancer 4.3) and FOXA3 (FPKM: pancreatic cancer 20.7) play transcription and transcription regulation roles. Among the group enriched genes, the following had the highest FPKM: TSPAN8 (colorectal cancer: 283.9; pancreatic cancer: 156.9; stomach cancer: 194.9), CEACAM6 (cervical cancer: 139.4; colorectal cancer: 500.8; lung cancer: 344.1; pancreatic cancer: 437.5; stomach cancer: 231.8), TFF2 (pancreatic cancer: 320.9; stomach cancer: 244.9) and TFF1 (breast cancer: 402.1; colorectal cancer: 268.3; pancreatic cancer: 805.7; stomach cancer: 744.5). These genes being identified as FDA approved drug targets, cancer-related genes and essential oncogenes. **(Table S4).**

4. Discussion

4.1 Pancreatic Cancer and Bile Acids

Bile acids play a significant role in many types of cancers along the gastrointestinal tract. In most cases, they play a role in the development and acceleration of cancer progression, and their effect largely depends on their hydrophobicity, the presence of BA transporters in the given tissue, and which organ-specific signaling pathways are activated by the BAs.

Pancreatic head cancer often leads to OJ and higher serum BA levels, indicating a strong association with PC. While BAs are known to be toxic to normal cells, their role in PC remains debated (Nagathihalli et al., 2014). FXR, also known as BA receptor, plays a significant role in various cancer types, such as breast, lung, esophageal, and pancreatic cancers, with overexpression of FXR associated with heightened cancer cell proliferation (You et al., 2019). Additionally, the activation of FXR was found to increase EMT in hepatocellular carcinoma by modulating EMT markers (Kainuma et al., 2018). Yu. J and colleagues reported that BAs play a role in inducing gastric intestinal metaplasia through activation of the FXR/nuclear factor-κB pathway, leading to increased expression of CDX2 and MUC2 (Yu et al., 2019). The process includes active or diffusion-based entry of BAs into cells, prompting increased p50/p65 expression and nuclear localization. Nuclear p50 interacts with the CDX2 promoter, activating CDX2. FXR's heightened expression, due to BAs, reinforces p50's binding to the CDX2 promoter.

Consequently, CDX2 links to the MUC2 promoter, leading to heightened MUC2 activation. This pathway triggers abnormal CDX2 and MUC2 expression in normal gastric epithelial cells through FXR/NF-κB signaling, potentially causing intestinal metaplasia (Yu et al., 2019). A prior study found that BAs enhance PDAC cell tumorigenicity by upregulating MUC4 expression.

Additionally, we observed a correlation between OJ and increased MUC4 expression in patients. Furthermore, our findings revealed a significantly worse 4-year overall survival rate for PDAC patients with OJ compared to those without (Gál et al., 2020). The high occurrence of PC near the biliary tract suggests a potential link between elevated BAs and PC progression (Joshi et al., 2016)(Gál et al., 2020). Our comprehensive study delved into the intricate relationship between TCDCA and PDAC progression. The significance of our research lies in the pressing need for innovative approaches to address the challenges posed by PDAC, including late-stage diagnoses and limited treatment options. Our investigation employed RNA sequencing techniques to explore the impact of TCDCA, a secondary BA, on the Capan-1 PDAC cell line. The Capan-1 cell line, derived from human PDAC, exhibits epithelial morphology during adherent tissue culture growth. These cells demonstrate resistance to 5-fluorouracil similar to that of the primary tumor. They carry an oncogenic K-Ras mutation (G12V) and an inactivating p53 mutation. Interestingly, they display heightened epidermal growth factor receptor (EGFR) expression but lack SMAD family member 4 (SMAD4) protein expression (Emily L. Deer et al., 2010).

4.2 BA influenced gene expression changes

This approach allowed us to uncover significant alterations in gene expression and associated pathways, shedding light on the potential implications of BA dysregulation in PDAC development and opening new avenues of therapeutic strategies. Our study's pivotal findings centered on identifying DEGs following a 24-hour TCDCA treatment of Capan-1 cells, with 1597 genes exhibiting upregulation and 1353 genes showing downregulation. To deepen our comprehension of the biological consequences of these DEGs, we conducted GSEA using GO and KEGG databases. Several highly upregulated genes were identified, such as DKK-1, SerpinB2, KRT80,

FST, FGFBP1, and UPLA. Several of these genes are well-established players in PDAC tumorigenesis, metastasis, and cell migration, further underscoring the potential relevance of BAs in driving PDAC progression. In addition to our findings, we reviewed other relevant research. Notably, DKK-1 emerged as a promising biomarker for PDAC, exhibiting high sensitivity and specificity (Han et al., 2015). Its overexpression in PDAC tissues correlates with disease progression and poor prognosis, highlighting its potential as a diagnostic and prognostic tool (Takahashi et al., 2010). DKK-1's role in promoting PDAC aggressiveness and tumor cell migration further underscores its significance in pancreatic carcinogenesis (Igbinigie et al., 2019)(Liu et al., 2017). Early tumor detection is crucial, and recent studies reveal that DKK-1 is a promising biomarker for PC (Han et al., 2015). With a sensitivity of 89.3% and specificity of 79.3% in detecting PDAC, DKK-1 has gained attention (Han et al., 2015). DKK-1 is frequently overexpressed in various cancers, including PDAC, and is linked to cancer aggressiveness and tumor cell migration (Takahashi et al., 2010). Its elevated expression in PC tissues suggests its role in tumorigenesis (Igbinigie et al., 2019). KRT80 also garnered attention due to its involvement in cellular differentiation and tumor progression (Wei et al., 2023). Elevated KRT80 expression in various cancer types suggests its role in promoting proliferation, migration, invasiveness, and poor prognosis (Li et al., 2018). Understanding how KRT80 influences cancer cell behavior could lead to novel therapeutic interventions. Proteases play a vital role in coagulation, inflammation, and extracellular remodeling, impacting cell migration, tumor growth, and metastasis (Brassart-Pasco et al., 2020). UPLA is a serine protease involved in cancer invasion and metastasis by regulating the plasminogen/plasmin system. UPLA amplifies proteolytic cascades necessary for cancer invasion, as evidenced by its overexpression, which correlates with lymphatic invasion and metastasis *in vivo*, and its requirement for cholangiocarcinoma cell invasion *in vitro*, suggesting

therapeutic potential (Thummarati et al., 2012). In PC, abnormal matrix remodeling and increased tumor growth are linked to elevated UPLA activity, correlating with worse survival in PDAC patients (Hosen et al., 2022)(K et al., 2015). UPLA and SerpinB2 were discussed regarding ECM degradation, cancer invasion, and metastasis (Harris et al., 2017).

Furthermore, SerpinB2, which inhibits serine proteases, plays a pivotal role in inflammation and coagulation while significantly influencing collagen remodeling (Harris et al., 2017). SerpinB2's impact on collagen remodeling and its correlation with UPLA activity in PDAC (Kumar et al., 2022). These discoveries emphasize the potential for targeting the UPLA/SerpinB2 axis as a therapeutic approach to treating PC (Kumar et al., 2022). Both proteins play crucial roles in these processes. However, UPLA's upregulation is associated with invasion and metastasis in various cancers (Emily L Deer et al., 2010). Upregulation of UPLA indicates an activation of the fibrinolytic system, which might be related to tissue remodeling and wound healing besides activating MMPs. This response could be linked to cellular migration, invasion, or tissue repair processes in pancreatic cells. Our pathway analysis discovered that UPLA plays a role in cellular migration and tumor development **(Figure S1 and Figure S2)**.

4.3 Implications in PDAC

Consequently, its upregulation could indicate an elevated risk of metastasis in PDAC. This finding suggests that TCDCA might influence signaling pathways that are essential for cell growth and survival in Capan-1 cells and offers persuasive evidence for the multifaceted influence of BAs on PDAC development. The positive regulation of pathways motility and migration hints at pathways BAs potentially enhancing cancer cells' migratory and invasive attributes. The upregulation of

pathways related to endothelial cell proliferation and angiogenesis further implies that BAs may promote the formation of new blood vessels to sustain tumor growth **(Figure S1 and Figure S2)**.

Taken together, we reported for the first time how bile acids influence the gene expression pattern in PC. Using RNA sequencing techniques on the Capan-1 cell line, we have identified some genes whose up- or downregulation by BA treatment presumably plays a role in the effect of bile acids on cancer progression. It also illuminates new avenues for therapeutic strategies targeting the interplay between BAs and PDAC progression. A deeper investigation is essential to elucidate the precise molecular mechanisms underlying these observed effects and explore their clinical implications within PC. Ultimately, our results may pave the way for new potential diagnostic markers and therapeutic interventions targeting BAs in the context of PC, offering promising strategies to combat this devastating disease.

5. Conclusion

In conclusion, our comprehensive investigation into the effects of TCDCA on Capan-1 PC cells has illuminated the intricate relationship between BAs and PDAC. These findings hold great significance in light of the urgent need for innovative approaches to address the challenges posed by PDAC, a disease characterized by late-stage diagnoses and limited therapeutic options.

We unveiled many DEGs in TCDCA-treated Capan-1 cells through RNA sequencing techniques, which are closely associated with PDAC tumorigenesis, metastasis, and cell migration. This underscores the potential relevance of BAs in driving PDAC progression through the modulation of these critical genes.

Furthermore, our gene set enrichment analysis (GSEA) revealed various pathways influenced by TCDCA treatment, ranging from coagulation and angiogenesis to cell migration and signaling

regulation. These findings suggest that BAs may create a microenvironment conducive to tumor growth and metastasis, thereby highlighting their multifaceted impact on PDAC development.

Moreover, our review of relevant literature reinforces the significance of specific genes such as DKK-1, KRT80, UPLA, and SerpinB2 in PDAC, shedding light on potential diagnostic and prognostic markers and therapeutic targets. Understanding the roles of these genes in cellular differentiation, tumor progression, ECM degradation, invasion, and metastasis could lead to innovative therapeutic interventions.

Collectively, our study, in conjunction with existing research, underscores the complex interplay between BAs and PDAC, opening up new avenues for research and offering promising directions for developing improved diagnostic and treatment strategies. The multifaceted effects of BAs on gene expression, signaling pathways, and the tumor microenvironment emphasize their pivotal role in PDAC progression. Further investigation is essential to unravel the precise molecular mechanisms and clinical implications of these discoveries, thereby advancing our knowledge of PDAC and instilling hope for enhanced diagnostic and therapeutic options in the future.

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Credit authorship contribution statement

Conceptualization: V.V., Z.V.; methodology: E.G., Z.V., M.V., and S.P.; validation: M.V., Z.V., and V.V.; formal analysis: S.P., V.M..; investigation: E.G., M.V., Z.V., V.V.; resources: Z.V., V.V. and H.P.; writing—original draft preparation: S.P., V.M., V.V., Z.V., and T.P.; writing—review and editing: Z.V., V.V., and S.P.; visualization: V.M., S.P.; supervision: V.V. and Z.V.; funding acquisition: V.V, P. H., L.K, and Z.V. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

Data availability

All data can be provided upon request.

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Figures and tables legends

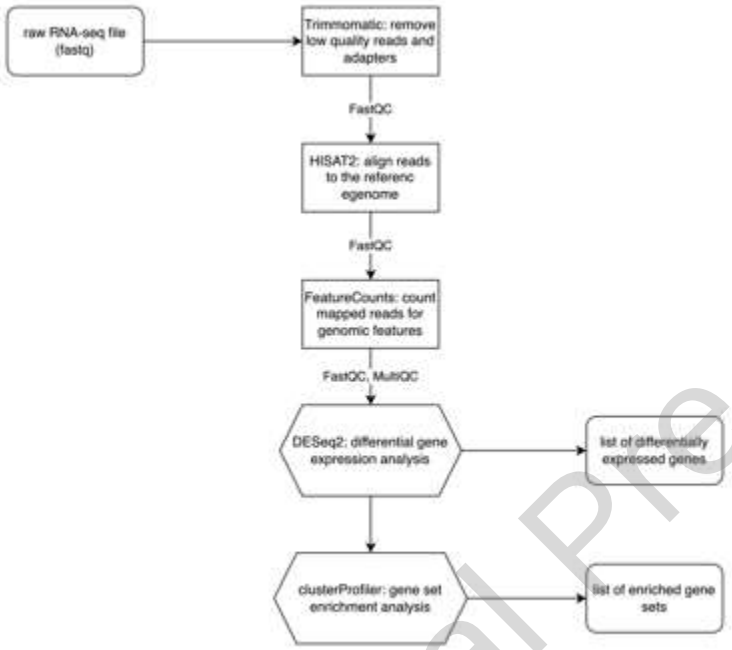

Fig 1. NGS Analysis Process.

The diagram outlines the progression of NGS data analysis, covering essential steps from initial sequencing data to the extraction of biological insights. *(FastQC: default settings, version 0.11.9, FeatureCounts: flags used: -s 0, subread version 2.0.3, HISAT2: default settings, version 2.2.1, MultiQC: default settings, version 1.11, R: version 4.3.2, DESeq2: version 1.44.0, clusterProfiler: version 4.4.0)*

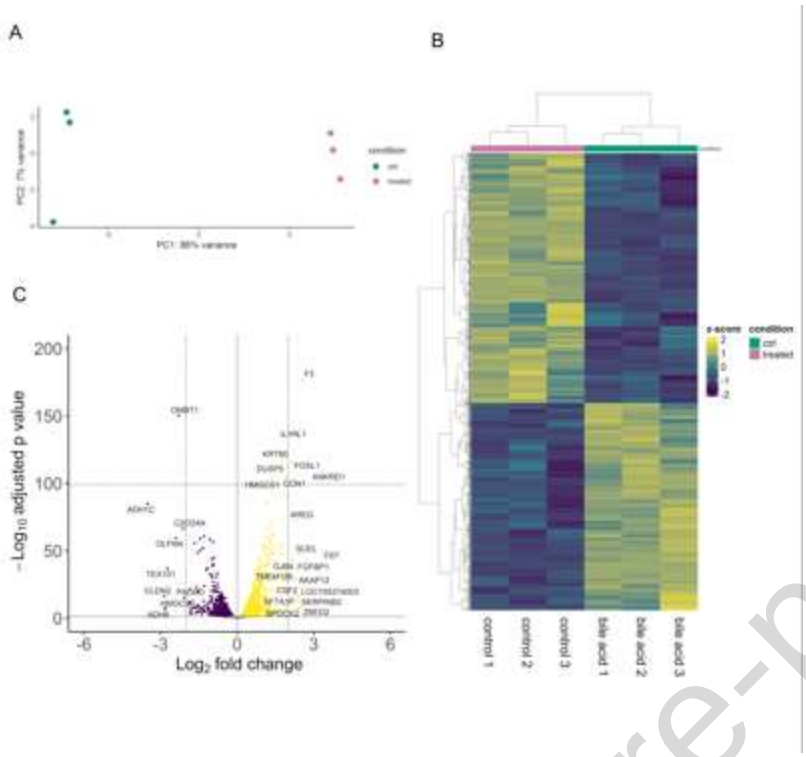

Fig 2. Representation of gene expression changes upon bile acid treatment

A) The PCA plot with PC1 explaining 86% and PC2 explaining 7% of the variance effectively distinguishes between the control and TCDCA-treated Capan-1 cell groups. **B)** Volcano plots show the genes with significantly increased or decreased gene expression based on log2fold changes of DEGs. Significantly up-regulated genes are shown in yellow, significantly downregulated genes are shown in blue, and genes that are not significant are represented with gray dots. **C)** The Heat map visualization of all DEGs of three TCDCA-treated capan-1 compared to untreated cells. **PCA:** the principal component analysis, **TCDCA:** taurochenodeoxycholic acid, and **DEG:** the differentially expressed gene. (Ctrl 1-2-3: untreated Capan-1 culture samples, TCDCA 1-3: treated Capan-1 cultures)

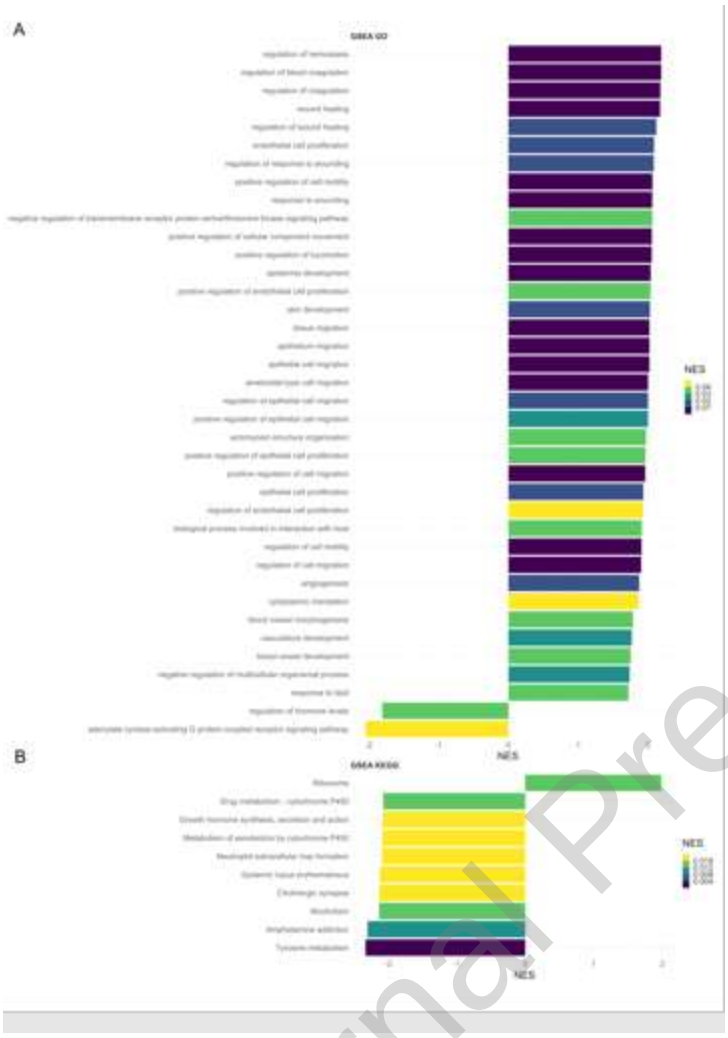

Fig 3. Significant GO terms and KEGG pathways are shown on bar charts.

The terms on the charts are ordered by their NES and colored based on the adjusted *p*-value. **A)** The results of the GSEA-GO revealed that 37 pathways showed upregulation, while 2 pathways exhibited downregulation. The input DEGs were ranked based on their log2 fold changes in the x-axis, and the color intensity of the columns depends on the set size and adjusted *p*-value. **B)** Compared to the control, the KEGG pathway analysis of all DEGs in TCDCA-treated Capan-1. The pathways are ordered according to their normalized enrichment score (NES), and the color intensity of the columns depends on the adjusted *p*-value. **GO:** gene ontology, **KEGG:** Kyoto Encyclopedia of Genes and Genomes, **GSEA:** gene set enrichment analysis**, NES:** normalized

enrichment scores, **DEG:** differentially expressed gene, **TCDCA:** taurochenodeoxycholic acid. Settings: minimum gene set size = 5, maximum gene set size = 200, adjusted *p*-value cutoff = 0.05.

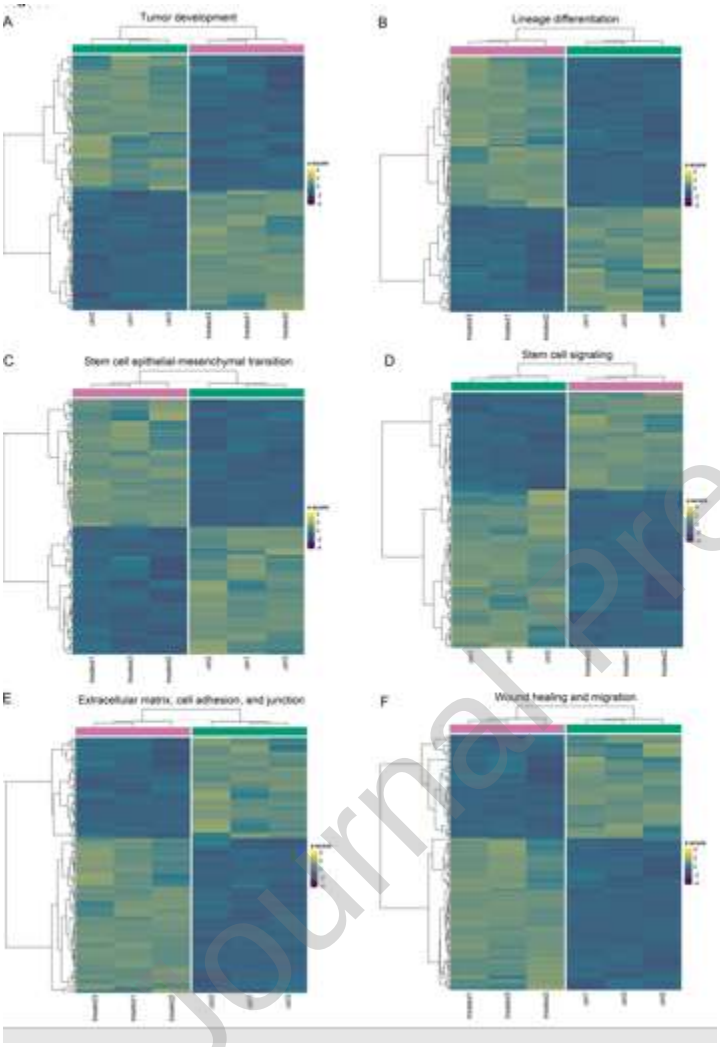

Fig 4. Detailed examination of pathway gene expression changes.

Using heat maps, this figure explores the gene expression alterations within the pathways featured in Figure 2A. The heat maps represent Differentiation lineage, DNA repair, Immune response, Cellular senescence, Cytokines growth factors, and ECM cell adhesion junction pathways. The control group is depicted in green and the TCDCA-treated group in pink. Z-scores were computed

from count data transformed with DESeq2's variance stabilizing transformation. Significantly upregulated genes are highlighted in yellow, while downregulated genes are indicated in blue

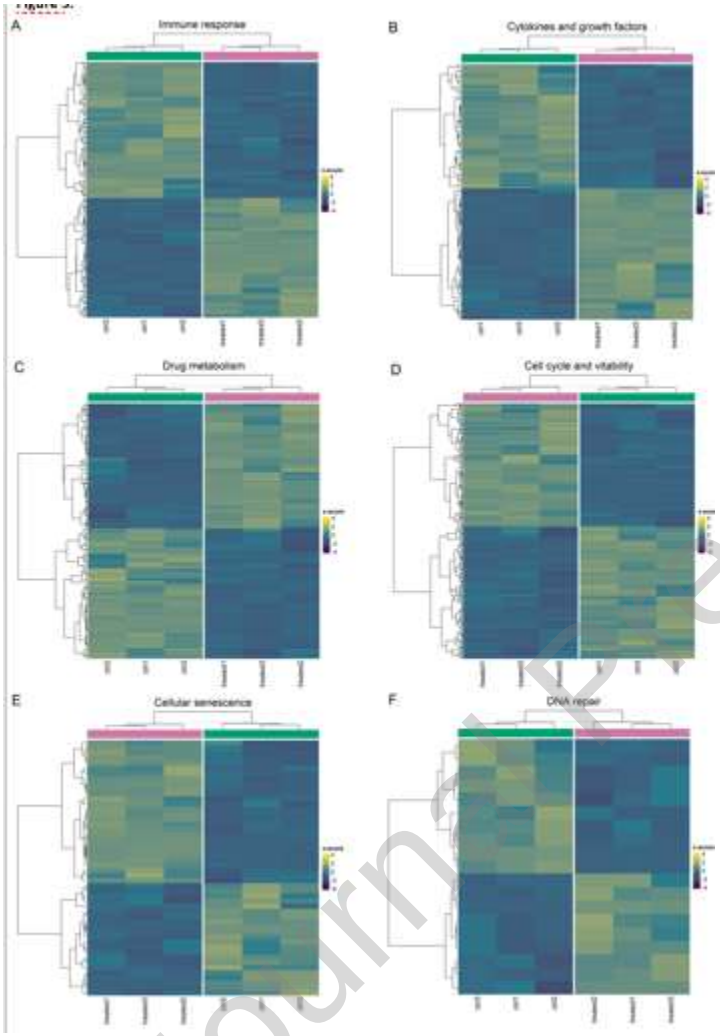

Fig 5. In-depth analysis of pathway gene expression alterations.

This figure provides a comprehensive exploration of gene expression changes within specific pathways. Heat maps represent the following pathways: Drug metabolism, Wound healing migration, Tumor development, Stem cell EMT, Stem cell signaling, and Cell cycle viability. The control group is represented in green and the TCDCA-treated group in pink. Z-scores were computed from count data transformed using DESeq2's variance stabilizing transformation.

Significantly upregulated genes are marked in yellow, while significantly downregulated genes are highlighted in blue.

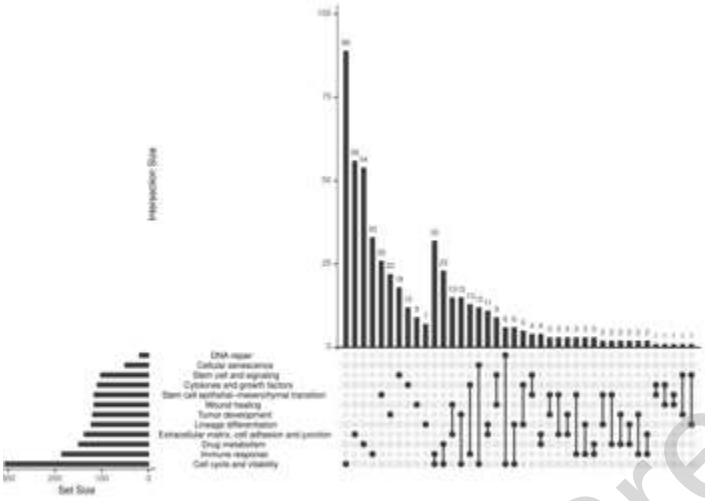

Fig 6. UpSet plots depict the intersection of the differentially expressed genes (DEGs) from each pathway gene set in TCDCA-treated Capan-1 Cells.

These UpSet plots visually represent the gene intersections within various pathways in the Capan-1 cell line following treatment with TCDCA. The vertical bars highlight the number of shared genes, denoting the intersection size, among specific pathway sets indicated by bottom-filled connected circles. Meanwhile, the horizontal bars indicate the number of DEGs in the gene sets obtained from each integration of the meta-analysis method used in this study. The most common DEGs are those linked to immune response and cell cycle viability.

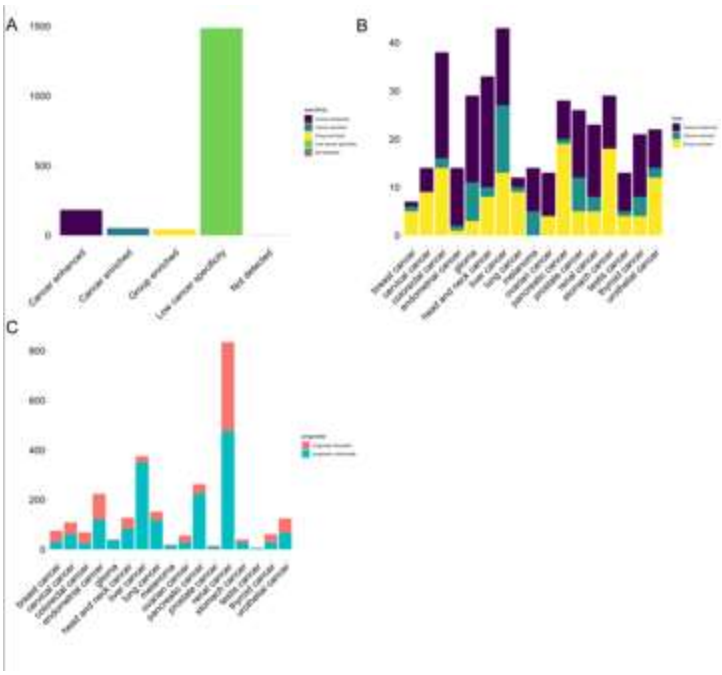

Fig 7. Differentially expressed genes (DEGs) of TCDCA-treated Capan-1 are categorized based on the protein atlas database

A) Bar plot illustrating the categorization of DEGs into the following groups: low cancer-specific, cancer-enhanced, cancer-enriched, group-enriched, and not-detected. **B)** Bar plot displaying the cumulative count of genes falling into categories of cancer-enriched, group-enriched, and cancer-enhanced genes within each specific cancer type. Genes for this analysis were selected based on their significant differential expression in Capan-1 cells. **C)** This figure features a bar plot illustrating DEGs within two distinct categories of prognostic genes: prognostic favorable and unfavorable genes. **TCDCA:** taurodeoxycholic acid.

Table 1. List of DEGs' highest upregulated and lowest downregulated genes (log2 fold change).

Top 20 genes with the highest log2fold changes		
Gene	log2FoldChange	adjusted P-Value

RPSAP52	1.91761748603617	6.11437192076265e-06
PLAU	1.97360283610232	2.48169637616083e-90
FOSL1	2.13275130322469	2.57763572053598e-116
GJB4	2.18171539753444	1.81520028197699e-43
TMEM156	2.26953820241796	1.23672603363859e-30
AREG	2.27168376315836	2.05194997274344e-73
CSF2	2.28504035076402	2.38491445732698e-17
AKAP12	2.30706055723608	2.49906145876908e-29
SFTA1P	2.34636945317727	5.38246382929021e-12
LOC105374003	2.41311667439154	6.72090193959893e-17
SPOCK2	2.41359729951972	1.47084504988416e-08
IL1RL1	2.44882697050403	1.6989449596199e-141
SCEL	2.45438215072186	3.59470131723638e-48
SERPINB2	2.46683912134666	9.70982528475217e-09
ZBED2	2.47538584334969	1.36436714554975e-08
F3	2.56767942267926	7.57848057833923e-178
FGFBP1	2.72662143216685	3.53350688991191e-43
DKK1	3.21874041131879	1.86268093823236e-291
ANKRD1	3.3342378339208	7.01457141961731e-110
FST	3.44877000557752	1.40552491430927e-51

Top 20 genes with the lowest log2fold changes

Gene	log2FoldChange	adjusted P-Value
ADH1C	-3.51067995768074	1.04980646149166e-85
CLDN2	-2.85854788668993	5.71302672805771e-17
ADH6	-2.8413629568822	1.06919198404025e-07
HMGCS2	-2.80783359858698	2.67768236893027e-08
TEX101	-2.73973144805931	1.17884901520272e-37
OLFM4	-2.39585772740399	2.47078310189859e-60
DMBT1	-2.28787580937837	4.09718601903283e-151
C2CD4A	-2.10988417599711	3.08329366212686e-67
PALMD	-2.08091091588025	5.03413482823346e-16

EGR1	-1.8839805327235	1.66426636709451e-11
CXCL10	-1.86202076858005	0.000282998659965532
BTG2	-1.84424223342062	3.55389605009985e-22
SLC38A4	-1.82013338628972	1.17279882810278e-12
GPRIN3	-1.80572063084712	4.04616010026662e-06
SLCO2A1	-1.79850459771961	5.2430610637314e-08
ADGRG7	-1.75550882900463	0.000341497352966066
C2CD4B	-1.69242794161865	2.00679635916326e-08
PRR15L	-1.6561714181394	2.03994445888457e-56
FOS	-1.61877792226112	1.78820923032861e-08
ADRA2A	-1.59118939622354	9.69715280092789e-05

Table 2. List of pathways and DEGs ratios of each gene set (DEGs/ Full set size)

Gene sets	Full set size	DEGs	Ratio
Cellular senescence	160	50	0.3125
Tumor development	447	119	0.2662192
Wound healing and migration	443	116	0.261851
Lineage differentiation	601	122	0.202995
Immune response	912	185	0.2028509
Stem cell signaling	508	102	0.2007874
Cell cycle and viability	1548	307	0.1983204
Stem cell EMT	599	116	0.1936561
Cytokines and growth factors	592	109	0.1841216
Drug metabolism	829	149	0.1797346
DNA repair	129	19	0.1472868
ECM cell adhesion and junction	1022	137	0.1340509

Fig S1. Volcano plots reveal gene expression alterations in pathways. This figure presents volcano plots illustrating changes in gene expression within pathways, including ECM cell adhesion junction, tumor development, stem cell EMT, Immune response, Wound healing migration, and Stem cell signaling. DKK1, UPLA, and MMP1 are consistently upregulated genes observed in all pathways. Significantly upregulated genes are depicted in yellow, significantly downregulated genes in blue, and genes without significant changes are shown in gray dots. **EMT**: epithelial-mesenchymal transition, **DKK-1:** Dickkopf-1, **UPLA**: urokinase plasminogen activator, **MMP1:** matrix metalloproteinase-1.

Fig S2. Volcano plots reveal gene expression alterations in pathways. This figure highlights volcano plots that visually represent gene expression alterations within several pathways, including cell cycle viability, Cellular senescence, Cytokines growth factors, Differentiation lineage, DNA repair, and Drug metabolism. UPLA, DKK1, and SerpinB2 emerge as the most prominently upregulated genes in specific pathways. Significantly upregulated genes are color-coded in yellow, significantly downregulated in blue, and genes without significant changes are denoted by gray dots. **DKK-1:** Dickkopf-1, **UPLA**: urokinase plasminogen activator.

Table S1. Significantly differentially expressed genes

Table S2. A list of gene set enrichment analysis (GSEA) results for gene ontology (GO) pathways.

Table S3. A list of gene set enrichment analysis (GSEA) results for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Table S4. Summary of Food and Drug Administration (FDA)- approved drug targets, cancer-related genes, and essential oncogenes.

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Zoltan Vereb reports financial support was provided by Hungarian Academy of Sciences. Viktoria Venglovecz reports financial support was provided by National, Research, Development and Innovation Office. Lajos Kemeny reports financial support was provided by Hungarian Centre of Excellence for Molecular Medicine. Zoltan Vereb reports financial support was provided by National, Research, Development and Innovation Office. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Highlights

- Pancreatic cancer (PC) presents a significant global health challenge, ranking as the fourth leading cause of cancer-related deaths due to its high mortality rate. The absence of specific symptoms

often results in late-stage diagnoses, with Pancreatic Ductal Adenocarcinoma (PDAC) being the predominant cause in most PC cases.Recent investigations have highlighted a promising correlation between heightened serum levels of bile acids (BAs) and the initiation of PDAC tumorigenesis. In our study, dedicated to unraveling this complex association, we examined the impact of taurochenodeoxycholic acid (TCDCA), a secondary bile acid, on PDAC using advanced RNA sequencing techniques applied to the Capan-1 cell line. Our research yielded a significant breakthrough, identifying 2,950 differentially expressed genes (DEGs) in response to TCDCA treatment. Among these, 1,597 genes exhibited upregulation, while 1,353 displayed downregulation, shedding light on the intricate molecular changes induced by TCDCA exposure.These DEGs were intricately linked to crucial PDAC pathways, including coagulation, angiogenesis, cell migration, and signaling regulation. This emphasizes the influential role of TCDCA in these vital processes and hints at potential avenues for targeted therapeutic interventions. Expanding our exploration into the genetic landscape, our study conducted an extensive literature review, spotlighting key genes such as DKK-1, KRT80, UPLA, and SerpinB2, acknowledged for their pivotal roles in PDAC tumorigenesis and metastasis.

- Our research unveils the intricate interplay between bile acids and PDAC, providing valuable insights into potential diagnostic markers and therapeutic targets. Identifying specific genes and pathways guides future investigations aimed at unraveling the precise mechanisms underlying these discoveries. While our study represents a significant advancement in comprehending the intricate connections between BAs and PDAC, it emphasizes the need for further research. A more in-depth exploration of these findings is essential to unveil precise mechanisms and clinical implications, offering the potential for transformative advancements in diagnosing and treating pancreatic cancer.