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RAPID COMMUNICATION

Systematic comparison of experimental and human obstructive cholestasis reveals conservation of canonical pathway activation and biomarkers relevant for cholestatic liver disease

Cholelithiasis-induced cholestasis is one of the most common causes of hospitalization due to gastrointestinal disease, yet considerable knowledge gaps exist in the pathogenesis of this disease. This can partially be explained by inadequate characterization of experimental cholestasis models. Here, we compared the transcriptional profile of commonly used mouse models for obstructive cholestasis and benchmarked them to human disease to identify the model(s) best suited for cholelithiasis-induced cholestasis research and to uncover conserved mechanisms involved in human and murine cholestasis. Selected mouse models included bile duct ligation (BDL) surgery and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet, and a drug-induced cholestasis model using cyclosporin A (CsA), in an acute and chronic setting. Human samples were collected from patients with cholelithiasis-induced cholestasis of an acute (HUMAC) and recurrent nature (HUMREC). RNA sequencing was performed on mouse and human liver tissue. Both the BDL and DDC models, but not the CsA model, were shown to be applicable for studying cholelithiasis-induced cholestasis, with transcriptomic profiles that highly correspond to acute cholestasis in human patients. In particular, the conservation of canonical pathways related to the inflammatory response and cytoskeleton organization, in which the Rho family GTPase is involved, were identified. Our study furthermore revealed promising mechanistic-based transcriptomic biomarkers relevant for murine and human cholestasis, which could potentially be useful for robust prediction and detection of diverse types of cholestatic liver disease.

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In this study, fresh liver tissue was collected from patients scheduled for cholecystectomy for treatment of acute cholecystitis (HUMAC1–3, $n = 3$) or recurrent symptomatic cholelithiasis (HUMREC1–4, $n = 4$), further referred to as acute and recurrent cholestasis, at the Ghent University Hospital between October 2020 and March 2021. The selected patients underwent liver biopsy at the time of surgery. For the collection of control liver tissue, patients ($n = 3$) without clinical signs of cholestasis that were scheduled for liver resection (HUMN1 and HUMN3) or gastric bypass (HUMN2) were selected. In mice, the BDL model was selected to induce extrahepatic obstructive cholestasis and the DDC diet to induce intrahepatic obstructive cholestasis. The CsA model was selected to be able to compare results against experimental drug-induced cholestasis. Liver samples from mice and patients were subjected to RNA sequencing. The general and clinical characteristics of the selected mouse models and human patients are described in Figure S1 and S2 and Table S1–3. The total amount of differentially regulated genes in mice and human cholestasis is illustrated in Figure S3. A principal component analysis was applied to visualize the resemblance and divergence in differentially regulated genes between acute and recurrent obstructive cholestasis in mice and humans as well as between the experimental mouse models, and to evaluate the variability within experimental groups. In human patients, 3 distinct clusters could be identified, namely control, acute and recurrent cholestasis (Fig. 1A). However, "HUMAC3", a liver sample from a patient suffering from acute cholestasis, clustered to the recurrent cholestasis samples. In this patient, sampling occurred during an acute episode in a patient with recurrent symptomatic episodes. Based on elevated serum biomarkers

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present in all samples with acute cholestasis, the sample was retained in the category of acute cholestasis. In the principal component analysis of mouse samples, 3 distinct clustering groups could be detected (Fig. 1B). The CsA model clustered together with the control groups (cluster A), whereas for the BDL and DDC model, acute and chronic samples clustered together per time point (Fig. 1B). Liver samples from the chronic CsA model only exhibiting 1 upregulated gene were compared to the control and therefore omitted from further analyses. To further elucidate conserved underlying mechanisms and conserved pathways involved in cholestasis, pathway analysis was performed on the RNA sequencing data. Accordingly, a comparison analysis was performed in IPA including all the canonical pathways that were significantly affected in at least 2 of the treatment groups (Fig. S4). An important divergence in the canonical pathways of murine and human cholestasis was the regulation of oxidative phosphorylation, which was predicted to be activated in human cholestasis samples, while inactivated in the mouse models. With regard to commonly affected pathways, a particular preponderance was found in pathways associated with the cytoskeletal reorganization (Fig. 1C). The latter included signaling by Rho family GTPases, Rho GDP-dissociation inhibitor signaling, RAC signaling, regulation of actin-based motility by Rho, actin nucleation by actin nucleation factor-Wiskott-Aldrich syndrome protein complex and Rho A signaling. Indeed, modifications in the intermediate filaments were presumed to play a role in cholestasis, particularly intermediate filaments containing cytokeratin 8 (*KRT8*) and cytokeratin 18 (*KRT18*). Accordingly, a strong upregulation of *Krt8* and *Krt18* was evidenced in the DDC and BDL models, already in the acute setting, and in patients with acute cholestasis, albeit only the upregulation

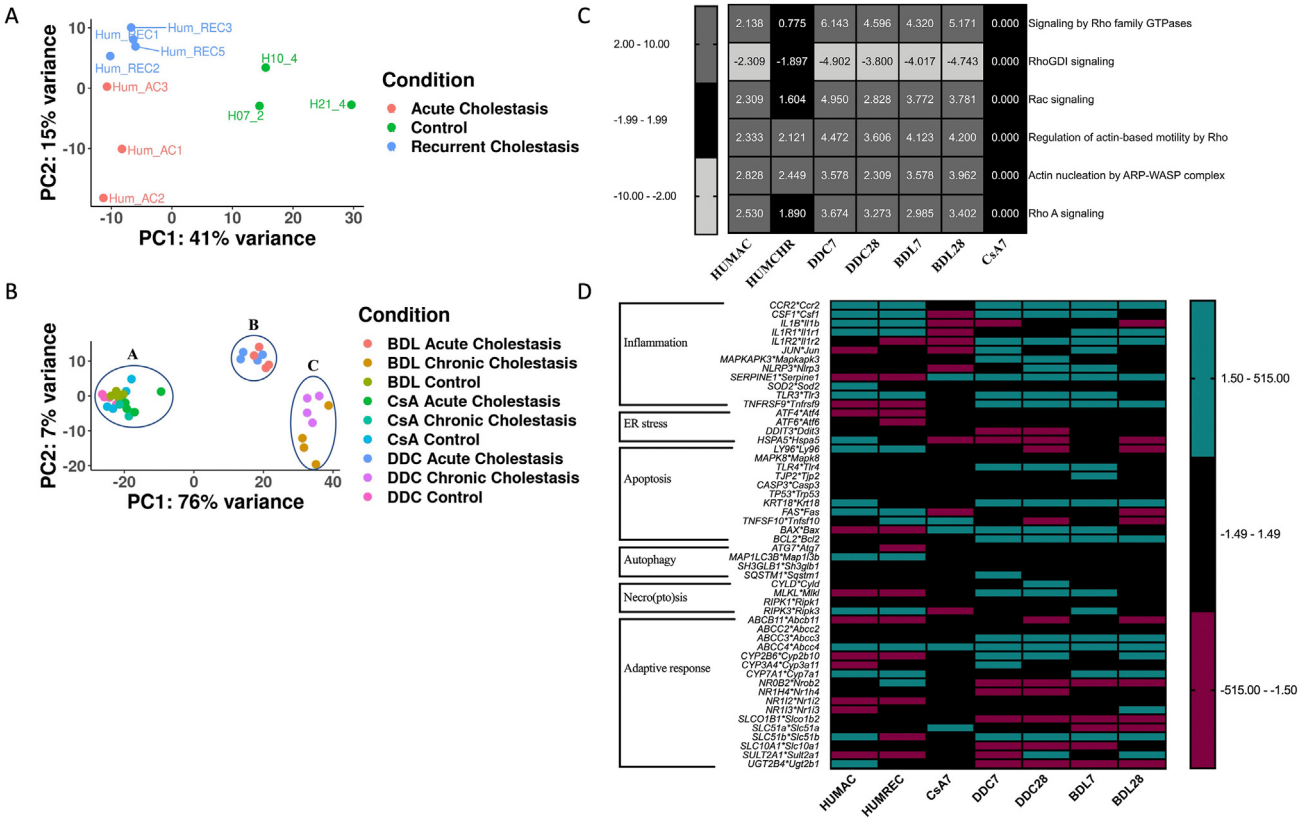

Figure 1 Transcriptional changes in human and experimental cholestasis. Human liver samples were collected from patients with acute and recurrent cholestasis and control patients and subjected to RNA sequencing analysis. Cholestasis was induced in mice, either by performing a BDL surgery, administering 15 mg/kg bodyweight CsA dissolved in olive oil via oral gavage or by feeding 0.1% wt/wt DDC diet. Control mice were subjected to sham surgery, olive oil via oral gavage or control diet. Samples were collected after 7 and 28 days in all models and subsequently subjected to RNA sequencing analysis. **(A, B)** Principal component analysis was applied to the variance stabilized counts to visualize and investigate the clustering of the RNA sequencing data. Genes were considered significantly differentially expressed if FDR $\leq$0.05 and an absolute value of the log2 fold change $\geq$1. FDR was calculated by means of a Wald test (DEseq2 package in R) followed by a Benjamini Hochberg correction ($n = 3-4$). **(C)** Further functional toxicological analysis was performed in the IPA software. Data related to cytoskeletal reorganization are expressed as z-score ($z \leq -2$ is predicted inhibited and $z \geq 2$ is predicted activated). Z-scores were calculated as a statistical measure for the similarity in expected relationship direction and observed gene expression via an algorithm in IPA. **(D)** Gene expression was assessed from an established gene list based on an updated adverse outcome pathway of cholestatic liver injury.[1] BDL, bile duct ligation; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; FDR, false discovery rate; HUMAC, human acute cholestasis; HUMREC, human recurrent cholestasis; IPA, ingenuity pathway analyzer; PC, principal component.

of *KRT8* was statistically significant (Fig. S5A). To corroborate the identification of pathways associated with cytoskeletal reorganization, gene expression levels of selected representative genes, including *RhoA, Rac1, Cdc42, Rock1, Mlc phosphatase* and *aSma*, were determined via RT-qPCR. All tested genes except *Rac1* showed mild but significant upregulation which was most pronounced in the acute setting in the BDL model. In contrast, increased gene expression in the DDC model tended to be most outspoken in the chronic setting. Notably, *Rac1* was downregulated in both models in a time-dependent manner (Fig. S5B).

Using transcriptomics, previous studies by Gijbels et al. report on a transcriptional blueprint based on the adverse outcome pathway (AOP) of cholestasis in an *in vitro* setting.[1,2] This AOP describes the inhibition of the bile salt export pump (BSEP) as the major molecular initiating event or triggering factor in addition to hepatocellular changes (e.g., cytoskeletal disorganization) and bile canalicular disturbances.[3] These molecular initiating events evoke a deteriorative response, characterized by inflammation, mitochondrial stress, oxidative stress, endoplasmic reticulum stress and cell death,[1,3] and an adaptive response which strives to counteract the deteriorative response by activating nuclear receptors that regulate genes involved in bile acid homeostasis.[3] Thus far, the AOP-based transcriptomic signature was merely focused on *in vitro* models of chemical-induced cholestasis and 1 single *in vivo* model of cholestasis.[1,2] In this study, we aimed to assess the robustness of the established transcriptomic blueprint for experimental and human (obstructive) cholestasis *in vivo,* and identify transcriptional biomarkers fit for detecting different types of cholestasis. In this regard, genes associated with inflammation and, to a lesser extent, apoptosis were upregulated in BDL and DDC mouse and human cholestasis in compliance with the transcriptomic signature (Fig. 1D). From these genes, toll like receptor 3, C—C motif chemokine receptor 2, colony stimulating factor 1 and interleukin 1 receptor 1 were commonly affected in mice and humans (Table S5). With respect to necroptosis, upregulation of receptor-interacting protein kinase 1 or 3 was identified in DDC models and the acute BDL model or human cholestasis patients, respectively. Related to the adaptive response, 4 genes encoding transporters of bile acids were upregulated in BDL and DDC models, being multidrug resistance associated protein 3 and 4 (*Mrp3/4*), organic solute transporter β (*Ostβ*) and sodium-taurocholate cotransporting polypeptide. Similarly, *MRP4* was also upregulated in human patients with acute and recurrent cholestasis, while *OSTβ* and *UDP glucuronosyltransferase 2B4* were only upregulated in liver tissue from patients with acute cholestasis (Fig. 1D and Table S5). Observed discrepancies with the transcriptomic signature include an upregulation of BSEP and cytochrome P450 7A1 in human cholestasis patients and BDL mice. Acute CsA-induced cholestasis showed minimal compliance with the transcriptomic blueprint, except from the upregulation of *Mrp4* and *Ostα*.

In conclusion, this work represents a transcriptional comparison analysis of different modes of cholestasis to identify the best suited mouse model to study human cholelithiasis-induced cholestasis, in which both BDL and DDC, but not CsA, mouse models showed fit-for-purpose. Conserved pathways were identified related to inflammation and cytoskeletal organization, with a potentially promising role of the Rho GTPase family. Abnormal activation of Rho kinase, an important downstream effector of Rho A GTPase, has been shown to play an essential role in liver fibrosis.[4] Our data show that upstream activation of Rho GTPases was already present in early obstructive cholestasis. Considering the described prominent role of Rho family GTPases in the retrieved canonical pathways of cholestasis (progression), targeting this group of enzymes might be a promising avenue in the treatment of cholestasis. Treatment studies including Rho GTPase modulators are already ongoing in other inflammatory diseases.[5] Studies devoted to cholelithiasis-induced cholestasis are awaiting.

Conflict of interests

The authors have no conflict of interests to declare.

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Availability of data and material

The transcriptomic data sets discussed in this manuscript have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE183899.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.02.003.

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