Expression of fibrosis-related genes in liver allografts: Association with histology and long-term outcome after pediatric liver transplantation

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Abstract

Background: Unexplained graft fibrosis and inflammation are common after pediatric liver transplantation (LT).

Objective: We investigated the graft expression of fibrogenic genes and correlated the findings with transplant histopathology and outcome.

Methods: Liver biopsies from 29 recipients were obtained at a median of 13.1 (IQR: 5.0–18.4) years after pediatric LT. Control samples were from six liver-healthy subjects. Hepatic expression of 40 fibrosis-related genes was correlated to histological findings: normal histology, fibrosis with no inflammation, and fibrosis with inflammation. Liver function was evaluated after a subsequent follow-up of 9.0 years (IQR: 8.0–9.4).

Results: Patients with fibrosis and no inflammation had significantly increased gene expression of profibrotic TGF-β3 (1.17 vs. 1.02 p = .005), CTGF (1.64 vs. 0.66 p = .014), PDGF-α (1.79 vs. 0.98 p = .049), PDGF -β (0.99 vs. 0.76 p = .006), integrin-subunit-β1 (1.19 vs. 1.02 p = .045), α-SMA (1.12 vs. 0.58 p = .013), type I collagen (0.82 vs. 0.53 p = .005) and antifibrotic decorin (1.15 vs. 0.99 p = .045) compared to patients with normal histology. mRNA expression of VEGF A (0.84 vs. 1.06 p = .049) was lower. Only a few of the studied genes were upregulated in patients with both fibrosis and inflammation. The gene expression levels showed no association with later graft outcome.

Conclusions: Altered hepatic expression of fibrosis-related genes is associated with graft fibrosis without concurrent inflammation.

KEYWORDS
fibrosis, gene expression, inflammation, liver transplantation, pediatrics

1 | INTRODUCTION

The long-term survival of patients after liver transplantation (LT) has improved during the last decades.1 The focus of interest has shifted from the early events to long-term patient health and graft survival. Several centers have reported the development of subclinical fibrosis and inflammation in long-term follow-up studies.2 The etiology and mechanism of this slow process remain unclear.
Subclinical portal/interface inflammation, often associated with fibrosis, is proposed to present a form of late antibody-mediated rejection, at least in some cases.\textsuperscript{2–4} Grafts with subclinical inflammation have histological features of chronic rejection, such as bile duct loss and pericentral fibrosis.\textsuperscript{5} The association of portal/interface inflammation with donor-specific antibodies is, however, controversial.\textsuperscript{6–8} Inflammation is closely related to fibrosis as the inflammatory reaction disturbs the balance of normal extracellular matrix turnover leading to fibrosis.\textsuperscript{9} Fibrosis may, however, also appear without inflammation.

The molecular and cellular basis of fibrosis in different liver diseases is actively examined, but less is known about grafts.\textsuperscript{10} Hepatic stellate cells (HSCs), activated by inflammatory cells and cytokines, are the major source of the extracellular matrix (ECM) producing myofibroblasts.\textsuperscript{11} The main activator of HSCs into ECM producing myofibroblasts is transforming growth factor-\(\beta\) (TGF-\(\beta\)).\textsuperscript{12} TGF-\(\beta\) induces ECM component production, promotes hepatocyte apoptosis, and inhibits ECM degradation.\textsuperscript{13} TGF-\(\beta\) is secreted as an inactive form that binds to latency-associated protein and latent TGF-binding protein-\(\beta\), which anchor it to the ECM, forming a reservoir.\textsuperscript{14} TGF-\(\beta\) can be released and activated by plasmin, matrix metalloproteinases, thrombospondin-1, and \(\alpha\)-integrins.\textsuperscript{15} Importantly, several other growth factors, such as the connective tissue growth factor (CTGF) and platelet-derived growth factor (PDGF), are also known to participate in liver fibrogenesis.\textsuperscript{16,17}

Multiple studies have described the gene expression of proinflammatory and profibrotic cytokines in allograft rejection.\textsuperscript{18,19} Less is known about the role of these genes in chronic graft fibrosis. In a recent study, the whole genome transcriptional analysis of the liver tissue was compared to graft histology in 133 pediatric LT recipients.\textsuperscript{20} In that study, liver grafts with fibrosis and no inflammation overexpressed genes involved in fibrogenesis and stellate cell activity. Proinflammatory gene sets were not over-presented. Livers with portal inflammation, interface activity, and variable degree of fibrosis showed overexpression of several gene sets known to be involved in T-cell mediated rejection.

The aim of this study was to analyze if the graft expression of fibrosis-related genes would associate with graft histology or later clinical outcome. In this work, the hepatic gene expression of 40 genes related to TGF-\(\beta\)-signaling, other growth factors, integrins, plasminogen system, HSC activation, and EMC components was analyzed in biopsy samples from asymptomatic recipients with a median of 13.1 years after pediatric liver transplantation. Hepatic gene expression of these mediators was correlated with histopathological findings and long-term graft outcome.

2  \textbf{PATIENTS AND METHODS}

2.1  Patients and controls

Between 1987 and 2007, a total of 99 pediatric LTs (recipients <18 years of age) from deceased donors were performed at the Helsinki University Hospital, Finland. At the time of this cross-sectional study, between 2009 and 2011, 66 of the 99 recipients were alive, and 56 consented to the study and underwent liver biopsy. Liver biopsies were used for conventional histology and immunohistochemical staining in other studies. The remaining tissue material was sufficient for gene expression analysis in 29 (29/56) patients. The median time from LT to liver biopsy was 13.1 (IQR: 5.0–18.4) years.

In October 2019, the clinical records of all 29 patients were evaluated. The median time from the liver biopsy to the last follow-up visit was 9.0 (IQR: 8.0–9.4) years. Patient characteristics divided into three groups according to histological findings are presented in Table 1.

Control liver samples for gene expression analysis were obtained from six patients undergoing cholecystectomy for cholecystolithiasis who consented to the biopsy (research protocol 434/13/03/03/2008).

2.2  Liver biopsies and histological assessment

Liver samples were percutaneous core needle biopsies taken under ultrasound guidance by an experienced pediatric radiologist. The biopsy sampling for controls was performed under visual control during laparoscopy. Biopsies included a median of nine portal areas (7–13). Two experienced pathologists blinded to the clinical data reviewed the biopsy samples to reach a consensus. Portal, interface, and endothelial inflammation were graded according to the Banff criteria.\textsuperscript{21} Fibrosis was staged according to the Metavir staging.\textsuperscript{22} Central and sinusoidal fibrosis were evaluated as absent or present. Microsteatosis was recorded as a percentage of hepatocytes affected and bile duct loss as a percentage of portal areas missing bile ducts.

2.3  Hepatic gene expression analyses

The expression of 40 genes related to fibrogenesis was investigated [Table 2]. The liver samples were embedded in RNAlater solution immediately after biopsy (Ambion, Life Technologies, Thermo Fisher Scientific Inc, Waltham, MA, USA). The samples were freeze-stored until analyzed. RNA was extracted using the RNeasy Mini Kit (QIAGEN, Frederick, Maryland, USA), and its integrity was assessed spectrophotometrically. Quantitative real-time polymerase chain reaction (rt-PCR) by the Human Fibrosis RT\(^2\) Profiler\textsuperscript{TM} PCR Array (QIAGEN SABiosciences, Frederick, Maryland, USA) was used for the analysis of mRNA levels in triplicate. ABI 7700 Sequence Detection System (Perkin-Elmer Life Sciences, Boston, MA, USA) was applied according to the manufacturer’s instructions (www.sabiosciences.org). Target-gene mRNA expression was quantified by the \(\Delta\Delta\text{Ct}\) method. After normalization to housekeeping genes, the mRNA levels were presented as fold change in relation to the mean of control subjects.

2.4  Laboratory measurements

Total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), \(\gamma\)-glutamyl transferase (GGT), platelet count, prealbumin, and thrombin time (TT) were measured at Helsinki University Hospital Laboratory (HUSLAB).
2.5 | Evaluation of the liver function

At follow-up, liver function was recorded as normal or abnormal. The latter group included patients who fulfilled one or more of the following findings: re-transplantation after gene analysis (2 patients), total bilirubin ≥25 µmol/L, ALT ≥60 U/L, GGT ≥50 U/L, or TT <70%.

2.6 | Ethics

This study was performed according to the requirements of the Helsinki Declaration. The ethics committee of the Children's Hospital, Helsinki, and Uusimaa Hospital District approved the study plan (IRB approval number 345/13/03/03/2008). All patients and control subjects (and parents in the case of minors) signed an informed consent form.

2.7 | Statistical analysis

Statistical analyses were performed with SPSS 25.0 statistics software (IBM, Somers, NY). Data are presented as medians and IQR, if not otherwise stated. Mann-Whitney U test was used for continuous variables and Fisher's exact test for categorical variables to compare differences between two groups. Kruskal-Wallis test and Fisher's test were used for comparison of difference across multiple groups. Correlations were calculated with the Spearman's test. Two-tailed p-values <.05 were considered statistically significant.

3 | RESULTS

The gene expression of 40 fibrosis-related molecules was analyzed in liver graft samples from 29 LT patients at a median of 13.1 years...
after LT [Table 1]. Considering the characteristics presented in Table 1, the patients included in the study (n = 29) differed only by the age at LT compared to patients excluded from the study due to insufficient liver biopsy (n = 27), 1.9 [1.1–8.45] vs. 8.9 [1.7–14.0] p = .043 Mann-Whitney U-test). Patients were divided into three groups according to their histological findings. Group 1 included patients without hepatic fibrosis or inflammation (n = 11), Group 2 included patients with fibrosis but no inflammation (n = 10), and Group 3 included patients with both fibrosis and inflammation (n = 8) [Table 3]. Control patients displayed normal liver function and had normal histology [Tables 1 and 3]. The three groups and controls differed in age, total bilirubin, and AP levels but were

| Gene name | Protein | Actions in liver fibrosis | Reference |
|-----------|---------|---------------------------|-----------|
| Transforming growth factor-β | Transforming growth factor-β1-β3 | Fibrogenic; Activation of HSCs, promotion of ECM production, induction of TIMP and reduction of MMP production, promotion of fibrogenic growth factor expression and hepatocyte apoptosis | 23–26 |
| TGFBR1 and 2 | Transforming growth factor-β receptor 1 and 2 | TGF-β superfamily receptors | 27 |
| ENG | Endoglin | Antifibrogenic; accessory co-receptor for TGF-β that inhibits TGF-β signaling pathway | 28 |
| SMAD2-4 and 6-7 | SMAD family member 2 | TGF-β superfamily intracellular signaling pathway proteins | 27 |
| DCN | Decorin | Antifibrogenic; extracellular inhibition of TGF-β | 29 |
| BMP-7 | Bone morphogenetic protein 7 | Antifibrogenic; inhibition of TGF-β intracellular signaling pathway | 30 |
| GREM1 | Gremlin 1 | Fibrogenic; BMP-7 antagonist | 31 |
| LTBP1 | Latent transforming growth factor-β binding protein 1 | TGF-β is secreted in an inactive form bind to LTBP | 32 |

**TABLE 2** List of the 40 genes whose mRNA expression in the liver grafts was measured, and the corresponding proteins

| Gene name | Protein | Actions in liver fibrosis | Reference |
|-----------|---------|---------------------------|-----------|
| CTGF | Connective tissue growth factor | Fibrogenic; Enhance TGF-β actions | 33 |
| EGF | Epidermal growth factor | Fibrogenic; inhibition of EGF receptor reduces fibrosis | 34 |
| HGF | Hepatocyte growth factor | Anti-fibrogenic; promotion of HSC apoptosis, inhibition of hepatocyte apoptosis | 35 |
| PDGFA | Platelet-derived growth factor subunit-α | Fibrogenic; Activation of HSCs, promotion of HSCs proliferation and ECM production, upregulates the expression of MMPs and TIMPs | 17 |
| PDGFB | Platelet-derived growth factor subunit-β | Fibrogenic; Activation of HSCs, promotion of HSCs proliferation and ECM production, upregulates the expression of MMPs and TIMPs | 17 |
| VEGFA | Vascular endothelial growth factor A | Anti- and pro-fibrogenic; Activation, but also inhibition of HSCs | 36,37 |

**Integrins and thrombospondins**

| ILK | Integrin-linked kinase | Fibrogenic; HSC activation | 38 |
| ITGA1-3 and ITGAV | Integrin subunit-α1-3 and αv | Fibrogenic; αv-integrins combined to β-subunits activate latent TGF-β | 39,40 |
| ITGB1, 3, 5, 6 and 8 | Integrin subunit-β1, β3, β5, β6 and β8 | Fibrogenic; activation of latent TGF-β (THBS1), increased in fibrosis (THBS2) | 41,42 |

**Plasminogen system**

| PLG | Plasminogen | Anti-fibrogenic; inactive precursor for plasmin that can degrade ECM components and activate latent TGF-β | 23,43,44 |
| PLAT | Tissue plasminogen activator | Anti-fibrogenic; activation of plasminogen into plasmin | 43 |
| PLAU | Urokinase plasminogen activator | Anti-fibrogenic; activation of plasminogen into plasmin | 43 |
| SERPINE1 | Plasminogen activator inhibitor-1 | Fibrogenic; inhibition of plasminogen activators | 43 |

**Hepatic stellate cell and extra cellular matrix**

| ACTA2 | Alfa-smooth muscle actin | Marker of HSCs activation | 45 |
| COL1A2 | Pro-α2(I) chain, component for type I collagen | Markers of ECM production; Type I and III collagens are the main overexpressed ECM components in liver fibrosis | 46,47 |
| COL3A1 | Pro-α1(III) chain, components for type III collagen | Markers of ECM production; Type I and III collagens are the main overexpressed ECM components in liver fibrosis | 46,47 |
otherwise similar [Table 1]. Gene expression of bone morphogenetic protein-7 (BMP7) and tissue plasminogen activator (PLAT) was mostly undetectable both in patients and controls, and they were not included in the analysis.

In the whole patient cohort fibrosis stage correlated positively with the gene expression of profibrotic TGF-β1 and -β3, PDGF-α, α-SMA, thrombospondins 1 and 2, and type I and III collagen and antifibrotic decorin [Figure 1].

Visual presentation of the expression of genes, which were found to differ between controls and the three patient groups, are presented in Figure 2A–E divided according to the main actions of the gene products. Altogether Group 2 patients (fibrosis without inflammation) showed most increased expression of fibrosis-related genes (Figure 2A–E).

The hepatic gene expression of the studied fibrosis-related genes was mostly lower in Group 1 (normal graft histology) than control subjects. Group 1 patients had lower gene expression of fibrogenic growth factor PDGF subunit-β (PDGFB) [Figure 2A], fibrosis markers α-SMA (ACTA2), type I collagen (COL1A2) and type III collagen (COL3A1) [Figure 2D], TGF-β1 signaling inhibitor endoglin (ENG) [Figure 2E] and higher expression of fibrogenic integrin-β3 (ITGB3) [Figure 2C] and TGF-β1 signaling related SMAD2 (SMAD2) [Figure 2B].

In Group 2 (fibrosis and no inflammation), the expression of the fibrosis-related genes was higher than that in controls, as Group 2 patients had higher expression of fibrogenic integrin-linked kinase (ILK), integrin-β1 (ITGB1), integrin-β3 (ITGB3) [Figure 2C], TGF-β1 signaling-related latent TGF-β1 protein (LTBP1), and SMAD2 (SMAD2) [Figure 2B].

Only a few genes differed between controls and Group 3 (both fibrosis and inflammation) patients, as Group 3 patients had lower TGF-β1 signaling inhibitor endoglin (ENG) [Figure 2E] and fibrogenic growth factor PDGF subunit-β (PDGFB) [Figure 2A].

Liver transplantation patients in Group 2 (fibrosis no inflammation) had significantly higher expression of fibrogenic growth factors TGF-β1, PDGF-α, PDGF-β, α-SMA (ACTA2), and type I collagen (COL1A2) [Figure 2D], antifibrotic TGF-β inhibitor decorin (DNC) [Figure 2E] and lower expression of antifibrotic vascular endothelial growth factor A (VEGFA) [Figure 2E] than Group 1 patients (normal graft histology). Patients in Group 3 (both fibrosis and inflammation) showed much fewer differences in the expression of the studied fibrogenic genes than patients in Group 1 (normal histology). Patients in Group 3 (both fibrosis and inflammation) had increased gene expression of only fibrotic growth factor TGF-β1 (TGFB1) [Figure 2A], fibrosis marker type I collagen (COL1A2) [Figure 2D], and antifibrotic TGF-β1 inhibitor decorin (DNC) [Figure 2E] and lower gene expression of fibrogenic integrin-β3 (ITGB3) [Figure 2C], than patients in Group 1 (normal histology).

We found few differences in mRNA levels between patients in Group 2 (fibrosis without inflammation) and group 3 (fibrosis and inflammation). Patients in the latter group had significantly lower hepatic gene expression of fibrogenic growth factors TGF-β3 (TGFB3), PDGF subunit-β (PDGFB) [Figure 2A], fibrogenic integrin subunit-β1 (ITGB1) and integrin subunit-β5 (ITGB5) [Figure 2C], and fibrogenic plasminogen activator inhibitor (SEPRINE1) [Figure 2F].

Ten patients’ clinical status was altered at the follow-up, median 9.0 years after the liver biopsies. Two had undergone re-LT (one from Group 1 and another from Group 2), and eight had altered laboratory findings at the follow-up. Clinical status was recorded as stable in 16 patients. Three patients were deceased due to extra-hepatic reasons during the follow-up and were excluded from the analysis. There were no differences in hepatic gene expressions of the investigated 40 genes between patients with stable or altered clinical status at the follow-up (Mann-Whitney U-test p-values ranging from 0.092 to 0.958).

4 | DISCUSSION

The fibrosis stage in liver grafts correlated positively with the gene expression of profibrotic TGF-β1 and -β3, PDGF-α, integrin-α3, thrombospondins 1 and 2, and antifibrotic decorin. Liver biopsies with fibrosis and no inflammation had higher mRNA levels of profibrotic TGF-β1, decorin, CTGF, PDGF-α, PDGF-β, and lower levels of VEGF-α than in patients with normal histology. Interestingly, the expression of fibrogenic genes in liver transplants with both inflammation and fibrosis was relatively similar to grafts with normal histology and control livers, as only a few significant differences were found. Altogether the expression of fibrosis-related genes had the strongest association with liver grafts with fibrosis and no inflammation. Gene expression levels showed no association with the long-term outcome of the grafts.

Hypotheses behind the etiology of subclinical graft inflammation and fibrosis include T-cell-mediated and antibody-mediated rejection.2 Besides immunological processes, fibrosis in the liver grafts may be caused by ischemia due to vascular problems, cholestasis, oxidative processes, and infections. After the insult, fibrosis can continue without inflammation as seen in many liver grafts. Further studies are needed to investigate the mechanisms behind subclinical lesions. The important question is whether the development of subclinical inflammation and fibrosis can be prevented and the prevention of these changes affects patient outcome.

In our work, mRNA expression of TGF-β1 was particularly related to fibrosis with inflammation and that of TGF-β3 to fibrosis without inflammation. TGF-β1 is the key mediator of liver fibrosis. It activates HSCs, induces epithelial to mesenchymal transformation (EMT), and induces hepatocyte apoptosis.12-14,23 Increased expression of TGF-β1 is also related to cirrhosis.48 In a study of adult kidney transplant patients, elevated mRNA levels of TGF-β1 were associated with interstitial fibrosis and tubular atrophy (IF/TA).49 TGF-β1 is inactivated by binding to decorin in the ECM.26

Decorin expression correlated positively with the stage of fibrosis, and the transcript levels increased in fibrotic grafts with or without inflammation. Decorin is an inhibitor of fibrotic grafts as
well as an extracellular inhibitor of TGF-β and has a protective role in liver fibrogenesis. Overproduced TGF-β, ECM deposition, and inflammatory activity (in chronic hepatitis) are all known to induce decorin expression, which probably explains the elevated decorin mRNA levels in the liver grafts.

The expression of SMAD2 and latent transforming growth factor-β–binding protein 1 was increased in patients with fibrosis but no inflammation when compared with controls. SMAD2 is a part of the TGF-β intracellular signaling pathway, and latent transforming growth factor-β–binding protein 1 is a protein that is bonded to TGF-β before its secretion from the cell. Our results are possibly reflecting increased TGF-β actions in these patients.

The mRNA levels of CTGF, PDGF-α, and PDGF-β were also elevated in liver grafts showing fibrosis without inflammation. PDGF and CTGF are important fibrogenic growth factors in liver disorders. PDGF participates in HSC activation, promoting ECM synthesis, and CTGF enhances TGF-β signaling. Similar to our finding, increased

| Histological findings | Group 1 (n = 11) | Group 2 (n = 10) | Group 3 (n = 8) | Controls (n = 6) |
|-----------------------|-----------------|-----------------|----------------|-----------------|
| **Fibrosis**          |                 |                 |                |                 |
| F0                    | 11 (100)        | 0 (0)           | 0 (0)          | 6 (100)         |
| F1                    | 0 (0)           | 8 (80)          | 6 (75)         | 0 (0)           |
| F2                    | 0 (0)           | 0 (0)           | 2 (25)         | 0 (0)           |
| F3                    | 0 (0)           | 2 (20)          | 0 (0)          | 0 (0)           |
| F4                    | 0 (0)           | 0 (0)           | 0 (0)          | 0 (0)           |
| **Inflammation**      |                 |                 |                |                 |
| Portal inflammation   |                 |                 |                |                 |
| 0                     | 11 (100)        | 10 (100)        | 1 (13)         | 6 (0)           |
| 1                     | 0 (0)           | 0 (0)           | 6 (75)         | 0 (0)           |
| 2                     | 0 (0)           | 0 (0)           | 0 (0)          | 0 (0)           |
| 3                     | 0 (0)           | 0 (0)           | 1 (13)         | 0 (0)           |
| Interface inflammation| 0               |                 | 5 (63)         | 0 (0)           |
| 1                     | 0 (0)           | 0 (0)           | 2 (25)         | 0 (0)           |
| 2                     | 0 (0)           | 0 (0)           | 1 (13)         | 0 (0)           |
| 3                     | 0 (0)           | 0 (0)           | 0 (0)          | 0 (0)           |
| Lobular inflammation  |                 |                 | 7 (88)         | 6 (0)           |
| 0                     | 11 (100)        | 10 (100)        | 0 (0)          | 0 (0)           |
| 1                     | 0 (0)           | 0 (0)           | 0 (0)          | 0 (0)           |
| 2                     | 0 (0)           | 0 (0)           | 1 (13)         | 0 (0)           |
| 3                     | 0 (0)           | 0 (0)           | 0 (0)          | 0 (0)           |
| Microsteatosis⁴        |                 |                 | 6 (75)         | 6 (0)           |
| 0%                    | 9 (82)          | 5 (50)          | 6 (75)         | 6 (0)           |
| >0 < 10%              | 1 (9)           | 2 (20)          | 1 (13)         | 0 (0)           |
| 10%–30%               | 1 (9)           | 2 (20)          | 1 (13)         | 0 (0)           |
| >30%                  | 0 (0)           | 1 (10)          | 0 (0)          | 0 (0)           |
| Loss of bile ducts⁵    |                 |                 | 7 (88)         | 6 (0)           |
| 0%                    | 11 (100)        | 8 (89)²         | 7 (88)         | 6 (0)           |
| >0 < 10%              | 0 (0)           | 0 (0)           | 0 (0)          | 0 (0)           |
| 10%–30%               | 0 (0)           | 0 (0)           | 1 (13)         | 0 (0)           |
| <30%                  | 0 (0)           | 1 (11)          | 0 (0)          | 0 (0)           |

Note: Data presented as number of patients in each group (percentage). Patients were divided in groups according to histological findings; group 1: Patients with near normal histology, Group 2: Patients with fibrosis, but no inflammation, Group 3: Patients with fibrosis and inflammation.

²Percentage of hepatocytes affected.
³Percentage of portal areas missing bile ducts.
⁴Data on bile duct loss is missing in one patient.
⁵<30% data is missing in one patient.
expression of CTGF has been associated with IF/TA lesions in adult kidney transplant patients. Patients with fibrosis without inflammation also had higher gene expression of integrin subunits β1 and β5 and integrin-linked kinase. Integrin subunit β3 also correlated with the fibrosis stage. The integrin receptor consists of different heterodimeric combinations of 18 α- and 8 β-subunits. Especially the αv-subunit that forms combinations with β1-, β3-, β5-, β6-, and β8-subunits is recognized as a regulator of tissue fibrosis, since αv-integrins combined to β-subunits activate latent TGF-β stored to ECM. Dosanjh et al found that the presence of IF/TA in kidney allografts was associated with upregulated gene expression of six β-integrin (1,2,3,5,6,8) and six α-integrin (v, μ, 1, 2,6,9) receptors. In our study of liver grafts, the association of fibrosis with different integrin subunits was less evident, as only three of the studied integrin subunit genes were upregulated. Integrin-linked kinase participates in the signal transduction between integrins and growth factor receptors, and its actions are profibrotic. Accordingly, its expression was increased in patients with fibrosis and no inflammation.

We also found an association of thrombospondins 1 and 2 with graft fibrosis, which is in line with their reported profibrotic properties. Thrombospondin 1 can activate latent TGF-β1 and thus promote liver fibrogenesis. Although thrombospondin 2 is less studied in liver fibrosis, its expression has been correlated with the fibrosis stage in patients with non-alcoholic fatty liver disease. In a previous study by Smith et al, the gene expression of proteins related to myofibroblasts, and myofibroblast-like cells were upregulated in LT recipients with early fibrogenesis. Similarly, we found an association between fibrosis and the α-SMA gene expression. The expression of type I and III collagens also correlated with the fibrosis stage.
FIGURE 2  (A) Graft gene expression of profibrotic growth factors (y-axis) in controls and the three patient groups (x-axis). Data presented as mean (central line), SD (bar borders), and range (outer lines). p-values calculated with Mann-Whitney U-test between two groups. Expression of transforming growth factor-β1 gene, TGFβ1 (A), transforming growth factor-β3 gene, TGFβ3 (B), platelet-derived growth factor subunit-α gene, PDGFA (D), connective tissue growth factor gene, CTGF (C), and platelet-derived growth factor subunit-β gene, PDGFB (E). (B) Graft gene expression of profibrotic TGF-β signaling–related factors (y-axis) in controls and the three patient groups (x-axis). Data presented as mean (central line), SD (bar borders), and range (outer lines). p-values calculated with Mann-Whitney U-test between two groups. Expression of latent transforming growth factor-β–binding protein 1 gene, LTBP1 (A) and SMAD2 gene (B). (C) Graft gene expression of profibrotic integrins (y-axis) in controls and the three patient groups (x-axis). Data presented as mean (central line), SD (bar borders), and range (outer lines). p-values calculated with Mann-Whitney U-test between two groups. Expression of integrin-linked kinase gene, ILK (A), integrin subunit-β1 gene, IGTB1 (B), integrin subunit-β3 gene, IGTB3 (C), and integrin subunit-β5 gene, IGTB5 (D). (D) Graft gene expression of fibrosis markers (y-axis) in controls and the three patient groups (x-axis). Data presented as mean (central line), SD (bar borders), and range (outer lines). p-values calculated with Mann-Whitney U-test between two groups. Expression of α-smooth muscle actin gene, ACTA2 (A), pro-α2(I) gene, a component for type I collagen gene, COL1A2 (B), pro-α1(III) gene, and a component for type III collagen gene, COL3A2 (C). (E) Graft gene expression of anti-fibrotic factors (y-axis) in controls and the three patient groups (x-axis). Data presented as mean (central line), SD (bar borders), and range (outer lines). p-values calculated with Mann-Whitney U-test between two groups. Expression of decorin gene, DCN (A), endoglin gene, ENG (B), and vascular endothelial growth factor-A gene, VEGFA (C). (F) Graft gene expression of profibrotic plasminogen activator inhibitor (y-axis) in controls and the three patient groups (x-axis). Data presented as mean (central line), SD (bar borders), and range (outer lines). p-values calculated with Mann-Whitney U-test between two groups.
Our work supports the recent findings of Feng et al. in their comprehensive study of 133 pediatric LT recipients, patients with interface inflammation and a varying degree of fibrosis had gene expression profiles characteristic to allograft rejection. These patients also had the highest prevalence of class II donor-specific antibodies (DSA). Genes specific for fibrogenesis and stellate cells were upregulated in patients with fibrosis without interface activity. In our study, we did not investigate the expression of genes related to allograft rejection. Still, as in the study of Feng et al., the gene expression of fibrogenic factors was related to graft fibrosis without inflammation. An earlier study of the same patient population as in this work showed that anti-HLA antibodies were associated with portal inflammation, which is in agreement with the findings of Feng et al. Our study supports earlier findings that fibrogenic hepatic gene expression signature is associated with histological graft fibrosis, especially when it occurs without concurrent inflammation. In our study, liver graft expression of fibrogenic genes had no prognostic value for later clinical outcome.

This work has several limitations. 1. The original national cohort of pediatric LT patients included 99 subjects, 33 of whom had died of variable reasons before this study. Of the remaining 56 patients, only 29 patients had enough liver biopsy material for the genetic analyses. Despite the fact that the characteristics of the included and excluded patients were quite similar, the low number of study patients may have caused a bias in the findings. 2. The liver graft samples showed mostly mild fibrosis, which interfered with the comparison of transcript levels and the fibrosis stage. 3. The time gap between LT and the gene expression studies as well as the ages of the patients in different histological groups showed variation causing a possible bias. 4. Patients in Group 2 (age range 4.8–13.4 years at the cross-sectional study) were somewhat younger than patients in the Group 1 (18.2–23.2 years) and Group 3 (11.9–29.3 years). How this affects the biopsy findings and outcome is not clear. However, from our experience, small children show very similar immunological response and histopathological changes in the graft as older patients after kidney and liver transplantation. How the age affects the gene expression, however, is poorly known.

Our results confirm the role of TGF-β, PDGF, and CTGF in the development of fibrosis in liver grafts. At present, there is no therapy for liver fibrosis available for clinical use. In experimental models, several types of antifibrotic therapies have been tested including antibodies against cytokines and their receptors, small-molecule inhibitors, and downregulation of gene expression by RNA interference. It seems important to suppress excessive profibrogenic TGF-β effects, while maintaining the desired wound healing and anti-inflammatory responses. Development of medications against TGF-β and other cytokines is clearly a challenge for the future.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHORS’ CONTRIBUTIONS
Voutilainen SH: Data collection, Statistics, Data analysis/interpretation, Drafting article, Critical revision of the article, Approval of article; Kosola SK: Data collection, Critical revision of the article, Approval of article; Lohi J: Data collection, Critical revision of the article, Approval of article; Pakarinen MP: Design, Funding, Critical revision of article, Approval of article; Jalanko H: Design, Funding, Data analysis/interpretation, Drafting article, Critical revision of the article, Approval of article.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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