Inflamm. Res. (2016) 65:355–365
DOI 10.1007/s00011-016-0921-6

Inflammation Research

ORIGINAL RESEARCH PAPER

# TGF-β signaling is activated in patients with chronic HBV infection and repressed by SMAD7 overexpression after successful antiviral treatment

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Received: 25 March 2015 / Revised: 7 November 2015 / Accepted: 27 January 2016 / Published online: 9 February 2016


**Abstract**
*Objectives* Although animal studies demonstrated that Smad7 induction ameliorates TGF-β/SMAD-mediated fibrogenesis, its role in human hepatic diseases is rather obscure. Our study explored the activation status of TGF-β/activin pathway in patients with chronic liver diseases, and how it is affected by successful antiviral treatment in chronic HBV hepatitis (CHB).
*Methods* Thirty-seven CHB patients (19 with active disease, 14 completely remitted on long-term antiviral treatment and 4 with relapse after treatment withdrawal), 18 patients with chronic HCV hepatitis, 12 with non-alcoholic fatty liver disease (NAFLD), and 3 controls were enrolled in the study. Liver mRNA levels of CTGF, all TGF-β/activin isoforms, their receptors and intracellular mediators (SMADs) were evaluated using qRT-PCR and were correlated with the grade of liver inflammation and fibrosis staging. The expression and localization of pSMAD2 and pSMAD3 were assessed by immunohistochemistry.
*Results* TGF-β signalling is activated in CHB patients with active disease, while *SMAD7* is up-regulated during the resolution of inflammation after successful treatment. *SMAD7* overexpression was also observed in NAFLD patients exhibiting no or minimal fibrosis, despite the activation of TGF-β/activin signaling.
*Conclusions* *SMAD7* overexpression might represent a mechanism limiting TGF-β-mediated fibrogenesis in human hepatic diseases; therefore, SMAD7 induction likely represents a candidate for novel therapeutic approaches.

**Keywords** TGF-β signaling · SMAD7 · Chronic HBV hepatitis · Non-alcoholic fatty liver disease

Responsible Editor: Artur Bauhofer.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00011-016-0921-6) contains supplementary material, which is available to authorized users.

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## Introduction

Chronic liver injury induces fibrosis that is characterized by accumulation of extracellular matrix (ECM) proteins, and is modulated by cytokines regulating the inflammatory response [1, 2]. Among many cytokines and growth factors, the transforming growth factor beta (TGF-β)/activin superfamily along with the major downstream mediator of its signaling, namely connective tissue growth factor

(CTGF), play a pivotal role in hepatic fibrogenesis and development of cirrhosis [3–6]. Blockade of TGF-β signaling in animal models inhibits the fibrotic response in the liver, whereas transcriptional activation of TGF-β induces plasminogen activator inhibitor-1 (PAI-1) and ECM proteins production [7]. Several recent studies have demonstrated increased mRNA and protein levels of TGF-β in the liver and serum of patients with chronic liver diseases that correlate with inflammatory activity and severity of liver injury [3, 8].

TGF-βs and activins transduce their signals by binding to specific transmembrane receptors and activating their intracellular mediators, namely the receptor-regulated SMAD (mothers against decapentaplegic drosophila homolog) proteins (R-SMADs), especially SMAD2 and/or SMAD3. After their activation, R-SMADs associate with the common-mediator SMAD (co-SMAD), namely SMAD4, resulting in the translocation of the SMAD complex into the nucleus, to regulate the expression of target genes [9]. The role of SMADs in the fibrotic process has been demonstrated by previous studies, since SMADs can modulate TGF-β-induced growth arrest through controlling the expression of c-Myc and $p21^{Cip1}$ and inducing retinoblastoma (Rb) phosphorylation [10, 11]. Especially in the liver, SMAD3 seems to be mainly activated during the fibrotic process, as it has been reported in ex vivo animal models [12]. Hepatitis viruses can also affect the TGF-β signaling through interaction with both receptors and SMADs [13]. For instance, the X protein of hepatitis B virus (HBV) can activate the TGF-β pathway through direct interaction with SMAD4 [14], while the nonstructural protein 5A (NS5A) protein of hepatitis C virus (HCV) can interact with TGF-β receptor type I (TGFβRI), known also as activin receptor-like kinase 5 (ALK5) [13].

The dominant intracellular antagonist (and regulator) of TGF-β/activin signaling is SMAD family member 7 (SMAD7) [15, 16]. Recent animal studies confirmed the protective function of Smad7 in attenuating TGF-β-mediated fibrosis in multiple organs, including the liver, through manipulation of its expression using transgenic overexpression, virus-mediated transfection, and genetic deletion by knockout technology [17–20]. In this context, Dong et al. have also observed that SMAD7 expression was low in cultured fibroblasts derived from skin biopsies of patients with systemic sclerosis, suggesting that deficient SMAD7 expression might represent a putative molecular defect in scleroderma [21]. On the other hand, Alatorre-Carranza et al. found a sixfold increase in *SMAD7* expression in liver that was not capable of attenuating the augmented serum expression of TGF-β and the fibrotic hepatic process after a bile duct injury [22].

Considering that the role of TGF-β/activin signaling and SMAD7 expression in human chronic liver diseases is still rather obscure, this study was scheduled to explore the activation status of this pathway in patients with chronic HBV hepatitis (CHB) at diagnosis and how it is affected by successful long-term antiviral treatment. For this purpose, we examined the expression levels of all TGF-β/activin ligands, namely the three TGF-β isoforms and the four types of activins (activin A, activin B, activin C, and activin E), their type I receptors (*TGFBR1/ALK5* and activin receptor-like kinase 4 (*ACVR1/ALK4)*, respectively), the intracellular transducers, *SMAD2*, *SMAD3* and *SMAD4*, the inhibitory protein *SMAD7*, as well as *CTGF*. We identified a specific pattern of expression of molecules involved in TGF-β/activin signaling and, thus, we further compared this pattern of expression with that observed in patients with other chronic liver diseases (chronic HCV hepatitis and steatohepatitis), and individuals with no hepatic pathology on histopathological examination. The expression pattern of the aforementioned genes was also correlated with the intensity of liver inflammation and fibrosis.

## Materials and methods

### Patients and biopsies

Liver biopsy specimens from a total of 67 patients with various liver diseases were evaluated. The original study group comprised 37 CHB patients, including 19 newly diagnosed (CHB/d), 4 responders under treatment for 48 weeks with pegylated-interferon alpha-2a and/or antivirals and relapse after treatment withdrawal for 24 weeks (CHB/nr), as well as 14 on maintained continuous antiviral treatment response and remission for at least 240 weeks (5 years) with entecavir (CHB/r). Considering that in the eastern Mediterranean region, the HBV genotype D and the HBeAg-negative serological form of CHB prevail (about 90 % of affected Greek patients) [23], all the enrolled patients had the abovementioned HBV genotype. Results were further compared with those obtained from the biopsies of 18 newly diagnosed patients with chronic hepatitis C (CHC) and 12 patients with non-alcoholic fatty liver disease (NAFLD). Moreover, liver biopsies from 3 individuals displaying a mild increase of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which revealed no pathology on histopathological examination, served as "healthy controls".

None of the patients had evidence of co-infection with other hepatotropic viruses (types A, D, and E) or superinfection with Human Immunodeficiency Virus (HIV), or had received any other antiviral or immunomodulatory treatment during the last 3 months prior to liver sampling. HBV DNA and HCV RNA quantification was assessed

using the bDNA assay V2.0 (Bayer, Siemens) and the COBAS Amplicor system (Roche Molecular Systems), respectively. Accordingly, the treatment efficacy of CHB patients at the fifth year included the biochemical response based on normalized ALT levels, and the complete virologic response defined as serum HBV DNA <169 copies/ml (29 IU/ml), namely the lower limit of quantification of the COBAS TaqMan assay (Roche Molecular Systems). Demographic, clinicopathologic and serologic data of the analyzed subjects are summarized in Table 1. All CHB patients and "healthy" controls, 14 out of 18 CHC patients and 11 out of 12 NAFLD were derived from previous studies of our group [24, 25], since their genetic material was also available for the analysis of all genes included in this study.

Liver biopsy specimens were separated in two parts, immediately after sampling. The main part was used for diagnostic purposes while the remaining tissue was snap frozen in liquid nitrogen and stored at −80 °C for subsequent analysis. Formalin-fixed, paraffin-embedded sections were stained with haematoxylin–eosin and Masson's trichrome. The diagnosis of each case was independently confirmed histologically by two liver pathologists and discrepancies were resolved by an expert hepatopathologist. In the patients with viral hepatitis, the modified histological activity index (HAI) score, introduced by Ishak et al. [26], was used to grade the inflammatory activity and the stage of disease. In patients with NAFLD, liver histology was graded according to the extent of steatosis, inflammation and fibrosis as proposed by Brunt et al. [27]. According to the grade of liver inflammation, patients were classified as I-0 (without inflammation), I-1 (chronic hepatitis: HAI score 1–4; NAFLD: minimal inflammation), I-2 (chronic hepatitis: HAI score 5–8; NAFLD: mild inflammation), I-3 (chronic hepatitis: HAI score 9–12; NAFLD: moderate inflammation), and I-4 (chronic hepatitis: HAI score 13–18; NAFLD: severe inflammation) (Table 1). The histologic findings pertaining to the grade of inflammation and the stage of disease (as determined by microscopic assessment of haematoxylin–eosin and Masson's trichrome stains) are summarized in Table 1.

Informed consent was obtained from all individual participants included in the study. The study was approved by the Institutional Review Board (Aristotle University of Thessaloniki, Greece) and all procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

## Molecular studies

RNA was extracted from homogenized liver specimens using the TRI reagent (Life Technologies, Invitrogen, Thessaloniki, Greece), according to the manufacturer's instructions. Afterwards, complementary DNA (cDNA) was reverse transcribed from 1 μg of RNA, using a random 6-mer oligonucleotide primer (Roche, Basel, Switzerland) and M-MLV reverse transcriptase (Life Technologies), according to manufacturer's instructions.

The mRNA expression levels of 14 genes were initially determined by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). These included: *TGFB1*, *TGFB2*, *TGFB3*, *ACVR1B/ALK4*, *TGFBR1/ALK5*, *SMAD2*,

**Table 1** Clinicopathological and serological data of the patients of the study

| | Controls | CHB/d | CHB/nr | CHB/r | CHC | NAFLD |
|---|---|---|---|---|---|---|
| No | 3 | 19 | 4 | 14 | 18 | 12 |
| Sex (male/female) | 2/1 | 9/10 | 2/2 | 11/3 | 14/4 | 7/5 |
| Age (median, range) | 61.0, 60–67 | 54.0, 24–64 | 57.0, 22–65 | 52.0, 23–60 | 41.5, 27–54 | 45.0, 21–71 |
| AST (U/μL), (median, range) | 42.0, 36–45 | 51.0, 17–1969 | 62.0, 39–277 | 29.5, 15–51 | 45.0, 24–237 | 31.5, 19–70 |
| ALT (U/μL), (median, range) | 32.0, 21–48 | 61.0, 15–1478 | 97.5, 70–332 | 31.5, 17–49 | 75.0, 32–213 | 54.0, 15–141 |
| Inflammation grade* | | | | | | |
| I-0 | 3 | – | – | 1 | – | 3 |
| I-1 | – | 4 | – | 10 | 2 | 4 |
| I-2 | – | 8 | 3 | 3 | 10 | 5 |
| I-3 | – | 5 | 1 | – | 6 | – |
| I-4 | – | 2 | – | – | – | – |
| Fibrosis stage (median, range)* | – | 4.0, 0–6 | 4.5, 1–5 | 2.0, 0–3 | 3.0, 1–6 | 0.5, 0–2 |
| HAI-score (median, range) | – | 8.0, 1–15 | 8.0, 5–11 | 2.0, 0–7 | 7.0, 2–12 | – |
| NAFLD stage (median, range) | – | – | – | – | – | 2.0, 0–5 |

* Inflammation grade (*I-0* without inflammation, *I-1* minimal, *I-2* mild, *I-3* moderate and *I-4* marked) and fibrosis stage were assessed as presented in the section of "Materials and methods"

*SMAD3*, *SMAD4*, *SMAD7*, *CTGF*, *INHBA* (encodes inhibin beta A), *INHBB* (encodes inhibin beta B), *INHBC* (encodes inhibin beta C), and *INHBE* (encodes inhibin beta E). Afterwards, targeted molecular analyses of *SERPINE1* (encodes plasminogen activator inhibitor type 1, PAI-1), *COL1A1* (encodes collagen type I, alpha I) and *COL3A1* (encodes collagen type III, alpha 1) were performed in 10 CHB patients, 5 with CHB/d and low *SMAD7* expression and 5 with CHB/r and high *SMAD7* expression. The qRT-PCRs were performed using Platinum SYBR-Green PCR Supermix UDG (Life Technologies) in the automated thermocycler RotorGene 6000 (Corbett Life Science, Sydney, Australia) for all genes except for activins/inhibins; the latter were carried out in the MJ Research Chromo 4 detector (Life Technologies) using the same Platinum PCR mix. The beta-2-microglobulin (*B2 M)* gene was used as internal control for the adjustment of relative expression data.

A 1/20 aliquot of the cDNA reaction product was used in duplicate qRT-PCRs, and all measurements were averaged. Primers for the amplification of *TGFB1, TGFB3, TGFBR1, SMAD2, SMAD3, SMAD4,* and *SMAD7* were commercially obtained from Qiagen (Venlo, Limburg, Netherlands). The primer pairs for the amplification of *INHBA*, *INHBB*, *INHBC*, *INHBE*, *ALK4, SERPINE1, COL1A1* and *COL3A1* have already been presented in previous manuscripts [28–30], while the primers for the amplification of *TGFB2* and *CTGF* were designed with the aid of the Oligo 6.0 software (NBI, Plymouth, MN, USA). Thermocycling conditions and primers of the analyzed genes are presented in Electronic Supplementary Material (Table 1). The efficiency of each qRT-PCR ranged between 0.9 and 1.09. To verify the specificity of the PCR products, melting curve analysis was performed from 65 to 95 °C with 0.1 °C/sec intervals and stepwise fluorescence acquisition. The relative quantification and the calculation of the range of confidence were performed using the comparative $\Delta\Delta^{CT}$ method [31]. The relative expression of each gene is presented as a multiple of the respective gene expression in a sample of a patient who underwent liver biopsy due to a mild increase of aminotransferases but without pathologic changes ("healthy control").

### Immunohistochemistry

Immunohistochemical stains for phosphorylated SMAD2 (pSMAD2) and pSMAD3 were performed on 4-micron-thick paraffin sections of liver biopsies with an automated staining system (Bond, Leica Microsystems, Newcastle, UK). In brief, after deparaffinization, the primary antibodies for either pSMAD2 or pSMAD3 were applied on the tissue sections in dilutions of 1:100. The subsequent steps were performed with the Bond polymer refine detection kit (Leica Microsystems, Newcastle, UK), according to the manufacturer's instructions. The primary antibodies to pSMAD2 and pSMAD3 were purchased from Cell Signaling Technology (Danvers, MA, USA). These are rabbit monoclonal antibodies (pSMAD2: 138D4; pSMAD3: C25A9) and detect phosphorylation at Ser465/467 and Ser423/425, respectively.

### Statistical analysis

For basic statistical calculations, all gene expression levels were treated as continuous variables. Differences of gene expression between disease groups were analyzed using the nonparametric Mann–Whitney *U* test. The possible associations of the above parameters with the grade of inflammation and the fibrosis stage were tested with the Kruskal–Wallis *H* test. Spearman's rank correlation coefficient was used to estimate the correlations of the expression levels among the aforementioned genes, as well as the correlations of gene expression with aminotransferase levels and viral load. All statistical calculations were performed with the use of SPSS (version 18.0, Chicago, Il, USA). A two-sided $p$ value of $p < 0.05$ was considered statistically significant.

## Results

As presented in Table 2, as well as Fig. 1a and b, CHB/d patients displayed a remarkable increased expression of *SMAD2* and *SMAD3* compared to CHB/r patients ($p < 0.001$). An increased *SMAD2* expression was also observed in NAFLD patients compared to "healthy controls" and CHB/r patients, although the differences were not significant (Table 2; Fig. 1a). Interestingly, the expression pattern of *SMAD2* and *SMAD3* in CHC patients was different than that observed in CHB/d and NAFLD ones and was rather indistinguishable with that observed in "healthy controls" (Table 2).

The expression pattern of pSMAD2 and pSMAD3 in liver tissues, which demonstrates more directly the localization of the activated TGF-β/activin signaling, was assessed by immunohistochemistry. Figure 2 is an example of active chronic inflammation and fibrosis in a liver biopsy with chronic hepatitis B. As presented in Fig. 3, there was extensive nuclear positivity of the inflammatory infiltrates in both CHB/d and steatohepatitis patients, as well as variable nuclear positivity of the hepatocytes and biliary epithelial cells. Thus, it is reasonable to speculate that TGF-β/activin signaling is activated in these pathologic conditions.

Additionally, we observed that CHB/d patients displayed significantly increased mRNA levels of *CTGF, INHBA*, *INHBE* and *ACVR1/ALK4* compared to controls

**Table 2** Relative expression of the examined genes

| Gene | "Healthy controls" (n.3) | CHB/d (n.19) | CHB/nr (n.4) | CHB/r (n.14) | CHC (n.17) | NAFLD (n.12) |
|---|---|---|---|---|---|---|
| | Mean ± SDEV | Mean ± SDEV ($p$ value)† | Mean ± SDEV ($p$ value)† | Mean ± SDEV ($p$ value)† | Mean ± SDEV ($p$ value)† | Mean ± SDEV ($p$ value)† |
| *TGFB1* | 0.90 ± 0.20 | 1.11 ± 0.84 | 0.61 ± 0.38 | 0.64 ± 0.43 | 0.62 ± 0.56 | 1.50 ± 1.03 |
| | | 0.962 | 0.289 | 0.131 | 0.132 | 0.470 |
| *TGFB2* | 1.44 ± 0.50 | 4.66 ± 5.12 | 5.44 ± 6.72 | 4.57 ± 4.48 | 4.56 ± 5.85 | 3.39 ± 3.09 |
| | | 0.191 | 0.480 | 0.257 | 0.576 | 0.139 |
| *TGFB3* | 0.39 ± 0.53 | 0.16 ± 0.26 | 0.05 ± 0.04 | 0.13 ± 0.12 | 0.09 ± 0.13 | 0.30 ± 0.39 |
| | | 0.667 | 0.157 | 0.529 | 0.159 | 0.773 |
| *ALK5* | 0.87 ± 0.15 | 1.63 ± 1.00 | 0.91 ± 0.50 | 1.69 ± 0.78 | 0.77 ± 0.38 | 2.30 ± 1.31 |
| | | 0.114 | 1.000 | **0.044** | 0.634 | 0.021 |
| *ALK4* | 0.70 ± 0.43 | 4.96 ± 6.15 | 2.38 ± 1.74 | 8.19 ± 5.32 | 2.79 ± 1.98 | 4.93 ± 3.79 |
| | | **0.037** | 0.355 | **0.039** | 0.101 | 0.079 |
| *SMAD2* | 0.41 ± 0.51 | 0.73 ± 0.40 | 0.54 ± 0.14 | 0.28 ± 0.24 | 0.35 ± 0.24 | 0.80 ± 0.30 |
| | | 0.271 | 0.480 | 0.313 | 0.791 | 0.194 |
| *SMAD3* | 0.61 ± 0.38 | 3.51 ± 3.58 | 0.98 ± 1.08 | 0.41 ± 0.31 | 0.72 ± 0.63 | 1.26 ± 0.82 |
| | | 0.114 | 0.724 | 0.313 | 0.920 | 0.149 |
| *SMAD4* | 1.07 ± 0.23 | 0.84 ± 0.59 | 0.64 ± 0.43 | 0.62 ± 0.27 | 0.88 ± 0.79 | 1.90 ± 1.15 |
| | | 0.197 | 0.157 | **0.032** | 0.186 | 0.386 |
| *SMAD7* | 2.36 ± 1.21 | 2.73 ± 1.74 | 1.39 ± 0.43 | 7.97 ± 3.99 | 1.76 ± 1.51 | 6.31 ± 4.24 |
| | | 0.962 | 0.480 | 0.059 | 0.366 | 0.083 |
| *INHBA* | 0.80 ± 0.28 | 6.22 ± 7.01 | 4.37 ± 3.65 | 4.70 ± 3.63 | 4.04 ± 5.72 | 29.69 ± 63.08 |
| | | **0.049** | 0.064 | 0.057 | 0.074 | **0.037** |
| *INHBB* | 0.53 ± 0.66 | 0.88 ± 0.65 | 0.63 ± 0.52 | 0.58 ± 0.34 | 0.50 ± 0.61 | 2.87 ± 2.75 |
| | | 0.399 | 0.643 | 0.751 | 0.874 | 0.117 |
| *INHBC* | 0.66 ± 0.49 | 1.84 ± 1.64 | 2.04 ± 1.73 | 2.90 ± 2.21 | 1.01 ± 0.72 | 3.73 ± 2.71 |
| | | 0.206 | 0.355 | 0.057 | 0.551 | 0.192 |
| *INHBE* | 0.80 ± 0.28 | 7.12 ± 10.04 | 7.93 ± 7.18 | 7.65 ± 5.42 | 3.57 ± 3.23 | 6.75 ± 7.41 |
| | | **0.049** | 0.064 | **0.026** | 0.101 | 0.068 |
| *CTGF* | 2.58 ± 2.76 | 15.98 ± 10.92 | 6.54 ± 5.19 | 7.46 ± 4.38 | 5.44 ± 3.80 | 10.52 ± 6.63 |
| | | **0.025** | 0.275 | 0.078 | 0.173 | 0.087 |

Statistically significant $p$ values are indicated in bold

† $p$ values correspond to Mann–Whitney $U$ test (comparisons with healthy controls)

(Table 2). CHB/r patients retained the increased mRNA levels of *INHBE* and *ACVR1/ALK4*, along with a decreased expression of *SMAD4* compared to controls (Table 2), but also displayed a significant reduction of mRNA expression of *CTGF* ($p = 0.010$), *TGFB1* ($p = 0.013$), *SMAD2* ($p < 0.001$) and *SMAD3* ($p < 0.001$) compared to CHB/d patients.

It is noteworthy that CHB/r patients displayed an approximately threefold increase of *SMAD7* expression compared to CHB/d patients ($p < 0.001$) (Fig. 1c). The expression levels of *SMAD7* were negatively correlated with the viral load ($r = -0.336$, $p = 0.012$), as well as with ALT ($r = -0.382$, $p = 0.004$) and AST levels ($r = -0.345$, $p = 0.010$). Interestingly, the expression pattern of *SMAD7* in CHB/r was similar to that observed in NAFLD patients (Fig. 1c). Unfortunately, our attempts to explore the localization of SMAD7 expression in liver tissue, using several monoclonal antibodies, were not successful.

In an attempt to further investigate possible relations between *SMAD7* expression and the intensity of inflammation and fibrosis, we considered all subjects of the study as a whole group. Related to the inflammation intensity, *SMAD7* mRNA levels were significantly increased in patients with minimal inflammation, followed by a decrease as inflammation became severe (Fig. 1d). This pattern of expression was rather similar for *INHBC, ACVR1/ALK4* and *TGFBR1/ALK5* (Fig. 4). The expression of the other analyzed genes was not affected by inflammation intensity ($p > 0.05$, in all cases). Considering the

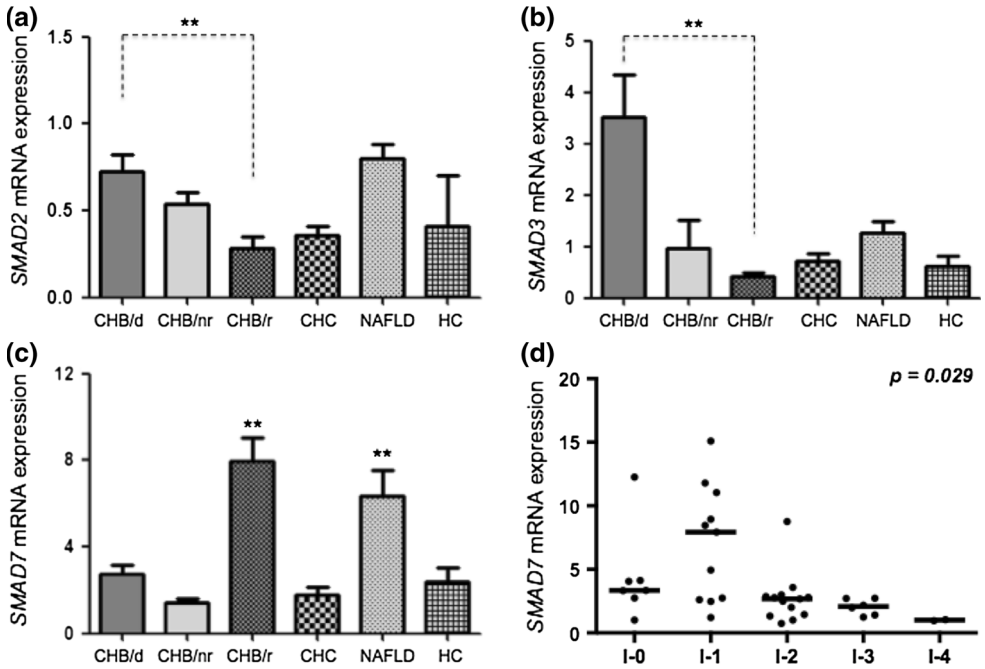


**Fig. 1** Liver mRNA levels of *SMAD2* (**a**), *SMAD3* (**b**), and *SMAD7* (**c**) in patients with chronic hepatic diseases. Bars demonstrate mean and SEM (standard error of mean) values. Significant differences with $p < 0.001$ are indicated by *double asterisks*. **d** Expression of *SMAD7* according to the intensity of liver inflammation: *lines* indicate median values and statistical significance ($p = 0.029$) refers to Kruskal–Wallis *H* test. *CHB/d* chronic HBV hepatitis at diagnosis, *CHB/nr* chronic HBV hepatitis non-responders, *CHB/r* chronic HBV hepatitis on maintained continuous antiviral treatment response and remission, *CHC* chronic HCV hepatitis, *NAFLD* non-alcoholic fatty liver disease, *HC* "healthy controls"

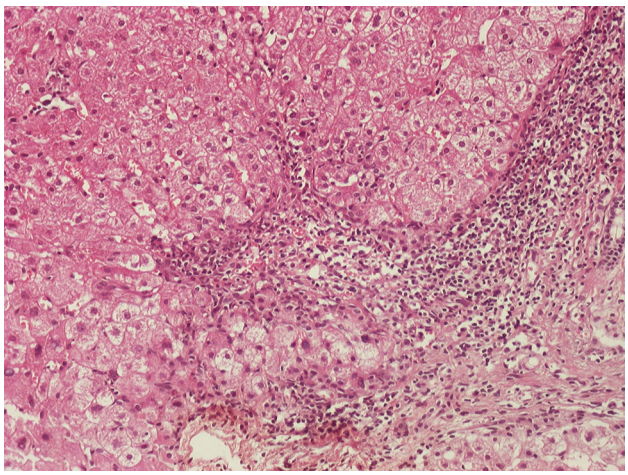

**Fig. 2** Representative area of active chronic inflammation and marked fibrosis in a liver biopsy from a newly diagnosed patient with chronic HBV hepatitis (haematoxylin–eosin stain)

expression of the analyzed genes in relation to fibrosis, we observed that *TGFB1, INHBB* and *SMAD4* mRNA levels are also significantly correlated with disease stage, following a similar expression pattern ($p = 0.039$, $p = 0.006$ and $p = 0.008$, respectively). It is noteworthy that no NAFLD patient presented with moderate or severe inflammation and/or fibrosis; therefore, all patients categorized as I-3 and I-4 and stage 2-6 had chronic viral hepatitis (Table 1).

As mentioned above, we observed increased mRNA levels of *SMAD2* and *SMAD3*, along with a remarkable decrease of *SMAD7* expression in CHB/d patients (compared to CHB/r ones), and these findings were validated by immunocytochemistry, demonstrating directly the expression pattern of pSMAD2 and pSMAD3 in liver tissues of CHB/d patients (Table 2; Fig. 3). In this context, we further investigated the expression of genes that represent direct targets of TGF-n activin signaling, namely *SERPINE1*, *COL1A1*, and *COL3A1*. Considering that the most robust finding in our study was that of *SMAD7* expression, we analyzed the expression pattern of the aforementioned genes in 5 patients with CHB/d and low *SMAD7* expression and 5 patients with CHB/r and high *SMAD7* expression. Although a statistical analysis was not performed for these genes due to the small numbers of patients in each group, it was clear that CHB/r patients displayed a notable reduction of *COL1A1, COL3A1* and *SERPINE1* mRNA levels compared to CHB/d ones (Fig. 5). Therefore, our data clearly demonstrate that TGF-β/activin signaling is activated in patients with chronic liver HBV inflammation and is down-regulated after successful response to long-term antiviral treatment.

Finally, correlation data of the expression of the analyzed genes in patients with chronic viral hepatitis (CHBd,

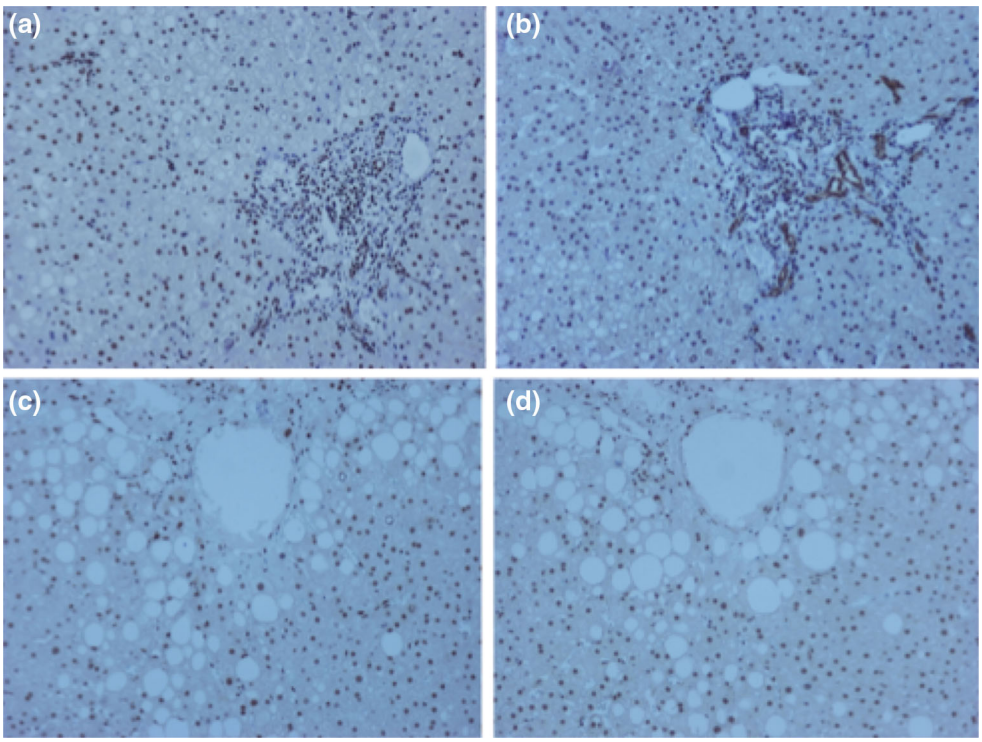


**Fig. 3** Immunohistochemical stains for pSMAD2 and pSMAD3 in liver biopsy specimens from a patient with chronic HBV hepatitis (**a**, **b**) and a patient with steatohepatitis (**c**, **d**). In chronic HBV hepatitis, the lymphocytes in the portal tracts and the lobules are positive for both pSMAD2 (**a**) and pSMAD3 (**b**). The biliary epithelial cells in the portal tracts are also positive, while the hepatocytes show positivity of variable intensity for both proteins (**a**, **b**). In steatohepatitis, there is also variable positivity of the hepatocytes for pSMAD2 (**c**) and pSMAD3 (**d**), while the inflammatory cells are positive for both proteins

CHB/r, CHB/nr, CHC) and NAFLD are presented in detail in Supplementary Material (Supplementary Figs. 1, 2, respectively). At the end, viral load (serum HBV DNA or HCV RNA) was positively correlated with the HAI score ($p < 0.001$, $r = 0.646$) and the disease stage ($r = 0.523$, $p < 0.001$), while the HAI score was also positively correlated with the stage ($r = 0.760$, $p < 0.001$).

## Discussion

Our study provides clear evidence that TGF-β signaling is activated in chronic HBV infection in the liver, while *SMAD7* is up-regulated during the resolution of inflammation after successful antiviral treatment. Moreover, in NAFLD, both an activation of TGF-β/activin signaling and an increased *SMAD7* expression were observed. These results may be related to the cell types involved in the inflammatory process in liver. Our immunohistochemical findings showed that predominantly lymphocytes and biliary epithelial cells and, to a lesser degree, hepatocytes expressed the pSMAD2 and pSMAD3 proteins, which are activated mediators of TGF-β signalling. On the other hand, *SMAD7* up-regulation is probably derived from hepatocytes. Taken together, the aforementioned data suggest that the *SMAD7* up-regulation might represent a mechanism capable of limiting the fibrotic process in chronic hepatic diseases (Fig. 6).

Several animal studies have demonstrated that TGF-β signaling through Smads results in the up-regulation of Smad7 expression [32, 33]. Smad7, in turn, is able either to hamper the TGFβRI binding with the R-Smads [34], or to interact with Smurf2 (SMAD specific E3 ubiquitin protein ligase 2) to form an E3 ubiquitin ligase, resulting in increased TGFβRI degradation [35]. Thus, Smad7 constitutes the most important feedback inhibitor of TGF-β signaling.

The potential contribution of Smad7 in the progression and/or resolution of inflammation has been studied in several animal models with contradictory results [36–40]. However, considering liver inflammation and fibrosis, Smad7 seems to possess anti-inflammatory effects, since tissue-specific *Smad7* deletion (in mice) results in spontaneous liver dysfunction and aggravates alcoholic hepatic injury [38]. Moreover, TGF-β signaling and its subsequent pro-fibrogenic effects can be abrogated after interferon-gamma (IFN-γ) treatment, since IFN-γ and tumor necrosis factor-alpha (TNF-α) can inhibit TGF-β signaling by

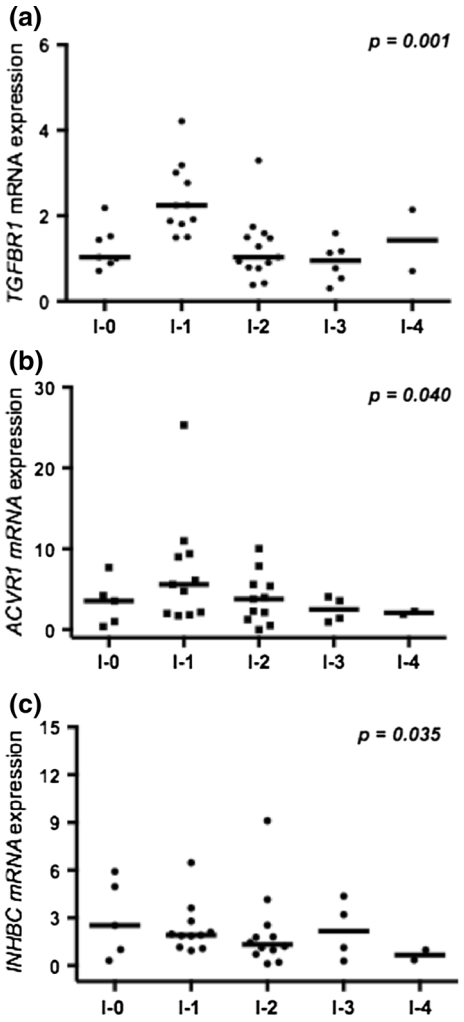


**Fig. 4** Significant differences of *TGFBR1* (**a**), *ACVR1* (**b**), and *INHBC* (**c**) expression among patients with chronic liver diseases, according to the grade of inflammation (following the classification presented in "Materials and methods"). *Lines* indicate median values and statistical significance refers to Kruskal–Wallis *H* test

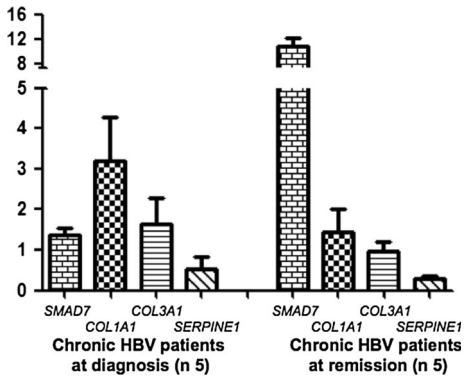


**Fig. 5** Liver mRNA levels of *COL1A1*, *COL3A1*, and *SERPINE1* in 10 patients with chronic HBV hepatitis: CHB/d patients ($n = 5$, at diagnosis) displayed also low *SMAD7* expression, while CHD/r patients ($n = 5$, after response to long-term treatment) exhibited high *SMAD7* expression. *Bars* demonstrate mean and SEM (standard error of mean) values

stimulating *Smad7* expression [41, 42]. In this context, Dooley et al., using an adenoviral expression system, demonstrated that Smad7 inhibits TGF-β signaling and prevents liver fibrosis [17], while Tahashi et al. found that Smad7 is increased in acute and decreased in chronic experimental liver injury in rats [18]. This notion is further supported by our findings in a human disease model, namely chronic HBV hepatitis, since we observed that as inflammation becomes severe, *SMAD7* expression decreases reaching lower levels than those of I-0 subjects, namely those without microscopic evidence of inflammation.

We further observed that NAFLD patients displayed a significant increase of liver *SMAD7* expression compared to untreated CHB/CHC patients. Moreover, NAFLD patients exhibit elevated mRNA levels of *TGFB1, TGFB3, INHBC, TGFBRI/ALK5,* and *SMAD4,* indicating an up-regulation of TGF-β signaling with a subsequent induction of *SMAD7* expression. In comparison, CHB/r patients exhibited also a significant increase of *SMAD7* expression compared to CHB/d, but without an up-regulation of TGF-β signaling, since they displayed significantly reduced levels of *TGFB1, CTGF,* and R-SMADs. This means that the up-regulation of *SMAD7* gene expression persists even after the down-regulation of the TGF-β signaling induced by long term (>5 years) antiviral treatment. In our recently published study in patients with CHB, we found that the expression of *FOXP3* (encoding forkhead box P3, FOXP3), *IL10* (encoding interleukin 10, IL-10) *TGFB1, PD1* (encoding programmed cell death protein 1, PD-1), *PDL1* (encoding programmed cell death-ligand 1, PD-L1), *FASL* (encoding fas ligand, Fas-L), and *CD8* (encoding CD8) were significantly down-regulated in the maintained on treatment long-term remission state; in contrast, liver expression levels of *IL2* (encoding interleukin-2, IL-2), IL1B (encoding interleukin 1β, IL-1β), *TNFA* (encoding TNF-α), and *IFNG* (encoding IFN-γ) were not changed significantly [25]. Hence, we could speculate that induced immune pathways, such as through IFN-γ and TNF-α, do not seem to contribute to the increased *SMAD7* expression. In this context, recent studies have shown that SMAD7 levels could be modulated by post-transcriptional mechanisms. In particular, high levels of microRNA-21 (miR-21) can lead to the abolishment of the negative feedback mechanisms of TGF-β signaling via repressing the translation of *SMAD7* mRNA, resulting in elevated pro-fibrogenic TGF-β signaling [43, 44]. Obviously, the contribution of miR-21 expression in CHB/r patients should be explored in further studies.

In conclusion, our study provides clear evidence that patients with chronic HBV hepatitis display activation of TGF-β/activin signaling, while patients on maintained

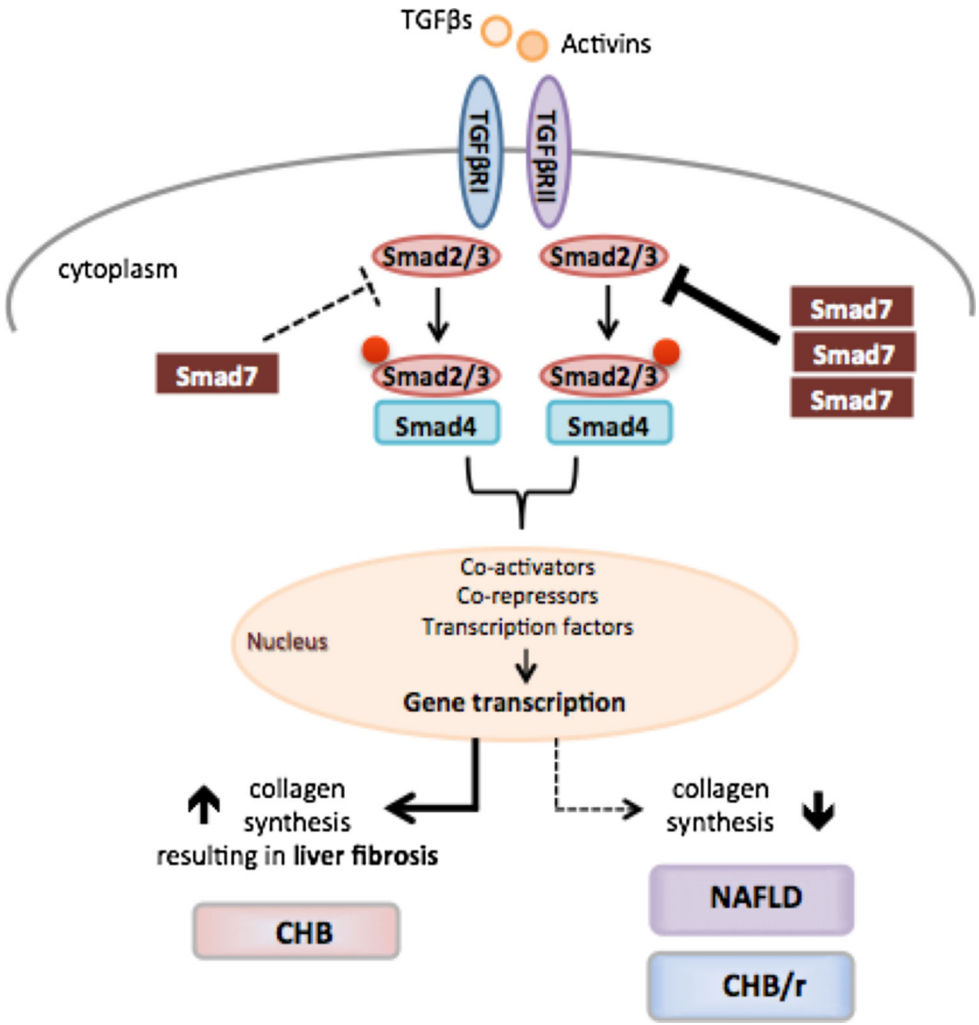


**Fig. 6** TGFβs and activins increase the expression of the extracellular matrix (ECM) proteins, including collagens and integrins. TGFbs bind to the receptor TGFβRII, which in turn recruits and phosphorylates TGFβRI, leading to the activation of intracellular SMAD2 and SMAD3 by phosphorylation (p) at their C-terminal SSXS motifs. Subsequently, activated SMAD2 or SMAD3 form heterodimers with Smad4 and translocate to the nucleus to initiate target gene expression of ECM genes, in collaboration with co-factors, co-repressors and other transcription factors. Sustained expression of ECM genes by TGFβs in the activated hepatic stellate cells and myofibroblasts leads to the accumulation of collagenous ECM proteins, resulting in progressive liver fibrosis, as observed in patients with active chronic HBV hepatitis (CHB) before treatment. This process might be inhibited by the increased expression of *SMAD7*, as found in patients with chronic HBV hepatitis maintained on continuous antiviral treatment response and remission (CHB/r), as well as in patients with non-alcoholic fatty liver disease (NAFLD), despite the increased expression of TGFβs and their cellular mediators in liver

remission after successful response to antiviral treatment exhibit *SMAD7* overexpression. The later might be a mechanism limiting the pro-fibrogenic effect of TGF-β signaling in various chronic liver diseases. Therefore, *SMAD7* induction may represent a candidate for novel therapeutic approaches, bearing in mind the considerable progress that has been achieved in well-performed translational studies [45].

**Acknowledgments** The authors want to thank Dr S. Anastasiadis for collecting patients' samples and Dr A. Stavropoulos for his excellent technical support.

This research has been co-financed by the ESF and Greek national funds through the Operational Program "Education and Lifelong Learning" of the NSRF–Research Funding Program: Heracleitus II. Investing in knowledge society through the European Social Fund, and was also granted by the Research Committee of Aristotle University of Thessaloniki. PS and EA were also supported by a grant (TGF-LUNG-CURE 3451) from the ARISTEIA II program which has been co-financed by the European Union (European Social Fund–ESF) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF).

**Authors' contribution** Study concept and design: MS, AEG; acquisition of data: AN, EA, PH; analysis and interpretation of data: NA, GG, PH, MS; drafting of the manuscript: NA, GG, MS; critical

revision of the manuscript for important intellectual content: GG, PS, PH, AEG; statistical analysis: AN, MS; obtained funding: NA, GG, TV, PS, MS; technical, or material support: GG, TV, KP; study supervision: MS.

**Compliance with ethical standards**

**Conflict of interest** None in connection with the submitted manuscript.

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