Overexpression of SMAD7 protects liver from TGFb/Smad-mediated fibrogenesis

Nikoletta Argentou [1], Georgios Germanidis [2], Eirini Apostolou [3], Themistocles Vassiliadis [3], Paschalis Sideras [3], Anastasios E. Germenis [1], Matthaios Speletas [1]

[1] Department of Immunology and Histocompatibility, Medical School, University of Thessaly, Biopolis 41110 Larissa, Greece
[2] A' Department of Internal Medicine , A.H.E.P.A Hospital, Aristotle University of Thessaloniki, 54636 Thessaloniki, Greece
[3] Center of Immunology and Transplantation, Biomedical Research Foundation, Academy of Athens, 11527 Athens, Greece
[4] Gastroenterology and Hepatology Division, Hippokration Hospital, Aristotle University of Thessaloniki, 54642 Thessaloniki, Greece

Background

Smad7 is a dominant intracellular inhibitor of TGFb/Activin signal transduction.[1] Recent animal model studies have corroborated the protective function of Smad7 in attenuating TGFb-mediated fibrosis in multiple organs, including liver, through manipulation of Smad7 expression.[2-5]

This study was scheduled in order to determine whether *Smad7* mRNA expression correlates with the expression of the molecules participating in the TGFb/Activin signal transduction pathway in liver tissue of patients with chronic hepatic diseases and to seek correlations with the status of liver inflammation, fibrosis and the effect of treatment.

Methods

Liver biopsies obtained from 67 patients with chronic hepatic diseases including a) 18 with chronic HCV hepatitis (CHC); b) 19 with chronic HBV hepatitis at diagnosis (CHB/d); c) 4 with CHB after antiviral treatment and relapse (CHB/nr) d) 14 with CHB after antiviral treatment response and remission for >5y (CHB/r); e) 12 with non alcoholic fatty liver disease (NAFLD). Three individuals submitted to liver biopsy due to a mild increase of aminotransferases but without liver architecture changes (served as controls). Demographic, clinicopathological and serological data of the analyzed subjects are summarized in **Table 1**.

The mRNA levels of *TGFBs (TGFB-1,-2,-3*), activins (*A,B,C,E*), *ALK4*, *ALK5*, SMAD molecules (*SMAD-2, -3, -4, -7*), and *CTGF* were determined in a quantitative reverse transcriptase PCR using SYBR-Green PCR Supermix (Invitrogen,UK). Primers were either designed or commercially obtained by SA Biosciences (USA). The sequences of the designed primers as well as the thermocycling conditions for all genes are summarized in **Table 2**. The *beta-2-microglobulin* (*B2M*) gene was used as a reference gene for sample normalization. Statistical analyses were performed using the SPSS ver. 18.0 software.

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

	Controls	CHB/d [a]	CHB/nr [b]	CHB/r [c]	CHC [d]	NAFLD [e]
No	3	19	4	14	18	12
Sex (M/F) [f]	2/1	9/10	2/2	11/3	14/4	7/5
Age (median, range)	61, 60-67	54, 24-64	57, 22-65	52, 23-60	41.5, 27-54	45, 21-71
AST [g] (U/μL), (median, range)	42, 36-45	51, 17-1969	62, 39-277	29.5, 15-51	45, 24-237	31.5, 19-70
ALT [h] (U/μL), (median, range)	32, 21-48	61, 15-1478	97.5, 70-332	31.5, 17-49	75, 32-213	54, 15-141
Inflammation grade [i]						
I-0 [i]	3	–	–	1	–	3
I-1 [i]	–	4	–	10	2	4
I-2 [i]	–	8	3	3	10	5
I-3 [i]	–	5	1	–	6	–
I-4 [i]	–	2	–	–	–	–
Fibrosis (median, range)[i]	–	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	2.0, 0-5
Viral load (median, range)	–	4 Meq/mL (0.009-699)	0.10 Meq/mL (0-44.5)	0 Meq/mL (0-0.008)	0.70 Meq/mL (0.10-6.25)	0, 0-0

Abbreviations: [a] CHB/d, newly diagnosed patients with Chronic HBV hepatitis; [b] CHB/nr, CHB patients 6 months after treatment withdrawal and no virologic/biochemical sustained response, [c] CHB/r, CHB patients after antiviral treatment response and remission for >5y, [d] CHC, Chronic HCV hepatitis; [e] NAFLD, non-alcoholic fatty liver disease; [f] M, male; F, female; [g] AST, aspartate aminotransferase; [h] ALT, alanine aminotransferase; [i] Inflammation grade (I-0: without inflammation, I-1: minimal, I-2: mild, I-3: moderate and I- 4: marked) and fibrosis

Table 2. Primers and PCR conditions for the amplification of the analyzed genes

Gene	Primers	Sequence	PCR conditions
TGFB1	forward reverse	commercially obtained by SABiosciences, Cat No PPH00508A	95^{0}C for 10 min, followed by 40 cycles (95^{0}C for 15 s, 60^{0}C for 60 s)
TGFB2	forward reverse	5'- AgAgTgCCTgAACAA -3' 5'- CCATTCgCCTTCTgCTCTT -3'	95^{0}C for 2 min, followed by 40 cycles (95^{0}C for 15 s, 53^{0}C for 15 s, 72^{0}C for 15 s)
TGFB3	forward reverse	commercially obtained by SABiosciences , Cat No PPH00531E	95^{0}C for 10 min, followed by 40 cycles (95^{0}C for 10 s, 58^{0}C for 10 s, 72^{0}C for 30 s)
ALK5	forward reverse	commercially obtained by SABiosciences, Cat No PPH00237B	95^{0}C for 10 min, followed by 40 cycles (95^{0}C for 15 s, 60^{0}C for 60 s)
ALK4	forward reverse	5'- CATTgACATTgCCCCgAATC -3' 5'- CgAgCAATCTCCCAATATACAAg -3'	95^{0}C for 2 min, followed by 50 cycles (95^{0}C for 15 s, 56^{0}C for 40 s), and 72^{0}C for 2 min
SMAD2	forward reverse	commercially obtained by SABiosciences, Cat No PPH01949E	95^{0}C for 10 min, followed by 40 cycles (95^{0}C for 15 s, 58^{0}C for 15 s, 72^{0}C for 15 s)
SMAD3	forward reverse	commercially obtained by SABiosciences, Cat No PPH01921B	95^{0}C for 10 min, followed by 40 cycles (95^{0}C for 10 s, 58^{0}C for 10 s, 72^{0}C for 30 s)
SMAD4	forward reverse	commercially obtained by SABiosciences, Cat No PPH00134B	95^{0}C for 10 min, followed by 40 cycles (95^{0}C for 15 s, 60^{0}C for 60 s)
SMAD7	forward reverse	commercially obtained by SABiosciences, Cat No PPH01905B	95^{0}C for 10 min, followed by 40 cycles (95^{0}C for 15 s, 60^{0}C for 60 s)
CTGF	forward reverse	5'- ACCAATgACAACgCCTCCTg -3' 5'- TTgCCCTTCTTA ATgTTCTCTTCC -3'	95^{0}C for 10 min, followed by 40 cycles (95^{0}C for 15 s, 60^{0}C for 60 s)
INHBA	forward reverse	5'- AgCAgACCTCggAgATCATC -3' 5'- TTggggACTTTTAggAAgAgC -3'	95^{0}C for 2 min, followed by 50 cycles (95^{0}C for 15 s, 56^{0}C for 40 s), and 72^{0}C for 2 min
INHBB	forward reverse	5'- AggAgCgCgTTTCCgAAATC -3' 5'- TggTTgCCTTCgTTggAgATg -3'	95^{0}C for 2 min, followed by 50 cycles (95^{0}C for 15 s, 56^{0}C for 40 s), and 72^{0}C for 2 min
INHBC	forward reverse	5'- AgAgCTgCTTTgAggACTgC -3' 5'- AAgACgAgTCTggTTGATggTg -3'	95^{0}C for 2 min, followed by 50 cycles (95^{0}C for 15 s, 56^{0}C for 40 s), and 72^{0}C for 2 min
INHBE	forward reverse	5'- gCAACAATTCCTggCgATACC -3' 5'- gCCCTCAATTTCCCCTCCAC -3'	95^{0}C for 2 min, followed by 50 cycles (95^{0}C for 15 s, 56^{0}C for 40 s), and 72^{0}C for 2 min
B2M	forward reverse	commercially obtained by SABiosciences, Cat No PPH01094E	95^{0}C for 10 min, followed by 40 cycles (95^{0}C for 15 s, 60^{0}C for 60 s)

Results

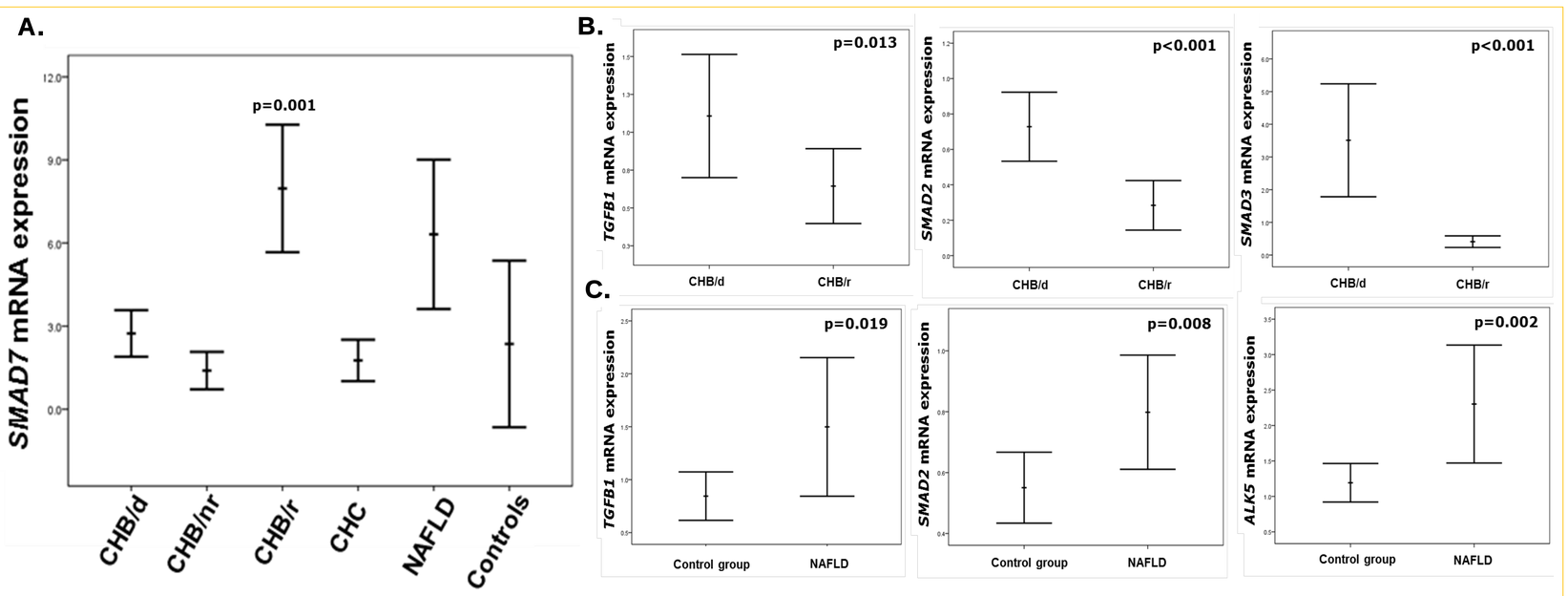


Figure 1. Error bar diagrams presenting the expression of genes, for which a significant alteration of their mRNA levels was observed. p values in each diagram refer to Mann-Whitney *U* test.

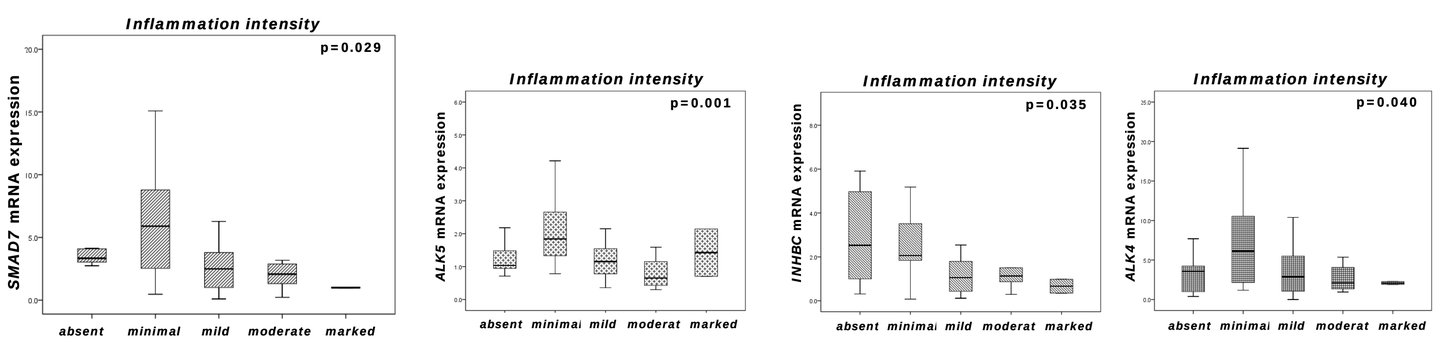


Figure 2. Boxplot diagrams presenting the expression of mediators of the TGFb/Activin signaling pathway according to the intensity of liver inflammation. p values in each diagram refer to Kruskal-Wallis H test.

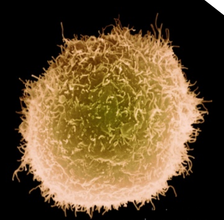
Patients with CHB/r exhibited a significant increase of *SMAD7* mRNA expression **(Fig.1A)** and reduced levels of *TGFB1*, *SMAD2*, *SMAD3*, and *CTGF* (p=0.010) as compared to CHB/d patients (**Fig.1B**). This pattern of expression of *SMAD7* was similar with that observed in patients with NAFLD, a disease characterized rarely by a fibrotic process **(Fig.1A)**.

SMAD7 expression was also found increased in NAFLD patients as compared to the control group including CHB/d, CHB/nr and CHC patients (p=0.001). Moreover, NAFLD patients were presented with elevated mRNA levels of *TGFB1*, *SMAD2*, *ALK4*, and *SMAD4* (p<0.001) (**Fig.1C**).

Considering the intensity of inflammation, *SMAD7*, *ALK5*, *INHBC*, and *ALK4* exhibited significant increased expression from absent to minimal inflammation with a gradual reduction as inflammation exacerbates (**Fig.2**).

Conclusion

Our data indicate that in cases with low grade fibrosis, such as NAFLD (characterized by a lower incidence of severe liver complications and fibrosis progression) and CHB/r, SMAD7 overexpression might be a mechanism limiting the fibrogenic effect of TGFb suggesting that its induction may provide a target for novel therapeutic approaches.



REFERENCES

1. Nakao A et al. Nature 1997; 389: 622-626
2. Dooley S et al. Gastroenterology 2003; 125: 178-191
3. Tahashi Y et al. Hepatology 2003; 35: 49-61
4. Dooley S et al. Gastroenterology 2008; 135: 642-659
5. Tang LX et al. PLoS One 2012; 7: e31350. Epub 2012 Feb 7

European Union
European Social Fund
OPERATIONAL PROGRAMME
EDUCATION AND LIFELONG LEARNING
investing in knowledge society
MINISTRY OF EDUCATION, LIFELONG LEARNING AND RELIGIOUS AFFAIRS
MANAGING AUTHORITY
NSRF 2007-2013
programme for development
EUROPEAN SOCIAL FUND
Co- financed by Greece and the European Union