

High Serum Transforming Growth Factor- β 1 Levels Predict Outcome in Hepatocellular Carcinoma Patients Treated with Sorafenib

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Abstract

Background: The TGF- β signaling pathway is crucial in the progression and metastasis of malignancies. We investigated whether the serum TGF- β 1 level was related to the outcomes of patients treated with sorafenib for advanced hepatocellular carcinoma (HCC).

Experimental Design: We selected patients who had received sorafenib-containing regimens as first-line therapy for advanced HCC between 2007 and 2012. Serum TGF- β 1 levels were measured and correlated with the treatment outcomes. The expression of TGF- β 1 and the sensitivity to sorafenib were examined in HCC cell lines.

Results: Ninety-one patients were included; 62 (68%) were hepatitis B virus surface antigen (+), and 11 (12%) were anti-hepatitis C virus (+). High (\geq median) pretreatment serum TGF- β 1 levels (median 13.7 ng/mL; range, 3.0–41.8) were associated

with high α -fetoprotein levels, but not with age, gender, or disease stage. Patients with high pretreatment serum TGF- β 1 levels exhibited significantly shorter progression-free survival (median, 2.5 vs. 4.3 months; $P = 0.022$) and overall survival (median 5.6 vs. 11.6 months; $P = 0.029$) than did patients with low serum TGF- β 1 levels. Compared with pretreatment levels, the serum TGF- β 1 levels were significantly increased at disease progression ($n = 29$, $P = 0.010$). In preclinical models of HCC, higher TGF- β 1 expression levels were associated with poorer sensitivity to sorafenib.

Conclusions: High pretreatment serum TGF- β 1 levels were associated with poor prognoses, and increased serum TGF- β 1 levels were associated with the disease progression of advanced HCC patients. TGF- β pathway may be explored as a therapeutic target for advanced HCC. *Clin Cancer Res*; 21(16); 3678–84. ©2015 AACR.

Introduction

The prognosis of advanced hepatocellular carcinoma (HCC), which is defined as HCC presenting with extrahepatic metastasis or locally advanced diseases that are unamenable to locoregional therapy, is poor because treatment options are limited. Sorafenib, a multikinase inhibitor, is the first and only therapy approved for patients with advanced HCC because it benefited patient survival in two phase III clinical trials; however, the efficacy of sorafenib in treating advanced HCC is moderate. The median time to disease progression in Western and Asian patients was only 4.1 and 2.8 months, respectively (1, 2). Factors that could predict the treatment efficacy of sorafenib or enable stratifying advanced HCC patients according to prognosis are urgently required, but have yet to be determined despite extensive exploration (3).

TGF- β is a family of growth factors that regulate various cellular processes, including cell growth, differentiation, embryologic morphogenesis, and immunosuppression (4–6). TGF- β ligands have 3 isoforms (TGF- β 1, TGF- β 2, and TGF- β 3), of which TGF- β 1 is the most abundant and is frequently upregulated in tumor cells (7). The TGF- β pathway exerts a dynamic effect on cancer cells. Early in the carcinogenesis process, TGF- β 1 suppresses tumors and arrests cell growth (8); in later and advanced tumor stages, TGF- β 1 potentiates epithelial-to-mesenchymal transition (EMT), angiogenesis, tumor progression, invasion, and metastasis (9–11).

Previous studies have reported that TGF- β 1 was overexpressed in HCC (12, 13). In established HCC cells, TGF- β 1 promoted EMT, triggered migration and invasion, and induced an aggressive phenotype by reducing E-cadherin expression (14, 15). In clinical studies, blood TGF- β 1 levels were higher in patients with HCC than in patients with chronic hepatitis or cirrhosis. Moreover, the serum TGF- β 1 level in metastatic HCC was higher than that in localized HCC (16–19).

Although blood TGF- β 1 levels have been evaluated in patients with chronic hepatitis, cirrhosis, and the early stages of HCC, the importance of blood TGF- β 1 levels in advanced HCC patients has seldom been explored. Therefore, in this study, we examined the serum TGF- β 1 levels in patients with advanced HCC, investigating its association with disease status and patient outcome. In addition, we also explored the significance of TGF- β pathway in several preclinical models of HCC treated with sorafenib.

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Translational Relevance

TGF- β signaling pathway plays a dynamic role in cancers. Activation of TGF- β signaling has been shown to promote tumor progression, especially in late and advanced tumor stages, by potentiating epithelial mesenchymal transition, angiogenesis, immune suppression, invasion, and metastasis. The significance of serum TGF- β 1 levels in advanced-stage HCC patients treated with sorafenib is unclear. The current study found that high pretreatment serum TGF- β 1 levels were associated with poor progression-free survival and overall survival of patients who received sorafenib-based treatment for advanced HCC. At disease progression, serum TGF- β 1 levels significantly increased. These findings support the notion that TGF- β signaling pathway may be explored as a therapeutic target for patients with advanced HCC.

Materials and methods

Study population

Advanced HCC patients, who were indicated for sorafenib treatment because they presented with extrahepatic metastasis or locally advanced diseases unamenable to locoregional therapies, such as transcatheter arterial chemoembolization or local ablation, were included in a prospective patient cohort at the Department of Oncology, National Taiwan University Hospital (NTUH), Taipei, Taiwan between 2007 and 2012. Patients who consented to participate in this biomarker study provided baseline blood samples, which were used to identify the potential predictive and prognostic biomarkers of sorafenib, within one week before sorafenib treatment. They were encouraged to provide blood samples when their disease progressed as well. This biomarker study was approved by the Research Ethical Committee of NTUH.

Patients were either treated with sorafenib alone or sorafenib in combination with tegafur/uracil. For the former, sorafenib was administered following the instructions listed in the package insert. For the latter, patients had been enrolled in a phase II clinical trial testing sorafenib in combination with metronomic chemotherapy, the uninterrupted administration of low-dose chemotherapeutic agents that exhibit antiangiogenic activity over a prolonged period (20). Continuous tegafur/uracil (125 mg/m² based on tegafur, twice daily) was chosen as the metronomic chemotherapy. Results of this phase II trial of sorafenib in combination with metronomic tegafur/uracil have been previously reported (21). Briefly, the study was conducted at NTUH. Patients were required to have pathologically proven or clinically diagnosed metastatic or locally advanced HCC not amenable to locoregional therapies. Other eligibility criteria included adequate liver reserve [Child-Pugh Class A, liver transaminases levels $\leq 5 \times$ upper limit of normal (ULN)] and organ functions (serum creatinine level $\leq 1.5 \times$ ULN; platelet counts $\geq 100,000/\mu\text{L}$).

All patients were examined and evaluated at least every 2 weeks, and tumor assessment was performed every 8 weeks following RECIST (Response Evaluation Criteria in Solid Tumors) 1.0. Pertinent clinical and laboratory parameters were collected at the baseline.

Study variables

Clinicopathologic variables were extracted from the cohort database. Overall survival (OS) was calculated from the beginning of sorafenib treatment to the date of death or the final follow-up, February 28, 2013. Progression-free survival (PFS) was determined from the beginning of treatment to the date of disease progression, death, or the final follow-up.

Measurement of TGF- β 1

Serums were aliquoted and stored at -80°C . TGF- β 1 serum levels were measured using an ELISA (eBioscience) according to the instructions of the manufacturer. We used the median pretreatment TGF- β 1 level to classify patients into high (\geq median) and low ($<$ median) TGF- β 1 levels patient groups.

Cell lines and reagents

Huh7, HepG2, Hep3B, PLC5, and SK-Hep1 were human HCC cell lines that were routinely maintained at our laboratory (22); SNU-387, SNU-423, SNU-449, SNU-475, and HLE cells were purchased from ATCC. A sorafenib-resistant subline of Huh7 cells was established from a subcutaneous xenograft of an immunocompromised mouse, that had been treated with sorafenib 7.5 mg/kg/day for 4 weeks but showed no tumor-suppressive effect. After primary culture, this Huh7 subline retained *in vitro* resistance to sorafenib with a higher 50% inhibitory concentration (IC₅₀) of $9.6 \pm 2.0 \mu\text{mol/L}$, compared with $3.7 \pm 1.8 \mu\text{mol/L}$ in parental Huh7 cell line (23). Cell lines were all maintained in DMEM (Biological Industries, Kibbutz Beit Haemek), supplemented with 10% (v/v) FBS (Biological Industries), 2 mmol/L L-glutamine, and antimicrobial PSA combination (penicillin 100 U/mL, streptomycin 0.1 mg/mL, and amphotericin 0.25 $\mu\text{g/mL}$; Biological Industries) at 37°C and 5% CO₂.

Sorafenib was purchased from LC Laboratories and prepared in DMSO as 10 mmol/L stock. LY2157299 was purchased from MedKoo Biosciences and prepared in DMSO as 10 mmol/L stock. TGF- β 1 recombinant protein was purchased from R&D system and prepared in sterile 4 mmol/L HCl as 2 $\mu\text{g/mL}$ stock.

Analysis of mRNA and protein expression

For analysis of mRNA expression, total RNA was extracted from each cell line with TRIzol Reagent (Life Technologies) according to the manufacturer's instructions, treated with DNase I (Promega), and subsequently reverse-transcribed to cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Expression of mRNA by qRT-PCR using Sybr Green (Roche Diagnostics) was performed at an Applied Biosystems 7900 HT Fast Real-Time PCR System (Applied Biosystems). Primers for TGF- β 1 were: forward, TGAGGGCTTTCGCCTTAGC and reverse, CGGTAGTGAACCCGTTGATGT; primers for GAPDH were: forward, TGAAGGACTCATGACCACAGT and reverse, GCCATCACGCCACAGTTTC.

For analysis of protein expression, we first collected cell lysates in lysis buffer [50 mmol/L Tris-HCl (pH = 8.0), 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, 1 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 $\mu\text{g/mL}$ aprotinin, and 1 $\mu\text{g/mL}$ leupeptin]. A 30- μg portion of each extracted protein sample was denatured at 100°C for 5 minutes and separated by SDS-PAGE, and then transferred to nitrocellulose membrane (Millipore Corp.). Membranes were washed, blocked, and incubated with indicated antibodies in TBST buffer [150 nmol/L Tris-HCl

(pH 8.0), 150 mmol/L NaCl, and 0.1% Tween 20] containing 5% nonfat dry milk at 4°C overnight. After washed with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies at room temperature for 1 hour, followed by extensive washing with TBST. The immunoblot analyses were detected with Immobilon Western Chemiluminescent HRP Substrate (Millipore). Primary antibodies against phospho-Smad2, Smad2, and Smad4 were from Santa Cruz Biotechnology, primary antibodies against phospho-Smad3, TGF- β receptor I, and TGF- β receptor II were from Cell Signaling Technology (Danvers), and primary antibody against β -actin was from Sigma-Aldrich.

Cell viability assay

Cells in logarithmic growth, seeded at 96-well plates, were exposed to indicate compounds 72 hours. Cell survival was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay, and was expressed as percentage of control.

Statistical analysis

Statistical analyses were performed using SAS statistical software (V9.1.3, SAS Institute). A two-sided P value ≤ 0.05 indicated statistically significance. Associations between the pretreatment TGF- β 1 level and basic clinicopathologic variables were analyzed using the χ^2 test (or Fisher exact test when appropriate) for nominal variables and the independent t test for continuous variables. Kaplan–Meier analysis was performed to estimate survival, and the log-rank test was used to compare the survival of patients with high TGF- β 1 levels and those with low TGF- β 1 levels univariately. A Cox proportional hazard model was applied to multivariately evaluate the association between the TGF- β 1 level and the survival outcome by adjusting for gender, age, hepatitis etiology, macrovascular invasion, extrahepatic metastasis, α -fetoprotein level, Barcelona-Clinic Liver Cancer stage, Eastern Coop-

erative Oncology Group performance status, and Cancer of the Liver Italian Program scores. To compare the TGF- β 1 levels before treatment with those measured upon disease progression, the paired t test was applied.

Results

Patient characteristics and treatment outcomes

The study cohort comprised 91 patients; 32 patients received sorafenib only, and 59 patients received sorafenib combined with metronomic tegafur/uracil chemotherapy. The median patient age was 55 years; the hepatitis etiology was the hepatitis B virus in 68% of the patients and the hepatitis C virus in 12% of the patients; and 89% of the patients exhibited either macrovascular invasion (59%) or extrahepatic metastasis (64%; Table 1). All patients except one had Child-Pugh class A liver reserve.

The best tumor responses to sorafenib-based treatment included 5 partial responses and 48 stable diseases, yielding a disease control rate (DCR; defined as the percentage of patients who exhibited complete response, partial response, or stable disease) of 58%. By the final follow-up date, 76 (84%) patients had died. The median PFS was 3.7 months (95% confidence interval; CI, 2.2–4.9), and the median OS was 7.4 months (95% CI, 5.6–11.3). The treatment outcomes of the 91-patient cohort were similar to those of other advanced HCC patient cohorts that had been reported from our institute (24, 25).

Pretreatment TGF- β 1 levels and treatment outcomes

Among the 91 patients, the median pretreatment serum TGF- β 1 level, which we used to classify patients into two groups, was 13.7 ng/mL (range, 3.0–41.8 ng/mL). Patients with high (\geq median) pretreatment TGF- β 1 levels were likely to have high (≥ 200 ng/mL) α -fetoprotein levels ($P = 0.028$). However, pretreatment TGF- β 1 levels were not associated with age, gender, disease stage, or performance status (Table 1).

Table 1. Patient Characteristics

Variable	Category	Total N (%)	Low TGF- β 1 N (%)	High TGF- β 1 N (%)	P
Total		91 (100)	45 (100)	46 (100)	
Gender	Male	82 (90)	40 (89)	42 (91)	0.739
	Female	9 (10)	5 (11)	4 (9)	
Median age (range, y)		56.9 (23.6–83.1)	56.9 (32.4–83.1)	57.3 (23.6–82.7)	0.2930
Etiology	HBV	62 (68)	31 (69)	31 (67)	0.626
	HCV	11 (12)	7 (16)	4 (9)	
	Both	7 (8)	3 (7)	4 (9)	
	Others	11 (12)	4 (9)	7 (15)	
Macrovascular invasion		54 (59)	25 (56)	29 (63)	0.467
Extrahepatic spread		58 (64)	30 (67)	28 (61)	0.565
AFP ≥ 200 ng/mL		47 (52)	18 (40)	29 (63)	0.028
Child-Pugh	A	90 (99)	44 (98)	46 (100)	0.4945
	B	1 (1)	1 (2)	0 (0)	
BCLC	B	9 (10)	4 (9)	5 (11)	1.000
	C	82 (90)	41 (91)	41 (89)	
CLIP score	0	11 (12)	8 (18)	3 (7)	0.177
	1	16 (18)	9 (20)	7 (15)	
	2	29 (32)	14 (31)	15 (33)	
	3	22 (24)	11 (24)	11 (24)	
	4	13 (14)	3 (7)	10 (22)	
ECOG PS	0	27 (30)	14 (31)	13 (28)	0.766
	1	64 (70)	31 (69)	33 (72)	

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α -fetoprotein; BCLC, Barcelona Clinic Liver Cancer; CLIP, Cancer of the Liver Italian Program; ECOG PS, Eastern Cooperative Oncology Group performance status.

The DCRs of patients with high and low TGF-β1 levels were not statistically different (52% vs. 64%; $P = 0.235$). Patients with high TGF-β1 levels exhibited significantly poorer PFS (median, 2.5 vs. 4.3 months; $P = 0.022$; Fig. 1A) and OS (median, 5.6 vs. 11.6 months; $P = 0.029$; Fig. 1B) than did patients with low TGF-β1 levels. However, in the multivariate analysis in which we adjusted for other potential prognostic predictors, a high pretreatment TGF-β1 level was not an independent predictor of either PFS (HR, 1.152; $P = 0.565$) or OS (HR, 1.242; $P = 0.396$; Table 2).

TGF-β1 levels and disease progression

Among the 91 patients, 29 had available sera upon disease progression after sorafenib-based treatment. The TGF-β1 levels upon disease progression significantly increased compared with the pretreatment levels (mean, 21.1 ng/mL vs. 14.8 ng/mL; $P = 0.010$; Fig. 2).

TGF-β1 signaling and sorafenib sensitivity in HCC cell lines

To explore whether the TGF-β1 pathway activity affected the sensitivity to sorafenib in HCC cells, we examined and correlated the expression levels of TGF-β1 mRNA and the IC₅₀s to the growth inhibitory effect of sorafenib in a panel of human HCC cells. HCC cells with higher IC₅₀ to sorafenib tended to have higher TGF-β1 mRNA expression (Fig. 3A). In a sorafenib-resistant HCC cell line (Huh7-SR) derived from a Huh7-xenografted mouse treated with sorafenib, we also found higher expression of TGF-β1 and downstream Smad molecules compared with the parental Huh7 cell line (Fig. 3B and C). Adding recombinant TGF-β1 increased resistance to sorafenib in Huh7 cells; while adding LY2157299, a TGF-β receptor I inhibitor, enhanced the sensitivity to sorafenib in Huh7-SR cells (Fig. 3D and E).

Discussion

In this study, we demonstrated that high pretreatment serum TGF-β1 levels were associated with poor survival outcomes in advanced HCC patients treated with sorafenib- and sorafenib-based combination therapy. The serum TGF-β1 levels increased upon disease progression. Although previous studies have shown that serum or plasma TGF-β1 levels are prognostic markers for patients with hepatitis, liver cirrhosis, or early-stage HCC, our study is the first to examine the associations between serum TGF-β1 levels and survival outcomes of patients with advanced HCC.

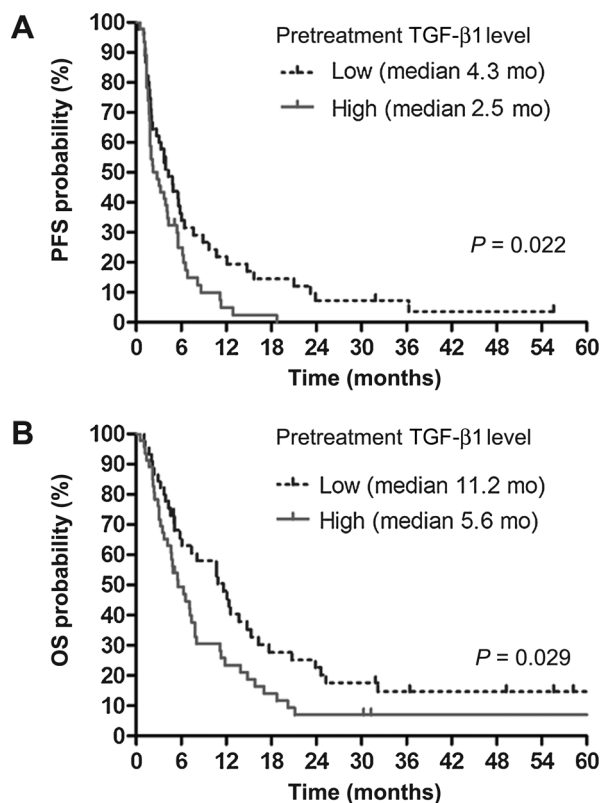


Figure 1. Kaplan-Meier analysis of (A) PFS, and (B) OS, grouped according to high (\geq median level) and low ($<$ median level) pretreatment TGF-β1 levels. The P values were determined using the log-rank test.

Table 2. Cox proportional hazards model for predictors of PFS and OS

Covariate	PFS		OS	
	HR (95% CI)	P	HR (95% CI)	P
High pretreatment TGF-β1 level	1.152 (0.712-1.864)	0.565	1.242 (0.753-2.050)	0.396
Male	2.676 (1.075-6.664)	0.035	1.689 (0.680-4.195)	0.259
Age	0.982 (0.961-1.003)	0.089	1.004 (0.983-1.026)	0.691
HBsAg positive	1.336 (0.768-2.326)	0.306	1.603 (0.886-2.898)	0.119
Macrovascular invasion	1.614 (0.822-3.170)	0.165	1.332 (0.650-2.727)	0.434
Extrahepatic involvement	1.478 (0.870-2.511)	0.149	1.199 (0.704-2.042)	0.505
AFP \geq 200 ng/mL	2.110 (1.113-4.000)	0.022	1.063 (0.559-2.020)	0.852
BCLC C (vs. B)	1.234 (0.481-3.169)	0.662	0.946 (0.366-2.445)	0.909
CLIP score \geq 3	0.808 (0.366-1.785)	0.598	3.789 (1.713-8.381)	0.001
ECOG PS 0 (vs. 1)	0.833 (0.500-1.388)	0.484	0.871 (0.507-1.495)	0.615

Abbreviations: HBsAg, hepatitis B virus surface antigen; AFP, α -fetoprotein; BCLC, Barcelona Clinic Liver Cancer; CLIP, Cancer of the Liver Italian Program; ECOG PS, Eastern Cooperative Oncology Group performance status.

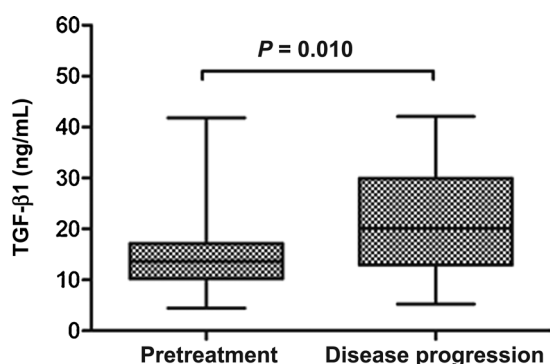


Figure 2. Levels of TGF-β1 before treatment and upon disease progression. The *P* values were obtained using the paired *t* test.

pretreatment serum TGF-β1 levels were associated with poor PFS and OS in advanced HCC patients.

We found that the serum TGF-β1 level can be associated with survival outcomes in univariate analysis, but not in multivariate analysis that adjusting for multiple clinicopathologic variables. These findings imply that serum TGF-β1 levels are associated with other clinicopathologic characteristics that are prognostically important in patients with advanced HCC. For example, we found that the high serum TGF-β1 level was associated with the high α-fetoprotein level, which has also been reported as a prognostic marker (28). Alternatively, the high serum TGF-β1 levels may not accurately reflect the activation of the TGF-β1 pathway in the tumor microenvironment, thereby contributing to its insignificant role as an independent prognostic factor.

In this study, we found that high TGF-β1 levels were associated with poor PFS, and TGF-β1 levels increased upon disease progression in advanced HCC patients treated with sorafenib. We

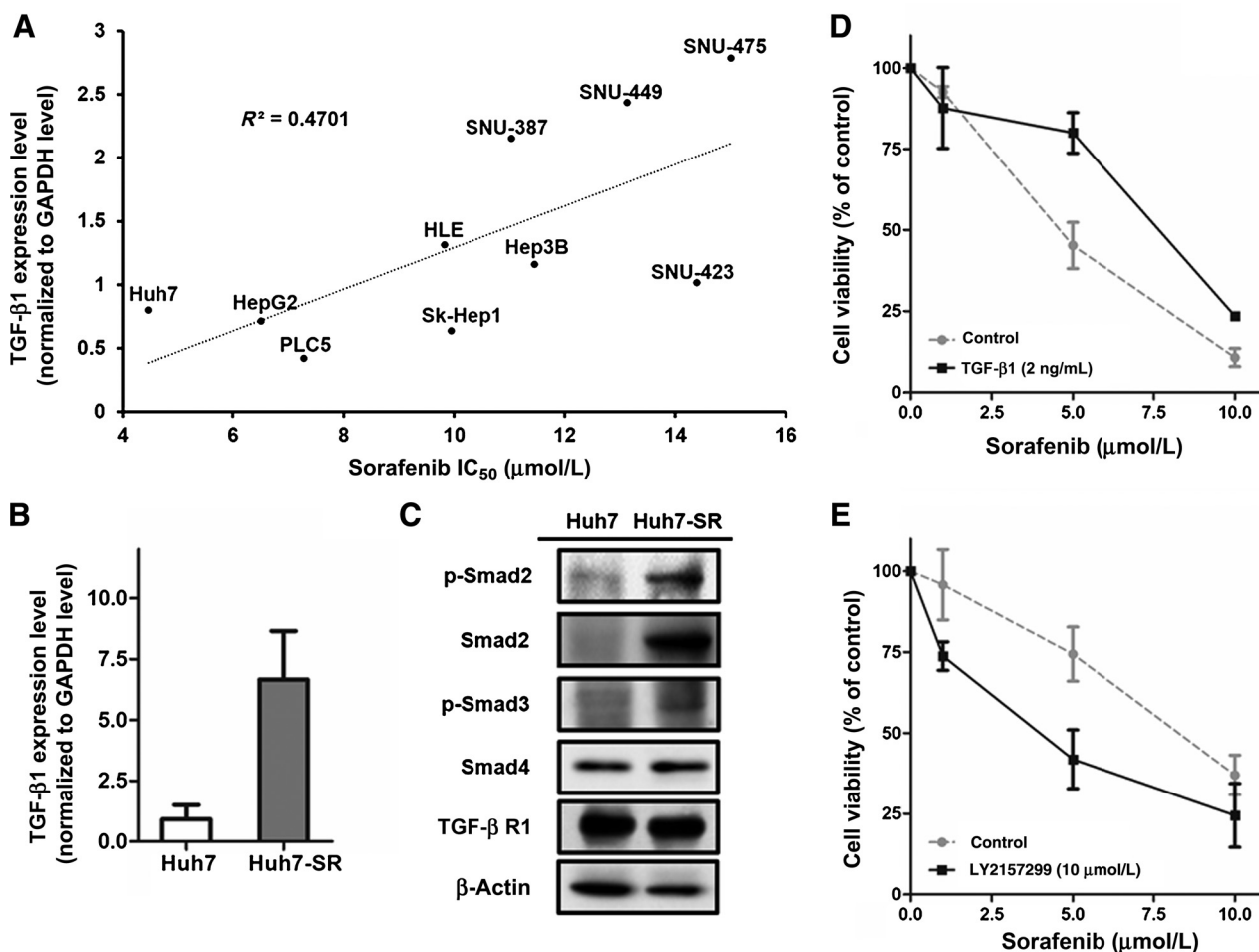


Figure 3. TGF-β1 signaling and sorafenib sensitivity in HCC cell lines. A, the antiproliferative effect of sorafenib in a panel of HCC cell lines determined by a 72-hour MTT assay and their mRNA expression level of TGF-β1, normalized by that of GAPDH, was evaluated by qRT-PCR. The plot shows the association between IC₅₀s of sorafenib and TGF-β1 mRNA expression level. Every value represents the mean derived from three independent experiments. B, the mRNA expression level of TGF-β1, normalized by that of GAPDH, in Huh7 and Huh7-SR cells. Huh7-SR was a subline derived from Huh7 and exhibited resistance to sorafenib. Data are presented as mean ± SD from three independent experiments. C, the protein expression of TGF-β signaling pathway molecules in Huh7 and Huh7-SR cells was determined by Western blotting. D, the antiproliferative effect of sorafenib, in the presence or absence of TGF-β1 (2 ng/mL), in Huh7 cells was determined by MTT assay. Data are presented as mean ± SD from three independent experiments. E, the antiproliferative effect of sorafenib, in the presence or absence of LY2157299 (10 μmol/L), in Huh7-SR cells was determined by MTT assay. Data are presented as mean ± SD from three independent experiments.

also found that TGF- β 1 expression levels were correlated with sensitivity to sorafenib in a panel of human HCC cells, and modulation of TGF- β activity could affect the sensitivity to sorafenib in Huh7 HCC cells. These data support the hypothesis that TGF- β signaling activity may contribute to resistance to sorafenib in HCC cells. TGF- β is a well-known signaling pathway promoting EMT. Previous studies have shown that EMT can induce resistance to EGFR-targeted therapy in HCC cell lines (29), and mesenchymal-type HCC cells have increased resistance to sorafenib (29, 30). In addition, TGF- β may contribute to resistance to molecularly targeted therapy through cross-talk with interleukin-6, as observed in lung cancer cells (31). Whether these or other mechanisms underlie TGF- β signaling activity contributing to sorafenib resistance in HCC cells requires further studies.

This study has several limitations. First, it was a retrospective analysis. Nevertheless, most of the clinicopathologic variables were prospectively collected and no patients who consented to donate blood for our study were excluded because of missing data. Second, two treatment regimens were used to treat patients: sorafenib only, and sorafenib plus metronomic tegafur/uracil chemotherapy. However, in a previous study, we reported that these two groups of patients exhibited similar PFS and OS (24). Finally, the number of patients in the current study was small, and the study did not include an independent cohort to validate the findings further.

In literature, the blood TGF- β 1 levels of patients with HCC significantly varied. Most studies used HCC patients with earlier or various stages. Only one study by Faivre and colleagues evaluated solely patients with advanced HCC as ours did (32). They reported a median serum TGF- β 1 level of 3.4 ng/mL, which was comparable with the median level found in our study (13.7 ng/mL). However, our maximum value clearly exceeded theirs (41.8 compared with 3.7 ng/mL). On the contrary, a Malaysian study reported a median TGF- β 1 level as high as 64.33 ng/mL (33). These results demonstrated that the range of blood TGF- β 1

levels in HCC patients can be huge. Whether ethnicity, disease status, or other factors may have impact on blood TGF- β 1 levels should be further explored.

In conclusion, we demonstrated that high pretreatment TGF- β 1 levels were associated with poor outcomes in advanced HCC patients treated with sorafenib or sorafenib-based combinations. TGF- β pathway may be explored as a therapeutic target for advanced HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T.-H. Lin, Y.-Y. Shao, C.-H. Hsu, A.-L. Cheng
Development of methodology: T.-H. Lin, C.-H. Hsu, A.-L. Cheng
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-H. Hsu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.-Y. Shao, S.-Y. Chan
Writing, review, and/or revision of the manuscript: T.-H. Lin, Y.-Y. Shao, C.-H. Hsu, A.-L. Cheng
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.-Y. Chan, C.Y. Huang, C.-H. Hsu
Study supervision: C.-H. Hsu, A.-L. Cheng

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