Regulation of transforming growth factor is involved in the efficacy of combined

5-fluorouracil and interferon alpha-2b therapy of advanced hepatocellular

carcinoma

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Abstract

Transforming growth factor-beta (TGF-β) is critical in cancer cell invasion and metastasis. The effects of a treatment that targets TGF-β using the combination of interferon alpha (IFNα)-2b and 5-fluorouracil (5-FU) are unknown. Here, we show that the serum levels of TGF-β1 prior to the therapy correlate with increased maximum tumor diameter, which is significantly ($p < 0.01$) decreased after the combination therapy. 5-FU increased both the expression and secretion levels of TGF-β1 in hepatoma cells, but not in normal hepatocytes. The combination of 5-FU and IFNα-2b synergistically affected cell death. However, a TGF-β1 specific inhibitor did not affect the anti-tumor activity of 5-FU. 5-FU inhibited the phosphorylation of SMAD2 and reduced the total protein levels of SMAD2, SMAD4, and pINK4b. Conversely, 5-FU stimulated the phosphorylation of extracellular signal-regulated kinase (ERK)1/2. Accordingly, the protein levels of E-cadherin and Claudin-1 were reduced in 5-FU-treated cells. The combination of 5-FU and IFNα-2b, and the inhibition of ERK1/2 by a specific inhibitor neutralized the effects of 5-FU on TGF-β-related signaling molecules and restored their protein levels to those observed in the control. Interestingly, the phosphorylated protein levels of SMAD2 and the total protein levels of

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E-cadherin and p15INK4b were increased in 5-FU-stimulated HuH-7 cells, but not in HepG2 cells. Our data suggest that despite its potent anti-tumor ability, 5-FU may induce epithelial–mesenchymal transition of hepatoma cells through TGF-β, and that the higher efficacy of the 5-FU and IFNα-2b combination therapy results from the inhibition of these effects of TGF-β.

Introduction

Common hepatocellular carcinoma (HCC) can be treated using diverse

approaches that include hepatectomy, transcatheter arterial chemo-embolization

(TACE), and radiofrequency ablation (RFA). However, there is no effective

therapy for advanced HCC with portal vein tumor thrombosis (PVTT)(Schwartz

et al., 2007). A combination therapy of interferon alpha (IFNα)-2b and

5-fluorouracil (5-FU), which was developed in the early 21th century(Obi et al.,

2006; Sakon et al., 2002), is being reconsidered as a treatment of advanced

HCC. We recently reported the improved survival rate of patients with advanced

HCC featuring portal venous invasion using this combination therapy (Kasai et

al., 2011; Kasai et al., 2012). However, the cumulative survival rate was < 50% at

36 months, which suggested that the treatment is not suitable or produces a

poor response for some patients. Better understanding of the mechanistic details

of this therapy would help in developing highly effective treatments for advanced

HCC and in predicting the causes of adaptation to the therapy.

Several reports have attempted to explain the mechanism of this combination

chemotherapy. IFN receptors may be involved in inducing the apoptosis of

cancer cells that has been observed with this combination chemotherapy(Nagano et al., 2007; Ota et al., 2005). Another possibility is the inhibition of the immunosuppression of cancer cells via tumor necrosis factor-related apoptosis-induced ligand (TRAIL) signaling(Nakamura et al., 2007; Yamamoto et al., 2004). Angiopoietin 2-related anti-angiogenesis might also have a role in the success observed with this treatment(Wada et al., 2007; Wada et al., 2009). However, these studies focused on the individual characteristics that appeared during the progression of advanced HCC, and did not determine the factors affecting the general efficacy of the combination therapy, such as targeting of key molecules or the associated signaling pathways.

As a member of the transforming growth factor beta superfamily, TGF-β activates the SMAD-dependent signaling pathway, which induces apoptosis and inhibits the proliferation of epithelial cells. Therefore, TGF-β has long been considered an anti-oncogenic molecule(Massague, 2012). However, recent research has revealed that mutations in certain components of the TGF-β signaling pathway exist in many cancers, especially in TGF-β receptor 2 and

SMADs(Nagaraj and Datta, 2010; Padua and Massague, 2009; Tesseur et al., 2006), which disrupt signal transduction and the subsequent inhibition of proliferation and apoptosis. In these mutants, TGF-β activates members of the mitogen-activated protein kinase (MAPK) pathway, such as extracellular signal-regulated kinase 1 and 2 (Erk1/2, or p44/42), c-Jun N-terminal kinases (JNKs), and p38 isoforms (p38 MAPK). This TGF-β-induced SMAD-independent mitogenic signaling protects cancer cells from apoptotic cell death and induces epithelial–mesenchymal transition (EMT), which renders the tumor cells metastatic(Akhurst and Hata, 2012; Miyazono, 2009; Padua and Massague, 2009). In addition, TGF-β is secreted excessively by several cancerous tissues, and contributes to the maintenance of the cancer stem cell population, immunosuppression, and angiogenesis(Pickup et al., 2013; Thomas and Massague, 2005).

Considering the importance of TGF-β in cancer development, we evaluated the relationship between the serum levels of TGF-β and the efficacy of the IFNα-2b/5-FU combination therapy. We also studied the effects of 5-FU with or without IFNα-2b on the regulation of TGFβ.

Materials and Methods

Subjects

Fifty patients with advanced HCC (stage IV-A/B) were treated using a subcutaneous administration of polyethylene glycol (PEG)-IFNα-2b (50-100 μg on day 1 of each week for 4 weeks) and intra-arterial infusion of 5-FU (250 mg/day for 5 h on days 1-5 of each week for 4 weeks). Blood was withdrawn before and after the therapy and centrifuged at 1,500 g for 10 min. The resulting serum was stored in aliquots at −80°C. The largest tumor diameter was also determined in the patients. Approval for the study was obtained from the institutional review board (H19-87) of Iwate Medical University, Morioka, Japan, and informed consent was obtained from the patients' relatives.

Cells

Human HuH-7 and Hep G2 hepatocarcinoma cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Rockford, IL, USA) containing 10% fetal bovine serum, (FBS; Thermo

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Fisher Scientific) and 100 U/mL penicillin–streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Mouse AML12 hepatocytes were kindly supplied by Professor Itaru Kojima, Gunma University, Japan. The cells were cultured in DMEM and Ham's F-12 nutrient mixture. (1:1, Thermo Fisher Scientific) containing 10% FBS until 80% confluency was reached. After starvation, the cells were treated with 5-FU (Kyowa Kirin, Tokyo, Japan), IFNα-2b (Funakoshi Co., Ltd., Tokyo, Japan), or the combination of 5-FU and IFNα-2b in the absence or presence of an inhibitor of TGF-β. Some experiments also included SB-431542, which is the specific inhibitor of receptor kinase (Sigma-Aldrich), and U0126, which inhibits ERK kinases (MEK; Promega Corporation, WI, USA).

Analysis of the levels of TGF-β1

TGFβ-1 levels in patients before and after the therapy, or in the cell culture supernatant of the cells were determined using the Quantikine Human LAP (TGF-β1) ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA).

Western blot analysis

Total protein was isolated from hepatocytes using a total protein extraction kit from the BioChain Institute (Newark, CA, USA) according to the manufacturer's instructions. Ten micrograms of protein from each sample was separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed using specific antibodies against TGF-β, phospho (p)-SMAD2, SMAD2, SMAD4, Claudin-1, E-cadherin, p15INK4b, p-ERK1/2 (Cell Signaling Technology Japan, K.K., Tokyo, Japan) and Beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immuno-reactive bands were visualized with an enhanced chemiluminescence reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and quantified with Image J software.

Cell viability assay

Serum-starved hepatocytes (5×10^4 cells/mL) were treated for 48 h under predetermined conditions. The number of viable cells was evaluated by adding SF cell counting reagent (Nacalai Tesque, Tokyo, Japan) directly to the cells.

Absorbance was measured at 450 nm (A_{450}) using an Immuno-Mini NJ-2300 microplate reader (Inter Med, Tokyo, Japan).

Results

Serum levels of TGF-β1 in patients with advanced HCC

On the basis of our previous results that the mean survival rate of patients is improved after the IFNα-2b/5-FU combination therapy (Kasai et al., 2011; Kasai et al., 2012), we first quantified the serum levels of TGF-β1 in patients with advanced HCC before and after the combination therapy to determine whether the efficacy of the therapy correlated with the regulation of TGF-β levels. Serum levels of TGF-β1 before the therapy showed a tendency towards correlation with increased maximum tumor diameter, which is used to evaluate the progress of HCC (Fig. 1a). Therapy was associated with significantly decreased serum levels of TGF-β1 ($p < 0.01$; (Fig. 1b). Seventeen patients had a background of hepatitis C virus (HCV) infection. Ten of the 17 patients improved and 7 worsened, as determined by a computed tomography (CT) scan one month after receiving the combination therapy. In these 17 patients, the serum levels of TGF-β1 decreased following the therapy, with no significant difference in the

therapy-related change in TGF-β1 levels between the patients who improved

and worsened (Supplemental Data).

Effects of 5-FU or IFNα-2b alone and in combination on TGF-β expression in

Hep G2 cells

The foregoing data prompted an *in vitro* assessment of whether the efficacy of

the combination therapy results from a synergistic effect of 5-FU and IFNα-2b on

the regulation of TGF-β. Unexpectedly, we found that 5-FU treatment stimulated

and increased the protein levels of TGF-β in the lysates of cultured Hep G2 cells

in a dose-dependent manner (Fig. 2a). In contrast, no TGF-β was detected in

cells treated with IFNα-2b (Fig. 2b). The combination of 5FU and 1 – 5 IU/mL

IFNα-2b completely inhibited the expression of TGF-β induced by 5-FU (Fig. 2b).

Since 5-FU is a potent anti-tumor reagent, we quantified cell viability to

determine whether the increased levels of TGF-β stimulated by the 5-FU

treatment contributed to the anti-tumor activity of 5-FU. 5-FU significantly

decreased cell viability, and the combination of 5-FU and IFNα-2b generated a

synergistic effect (Fig. 2c), consistent with previous descriptions of the

anti-tumor activity of 5-FU(Hu et al., 2016; Longley et al., 2003; Sui et al., 2013).

However, SB 431542, a specific inhibitor of TGF-β, showed no effects on the 5-FU-induced effects on Hep G2 cells.

Effects of 5-FU on TGF-β-related signaling molecules

The effect of 5-FU on TGF-β-induced apoptosis signals was investigated. 5-FU inhibited the phosphorylation of SMAD2 and reduced the total protein levels of pINK4b (Fig. 3a). Conversely, 5-FU stimulated the phosphorylation of ERK1/2, a TGF-β-induced EMT molecule. During the progression of EMT, tight junction proteins, like Claudins and adherens junction proteins like E-Cadherin are usually down-regulated(Lamouille et al., 2014; Shiozaki et al., 2012). Consistent with these observations, we observed that the protein levels of E-cadherin and Claudin-1 were reduced in the cells treated with 5-FU. On the other hand, IFNα-2b stimulated the phosphorylation of SMAD2, but not ERK 1/2, increased the total protein levels of pINK4b, and did not affect the levels of E-cadherin and Claudin-1. The combination of 5-FU and IFNα-2b inhibited the effects of 5-FU on the aforementioned TGF-β-related signaling molecules (Fig. 3b). In addition, similar to the effects of IFNα-2b, U0126 (a MEK-specific inhibitor and an upstream effector of ERK1/2) inhibited the negative effect of 5-FU on the

regulation of E-cadherin and Claudin-1 (Fig. 3c).

5-FU increases expression and secretion levels of TGF-β1 in Hep G2 and HuH-7 cells

We also compared the effects of 5-FU on the expression and secretion of TGF-β in Hep G2 and HuH-7 hepatocarcinoma cells, and normal alpha mouse liver (AML) 12 hepatocytes. Time- and dose-course experiments revealed increased TGF-β levels in 5-FU treated Hep G2 and HuH-7 cells. In HuH-7 cells, the levels of TGF-β levels peaked upon treatment with 30 µg/mL 5-FU (Fig. 4a), whereas there was a progressive increase in TGF-β levels in Hep G2 cells. Conversely, a TGF-β peak appeared in Hep G2 cells that were treated for 24 h with 5-FU, whereas there was a progressive increase in TGF-β levels for up to 48 h in HuH-7 cells (Fig. 4b). We further compared the TGF-β secretion pattern of these hepatoma cell lines. The baseline level of TGF-β in the supernatant fraction of cultured HuH-7 cells was higher than that of Hep G2 cells. The peak in TGF-β secretion occurred upon a 48-h treatment of either cell line with 15 µg/mL 5-FU (Fig. 4c, 4d). In contrast, there was no expression (Fig. 4a, 4b) or secretion (Fig. 5b) of TGF-β in normal AML 12 hepatocytes with or without 5-FU treatment.

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Differences in the effects of 5-FU and IFNα-2b combination therapy on TGF-β regulation between Hep G2 and HuH7 cells

The combination of 5-FU and IFNα-2b decreased both the expression and secretion of TGF-β in Hep G2 cells compared to cells treated with 5-FU alone. However, in HuH-7 cells, the combination therapy decreased only the secretion of TGF-β, with no effect on the protein levels (Fig. 5a, 5b). Interestingly, the phosphorylated protein levels of SMAD2 and the total protein levels of E-cadherin and p15INK4b were increased in 5-FU-stimulated HuH-7 cells, in contrast to the inhibition of these TGF-β-induced signaling molecules in 5-FU-stimulated HepG2 cells (Fig. 5c).

Discussion

Elevation in the serum levels of TGF-β or TGF-β expression have been reported in various cancers, and particularly during the progression of advanced HCC(Barcellos-Hoff and Akhurst, 2009; Lin et al., 2015; Pang et al., 2016; Reis et al., 2011; Song et al., 2002; Xu et al., 1999). TGF-β1 is a useful biomarker of prostate cancer and small HCC(Reis et al., 2011; Song et al., 2002).   Presently,

although no significant difference was found, possibly because all the subjects were stage IV, serum TGF-β levels tended to correlate positively with maximum tumor diameter. More importantly, TGF-β levels decreased in patients who were administered a combined chemotherapy of 5-FU and IFNα-2b, accompanied by a high efficacy of the therapy with a mean survival rate of 29.9 months(Kasai et al., 2012). The data suggest that the reduction in TGF-β levels may be useful to predict the efficacy of the combination therapy.

Surprisingly, 5-FU increased both the protein and secretion levels of TGF-β, and regulated the TGF-β-induced signaling in HepG2 and HuH-7 hepatoma cells, but not in normal hepatocytes. A recent study reported that 5-FU can activate the TGF-β pathway and reasonably upregulate the expression of TGF-β in an autocrine manner in drug resistant colorectal carcinoma cells(Romano et al., 2016). The association between 5-FU and TGF-β signaling has also been described in human HCC cells(Nomura et al., 2017). It is possible that 5-FU regulates TGF-β signaling indirectly via poly (ADP-ribose) polymerase-1 (PARP-1), a nuclear enzyme that is conventionally linked to DNA repair (Akpinar et al., 2015; Zhang et al., 2013). How 5-FU regulates TGF-β and its pathway in

HCC cells, and whether its function is limited only to cancer cells is unclear and requires further investigations.

TGF-β regulates both apoptosis and EMT of hepatocytes. However, resistance of hepatoma cells to TGF-β-induced apoptosis has been reported(Buenemann et al., 2001). Presently, a specific TGF-β inhibitor did not antagonize the effects of 5-FU on Hep G2 cell viability. Additionally, although the combination of 5-FU and IFNα-2b was more lethal to cancer cells than 5-FU alone, TGF-β expression and secretion decreased in cells treated with the drug combination compared to cells treated with 5-FU alone. Our data indicate that the increase in TGF-β levels does not contribute to the anti-cancer activity of 5-FU in Hep G2 cells. Moreover, we found that 5-FU inhibited TGF-β-mediated apoptosis signaling but stimulated the activation of ERK1/2 and inhibited the protein levels of E-cadherin and Claudin-1. In addition, inhibition of ERK1/2 neutralized the effects of 5-FU on the protein levels of E-cadherin and Claudin-1. These events are indications of prompt TGF-β-mediated EMT signaling. In fact, we have found that the supernatant medium of cultured Hep G2 cells can stimulate cell invasion/migration (data not shown). 5-FU is a known anti-tumor reagent.

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However, the present findings indicate the possibility that 5-FU upregulates TGF-β, which may contribute to the increase in the biological grade of liver cancer.

On the other hand, as we presently confirmed, IFNα inhibits the expression of TGF-β(Yu et al., 2016). We provide the first evidence that IFNα-2b can correct the inhibitory effects of 5-FU on TGF-β-mediated apoptosis signaling, and inhibits the 5-FU induced EMT signaling in Hep G2 cells. Therefore, it can be assumed that the higher efficacy of the combination therapy for advanced HCC results from the important contribution of IFNα-2b in synergizing 5-FU by anti-tumor activity and by inhibiting TGF-β-mediated EMT that is usually elevated in advanced HCC, and which may be induced by 5-FU treatment alone. Aside from the present finding, evidence for a benefit of IFNα-2b in protecting against EMT is scant. Indeed, medical insurance does not cover the use of IFN alpha 2b in treatment of advanced HCC. Cisplatin(Park et al., 2006), sorafenib(Keating and Santoro, 2009) and more recently, the TGF-β receptor I kinase inhibitor, LY2157299 monohydrate(de Gramont et al., 2017), have good efficacy against advanced HCC. The present data indicate that, except for

IFNα-2b, co-treatment involving 5-FU and other compounds or inhibitors of TGF-β and its signaling will be efficacious. This is consistent with a prior study that described the therapeutic value of the combination of a TGF-β receptor kinase inhibitor with the 5-FU analog, S1, for the treatment of scirrhous gastric carcinoma with lymph node metastasis(Shinto et al., 2010). An investigation to identify a replacement for IFNα-2b in a combination treatment involving 5-FU against advanced HCC is underway.

Presently, TGF-β expression and secretion were unaffected by 5-FU treatment of normal hepatocytes, compared to Hep G2 and HuH-7 hepatoma cells. Moreover, the two hepatoma cell lines responded differently to treatment with 5-FU alone or in combination with IFNα-2b. Although the combination therapy has therapeutic value, patients may respond poorly or not at all. This variation in response could be connected with our observations concerning the differential effect of 5-FU monotherapy and combined therapy on the TGF-β levels of the two hepatoma cell lines used in this study. Of note, although both Hep G2 and HuH-7 are hepatoma cell lines, they differ appreciably in the pattern of TGF-β expression and secretion. Western blot analysis of TGF-β protein revealed a

difference in the banding pattern between the two cell types. Additionally, the combination of 5-FU and IFNα-2b significantly blocked the increase in TGF-β levels in Hep G2 cells, but not in HuH-7 cells. The collective data indicate a cell-dependent function of 5-FU. Interestingly, the Hep G2 cell line was established from a HBV-positive male with a primary hepatoma(Aden et al., 1979; Huber et al., 1985), while HuH-7 cells possess HCV replicons capable of auto-replication(Kapadia and Chisari, 2005). Thus, our in vitro data might explain the variation in the clinical efficacy of the combination therapy. In other words, the efficacy of the therapy may differ between patients with HCV or HBV background. The small number of patients with HBV background precluded us from an in vivo analysis of the relationship between the variation of therapeutic efficacy and viral background. However, it has been reported that patients with HCC and a HCV-positive background had higher survival rates and longer survival periods than patients who were HCV-negative or produced higher levels of TGF-β receptor 2(Sakabe et al., 2013). Furthermore, in contrast to Hep G2 cells, 5-FU increased the levels of p-SAMD and p15INK4b, and maintained or even increased the level of E-cadherin in HuH-7 cells. These observations could indicate that TGF-β and its signal induced by 5-FU in HuH-7 cells tend to

mediate apoptosis rather than EMT. In cases with an HCV background that were analyzed, although the serum levels of TGF-β decreased after the combination therapy in all the patients, 41.2% of the cases responded poorly to the therapy with a continued worsening of their condition, and there were no significant differences in TGF-β levels between patients who worsened and improved. These data suggest that although co-treatment with 5-FU and IFNα-2b may avoid the risk of TGF-β-mediated EMT, excessive inhibition of TGF-β and its signal is unnecessary or may even weaken the apoptotic function of 5-FU against advanced HCC in patients with an HCV background, rather than HBV background, which could lead to poor or no response to the combination therapy.

In Japan and China, the majority of HCC patients have an HCV and HBV background, respectively. Proper treatment of these patients will undoubtedly determine the efficacy of HCC treatment, especially for those in advanced stages. The present data clarify the various effects of 5-FU and IFNα-2b applied alone and in combination, on the regulation of TGF-β levels and its signals in two hepatoma cells. The knowledge gained will aid in determining the mechanism of

the combination chemotherapy, which will in turn drive the formulation of better therapies for advanced HCC.

Conflict of Interest

The authors declare no conflicts of interest associated with this manuscript.

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Figure legends

Fig. 1. Detection of TGF-β1 serum levels in patients with advanced HCC.

(a) TGF-β1 levels before the treatment were used to analyze the correlation with the maximum tumor diameter. A p value and its correlation were calculated in Microsoft Excel 2010. (b) TGF-β1 serum levels in patients before and after treatment were determined according to the manufacturer's instructions. Fifty patients were analyzed. Statistical comparisons were performed using a paired t-test. A p value <0.05 was considered to be significant.

Fig. 2. Effects of 5-FU and IFNα-2b alone and in combination on TGF-β levels in Hep G2 cells. Cells were treated with 0, 15, 30, and 100 μg/ml 5-FU for 24 h (a) or with 30 μg/ml 5-FU, IFNα-2b (1, 2, 5 IU/ml), or 5-FU and IFNα-2b for 24 h (b). TGFβ levels in the cell lysates were determined by western blotting. (c) Cells were treated with 15 μg/ml 5-FU, 2 IU/ml IFNα-2b, or a combination of 5-FU and IFNα-2b for 48 h. 10 μM SB 431542 (TGF-βi) was used with or without 5-FU. The number of viable cells was evaluated as the value of the absorbance at 450nm. The statistical analysis was carried out using t-test for unpaired comparisons. A p value <0.05 was considered to be significant. Results are expressed as the means ± SD (n=3).

Fig. 3. Effects of 5-FU and IFNα-2b alone and in combination on TGF-β-mediated signaling. Hep G2 cells were treated with 5-FU and/or IFNα-2b at the indicated concentrations. (a) The protein levels of p-SMAD2, SMAD2, SMAD4, and p15INK4b were detected by western blotting. (b) In another experiment, E-cadherin, Claudin-1, and p-ERK1/2 levels were detected by western blotting. (c) Besides the treatment of 5-FU and IFNα-2b alone and in combination, the ERK1/2 inhibitor U0126 was used to clarify the role of ERK1/2 in the regulation of E-cadherin and Claudin-1 by 5-FU. Actin was detected as an internal control. The quantitative data are presented as the means ± SD (n=3).

Fig. 4. Cell-dependent regulation of TGF-β expression and secretion by 5-FU. HuH-7, Hep G2, and AML 12 cells were treated with different concentrations of 5-FU as indicated (a), and 30 μg/ml 5-FU for the indicated time periods (b). TGF-β was detected by western blotting. TGF-β secretion was measured in HuH-7 and Hep G2 cells as described in Materials and Methods (c, d). Results are expressed as the means ± SD (n=3).

Fig. 5. Various responses of different kind of cells to 5-FU treatment combined with IFNα-2b and/or 5-FU alone on TGF-β levels and TGF-β mediated signaling. Hep G2, HuH7 and AML-12 cells were individually treated with 5-FU, IFNα-2b, and a combination of 5-FU and IFNα-2b for 24 h. TGF-β levels in cell lysates (a) and culture supernatants (b) were detected. (c) Both Hep G2 and HuH7 cells were treated with 5-FU for different periods as indicated. The levels of E-cadherin, p-SMAD2, and p15INK4b were detected by western blot. Actin was detected as an internal control. Results are expressed as the means ± SD (n=3).

Supplemental Data. The change in serum levels of TGF-β1 with the combination therapy in the patients with an HCV background whose condition worsened (n=7) or improved (n=10). The change in serum levels of TGF-β1 was determined by calculating the ratio of serum level of TGF-β after the therapy with that before the therapy. The efficacy of the therapy was evaluated based on the results of CT scans one month after the therapy. The statistical analysis was carried out using t-test for unpaired comparisons. A *p* value <0.05 was considered to be significant. Results are expressed as the means ± SD. Seventeen patients were analyzed.