Identification of the Genes Chemosensitizing Hepatocellular Carcinoma Cells to Interferon-α/5-Fluorouracil and Their Clinical Significance

Tomohiko Sakabe¹, Hiroyuki Tsuchiya², Keita Kanki¹, Junya Azumi¹, Kazue Gonda¹, Yusuke Mizuta¹, Daisaku Yamada³, Hiroshi Wada³, Kohei Shomori⁴, Hiroaki Nagano³, Goshi Shiota¹*¹

¹Division of Molecular and Genetic Medicine, Department of Medical and Regenerative Therapeutics, Graduate School of Medicine, Tottori University, Yonago, Japan, ²Department of Biophysical Chemistry, Kyoto Pharmaceutical University, Kyoto, Japan, ³Department of Surgery, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan, ⁴Department of Microbiology and Pathology, Faculty of Medicine, Tottori University, Yonago, Japan

Abstract

The incidence of advanced hepatocellular carcinoma (HCC) is increasing worldwide, and its prognosis is extremely poor. Interferon-alpha (IFN-α)/5-fluorouracil (5-FU) therapy is reportedly effective in some HCC patients. In the present study, to improve HCC prognosis, we identified the genes that are sensitizing to these agents. The screening strategy was dependent on the concentration of ribozymes that rendered HepG2 cells resistant to 5-FU by the repeated transfection of ribozymes into the cells. After 10 cycles of transfection, which was initiated by 5,902,875 sequences of a ribozyme library, three genes including protein kinase, adenosine monophosphate (AMP)-activated, gamma 2 non-catalytic subunit (PRKAG2); transforming growth factor-beta receptor II (TGFBR2); and exostosin 1 (EXT1) were identified as 5-FU-sensitizing genes. Adenovirus-mediated transfer of TGFBR2 and EXT1 enhanced IFN-α/5-FU-induced cytotoxicity as well as 5-FU, although the overexpression of these genes in the absence of IFN-α/5-FU did not induce cell death. This effect was also observed in a tumor xenograft model. The mechanisms of TGFBR2 and EXT1 include activation of the TGF-β signal and induction of endoplasmic reticulum stress, resulting in apoptosis. In HCC patients treated with IFN-α/5-FU therapy, the PRKAG2 mRNA level in HCC tissues was positively correlated with survival period, suggesting that PRKAG2 enhances the effect of IFN-α/5-FU and serves as a prognostic marker for IFN-α/5-FU therapy. In conclusion, we identified three genes that chemosensitize the effects of 5-FU and IFN-α/5-FU on HCC cells and demonstrated that PRKAG2 mRNA can serve as a prognostic marker for IFN-α/5-FU therapy.

Introduction

Hepatocellular carcinoma (HCC) is the third most common cancer, the incidence of which is reportedly increasing worldwide [1]. Moreover, cases of advanced HCC, which is characterized by an extremely poor prognosis, are increasing in number. Recently, sorafenib, an oral multikinase inhibitor, was reported to improve the median overall survival rate in advanced HCC patients [2,3]. However, it did not improve overall survival and prognosis in advanced HCC patients with portal vein tumor thrombosis (PVTT) [4]. Although interferon-alpha (IFN-α)/5-fluorouracil (5-FU) combination therapy showed favorable effects in advanced HCC patients [5], especially compared to those with PVTT [6,7,8], its maximum efficacy was only 25%–50% in some countries including Japan and USA, suggesting that chemoresistance limits the therapeutic potential of IFN-α and 5-FU. To achieve more favorable outcomes in advanced HCC patients, advances in IFN-α/5-FU therapy are urgently required. However, limited information is currently available on the genes involved in enhancing chemosensitivity to this therapy. Several screening strategies using DNA microarray and RNA interference technologies are developed to identify genes with unrecognized functions. Although predictive molecular markers, including IFN-α receptor 2 [9], Wnt/b-catenin [10], and CD133 [11], are reported in association with IFN-α/5-FU therapy, little is known about the enhancing mechanisms. Therefore, we focused on ribozyme-based functional screening. Ribozymes are small catalytic RNA molecules comprising target recognition sequences and a catalytic center with RNase activity. Ribozyme libraries have been used to identify genes that are associated with several pathways [12]. Additionally, OZ1, a vector containing a ribozyme targeting the reading frames of HIV-1, was used in a clinical trial [13].

In the present study, we hypothesized that if the genes sensitizing HCC cells to 5-FU are identified, they could be applied to IFN-α/5-FU therapy and used as prognostic markers. To confirm this hypothesis, we used ribozymes to perform reverse genetics-based functional screening to identify genes that augment the efficacy of IFN-α/5-FU therapy.
Materials and Methods

Ethics Statement
All animal experiments were approved by the Institutional Animal Care and Use Committee of Tottori University (the permit number: 10-Y-54). The mice received humane care in accordance with the study guidelines for the care and use established by the Tottori University. All mice were kept under pathogen-free conditions and were maintained in a temperature-controlled room with a 12 h light/dark illumination cycle.

Materials and Cell Culture
IFN-α and 5-FU were provided by MSD (Tokyo, Japan) and Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan), respectively. HepG2, HuH7, and HLF human HCC cells were obtained from the Japanese Collection of Research Bioresources and maintained in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (MBL, Nagoya, Japan), L-glutamine, and glucose in a humidified atmosphere of 5% CO₂ at 37°C.

The Water Soluble Tetrazolium Salt (WST) Assay
The IFN-α and 5-FU antitumor effects were assessed using the WST assay (Seikagaku Corporation, Tokyo, Japan). The cells were cultured in various concentrations of IFN-α and 5-FU for 72 h. The viability of cells treated with dimethyl sulfoxide was defined as 100%.

Screening for Genes Involved in Enhancing the Effect of 5-FU
We constructed a plasmid DNA (pDNA) library expressing ribozyme genes (Figure S1 and Table S1). Screening was performed on the basis of the ability of 5-FU to eliminate cells expressing ribozymes that target unrelated chemosensitive genes, as described in File S1.

Subcutaneous Xenograft Model in Mice
Four-week-old male non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). We subcutaneously transplanted 6.8×10⁶ cells HepG2 cells in 0.1 mL PBS into the right flank of each mouse. The mice were randomly assigned to the 4 following groups: (i) LacZ-adenovirus and PBS (instead of IFN-α and 5-FU); (ii) LacZ-adenovirus and IFN-α/5-FU; (iii) TGFBR2-adenovirus and IFN-α/5-FU; and (iv) EXT1-adenovirus and IFN-α/5-FU. At 3 weeks after transplantation, 2.5×10⁶ plaque forming unit (PFU)/tumor adenoviruses carrying either the TGFBR2, EXT1, or LacZ genes were intratumorally injected and this was repeated every 3 d. On the next day, IFN-α and 5-FU combination treatment was started and repeated every 3 d. We administered 20,000 U/body IFN-α subcutaneously and 30 mg/kg 5-FU was administrated intraperitoneally. Administration of adenovirus carrying TGFBR2, EXT1, or LacZ and IFN-α/5-FU were repeated for 4 weeks according to the previous reports [14]. During the 4 weeks of treatment, tumor size was measured every week. The volumes were calculated using the following equation: (tumor volume; mm³) = (length; mm)×(width; mm)²×0.5³.

RNA Isolation and Real-time Reverse Transcription-polymerase Chain Reaction
Total RNA from HCC cells and resected HCC specimens was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed using SuperScript II (Invitrogen) and oligo(dT) primers. mRNA expression levels were determined using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Applied Science, Basel, Switzerland) and gene-specific primers (Table S1).

Western Blotting
Whole-cell lysates were prepared using a protease inhibitor cocktail (Roche Applied Science) and phosphatase inhibitor cocktail (Roche Applied Science). Actin was used as an internal control. The antibodies against PRKAG2 (Cell Signaling Technology, Danvers, MA, USA), TGFBR2 (Santa Cruz Biotechnology, CA, USA), EXT1 (Santa Cruz Biotechnology), SAPK/JNK (Cell Signaling Technology), Phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling Technology), Phospho-p38 MAP Kinase (Thr180/Tyr182) (Cell Signaling Technology), BAX (Santa Cruz Biotechnology), BCL-xl (Santa Cruz Biotechnology), BCL-2 (Santa Cruz Biotechnology), ATF4 (Santa Cruz Biotechnology), Bif/GRP78 (Cell Signaling Technology), CHOP (Cell Signaling Technology), LC3B (Cell Signaling Technology), and actin (Santa Cruz Biotechnology) were used in western blot analysis.

Immunofluorescence Analysis
Immunofluorescence analysis was performed according to previous report [15]. HepG2 cells infected with adenoviruses were treated with 5-FU alone, IFN-α/5-FU or tunicamycin for 48 h. Cells were incubated with LC3B antibody (Cell Signaling Technology) followed by incubation with Alexa Fluor 488 conjugated secondary antibody (Molecular Probes, Leiden, Netherlands). DAPI was used for nuclear counterstaining.

Patients and Clinical Specimens
Subjects included 17 multiple advanced HCC patients admitted to Osaka University Hospital between 1999 and 2004, who agreed to undergo palliative reduction surgery and IFN-α/5-FU therapy after providing written informed consent. The present study was approved by the Institutional Review Board of Tottori University and Osaka University. The criteria of selection for IFN-α/5-FU...
Figure 2. Enhancing effects of genes on IFN-α/5-FU treatment. (A–C). Viability of HepG2 cells infected with adenovirus-carrying protein kinase, adenosine monophosphate (AMP)-activated, gamma 2 non-catalytic subunit (PRKAG2) (A), transforming growth factor-beta receptor II (TGFBR2) (B), and exostosin 1 (EXT1) (C) with or without 5-FU. Adenovirus-carrying LacZ served as a negative control. Cell viability was determined using the WST assay at 72 h. *P<0.05 and **P<0.01, between the two groups. (D) Viability of HepG2, HuH7, and HLF cells infected with adenovirus-carrying TGFBR2 and EXT1 with or without agents. Data are expressed as mean ± standard deviation (SD) (n=3). Statistical significance was determined using Student’s t-test. *P<0.05 between two groups. (E) In vivo studies using NOD/SCID mice subcutaneously transplanted with HepG2 cells. In adenovirus-administered mice, tumor growth was assessed during IFN-α/5-FU treatment. Data are shown as means ± SD (n=4–7).

Figure 3. Enhancement of IFN-α/5-FU-induced apoptosis by TGFBR2 and EXT1 overexpression. (A) Evaluation of nuclear condensation. The arrows indicate cells with apoptosis-specific nuclear condensation and fragmentation. (B) Measurement of intracellular caspase 3/7 activity. After adenovirus infection, cells were treated with the indicated concentrations of IFN-α and 5-FU. Activity was expressed as the fold increase relative to that at 0 h. Data are expressed as mean ± standard deviation (n=3). Statistical significance was determined using Student’s t-test. *P<0.05 compared to LacZ at 48 h.
therapy were based on the previous report [8]. Clinical responses of the 17 patients to IFN-α/5-FU therapy were evaluated according to the criteria of the World Health Organization [16,17], and were classified into four categories as complete response (CR), partial response (PR), no change (NC), and progressive disease (PD). On the basis of their clinical responses, the patients were classified into the following two types: responder group (CR or PR), and non-responder group (NC or PD). Moreover, these patients were further classified into other two groups based on the presence or absence of hepatitis C virus (HCV) antibody, regardless of hepatitis B virus (HBV) infection: HCV negative group, which includes one patient with NonB/NonC and nine patients with HBV, and HCV positive group, which includes three patients with HCV and four patients with HBV/HCV.

Statistical Analysis

For statistics, Excel spreadsheet software (Microsoft Corporation, Redmond, WA, USA) and predictive analytics software (SPSS Inc., Chicago, IL, USA) were used. Statistical comparisons were made using Student’s t-test, one-way analysis of variance, Tukey’s HSD test and the Mann–Whitney U test. The association between survival and gene expression was assessed by Spearman’s rank correlation test. P<0.05 was considered statistically significant.

Results

Identification of Protein Kinase, Adenosine Monophosphate (AMP)-activated, Gamma 2 Non-catalytic Subunit (PRKAG2); Transforming Growth Factor-beta receptor II (TGFBR2); and Exostosin 1 (EXT1) as 5-FU-sensitizing Genes

We first tried to identify genes sensitizing to 5-FU instead of those sensitizing to both IFN-α and 5-FU because it seemed difficult to identify genes sensitive to both agents due to their multiple apoptotic pathways [18–20]. Functional screening was performed using a random ribozyme library. Briefly, HepG2 cells were first transfected with the ribozyme library containing 5,902,875 sequences, following which they were treated with 5 μg/mL of 5-FU, which is a sufficient concentration to kill HepG2 cells, for 72 h (Figure 1A). To avoid false-negative and false-positive results, we decided to adopt a moderate concentration of 5-FU for the present screening. The cells were harvested to recover ribozymes and were transfected again with the recovered ribozymes. The screening included repetition of this process for 10 rounds (Figure 1B). The cells were transfected with pDNAs that recovered from 6 (Rz-C6), 8 (Rz-C8), and 10 (Rz-C10) cycles of screening and treated with the indicated 5-FU concentrations for 72 h. The viability of cells transfected with the recovered pDNAs significantly increased during the progression from 0 to 10 cycles of screening at 1–10 μg/mL of 5-FU, suggesting that ribozymes become densely concentrated by the screening (Figure 1C). Five genes were selected as candidates because the number of ribozymes targeting the Rz-C10-transfected genes increased to more than four, whereas that of ribozymes targeting the Rz-C0-transfected genes was zero (Table S2). Of the five genes, four, with the exception of FOXP2, were expressed in HepG2 cells (Figure 1D). POLR2J4 was excluded because it is a pseudogene. Since selective knockdown of the genes by specific small interfering RNA (siRNA) induced resistance to 5-FU, we subsequently focused on PRKAG2, TGFBR2, and EXT1 (Figure 1E and 1F).

Enhancing the Effect of the Identified Genes on IFN-α/5-FU Treatment

To investigate whether these genes actually enhance 5-FU- and IFN-α/5-FU-induced cytotoxicity, PRKAG2, TGFBR2, and EXT1 were exogenously expressed in HepG2 cells and cell viability was examined at various 5-FU concentrations (Figure 2A–C). Adenovirus-mediated expression of PRKAG2, TGFBR2, and EXT1 proteins was confirmed in these cells (Figure S2A and S2B) and mCherry fluorescent protein expression, which is used as a marker, were detected flow cytometry analysis (Figure S2C–H). Adenovirus-mediated PRKAG2 expression enhanced the effects of 5 μg/mL of 5-FU (Figure 2A). In contrast, adenovirus-mediated TGFBR2 expression enhanced effects at all 5-FU concentrations (1–40 μg/mL) (Figure 2B). Additionally, adenovirus-mediated EXT1 expression accelerated the effects of 5-FU from 2.5 to 20 μg/mL (Figure 2C).

Furthermore, we focused on the effects of TGFBR2 and EXT1 on IFN-α/5-FU because the enhancement of TGFBR2 and EXT1, but not PRKAG2, was effective within wide concentrations of 5-FU (Figure 2D). In HepG2 cells, 1000 U/mL of IFN-α without 5-FU was effective; however, adenovirus-mediated expression of TGFBR2 or EXT1 did not enhance its effects. IFN-α/5-FU greatly suppressed cell viability compared with IFN-α alone. Exogenous overexpression of TGFBR2 and EXT1 clearly enhanced chemosensitivity to IFN-α/5-FU as well as 5-FU. IFN-α did not kill Huh7 cells; however, adenovirus-mediated transfer of EXT1 enhanced the effects of IFN-α, while that of TGFBR2 also tended to be effective. The effects of IFN-α/5-FU and 5-FU were much greater than those of IFN-α alone. Moreover, adenovirus-mediated expression of TGFBR2 and EXT1 chemosensitized the cells to the effects of IFN-α/5-FU and 5-FU. In addition to HepG2 cells and Huh7 cells, TGFBR2 and EXT1 also enhanced the effects of 5-FU and IFN-α/5-FU in HLF cells. Then, to evaluate the significance of identified genes in IFN-α/5-FU combination treatment, we performed statistical comparisons between 5-FU alone and IFN-α/5-FU. When compared to the treatment with 2.5 μg/mL 5-FU alone, a combined effect of 200 U/mL IFN-α and 2.5 μg/mL 5-FU was observed in TGFBR2-overexpressing Huh7. Similarly, 200 U/mL IFN-α and 5 μg/mL 5-FU showed statistical significance in TGFBR2 or EXT1-overexpressing HLF cells.

Additionally, in vivo studies were performed using NOD/SCID mice subcutaneously transplanted with HepG2 cells. In mice administered adenoviruses expressing either TGFBR2 or EXT1, tumor growth suppression by IFN-α/5-FU was significantly enhanced compared with that in controls (Figure 2E). These findings suggest that overexpression of TGFBR2 and EXT1 enhance chemosensitivity to IFN-α/5-FU in vitro and in vivo and support our hypothesis that if the genes sensitizing HCC cells to 5-FU are identified, they could be applied to IFN-α/5-FU therapy.

Molecular Mechanisms Underlying the Enhancement of Antitumor Effects by TGFBR2 and EXT1

Although the genes greatly enhanced the antitumor effects of IFN-α/5-FU, the underlying mechanisms remained unclear. The principal mechanism underlying the antitumor effects of IFN-α/5-FU or 5-FU alone in several HCC cells is apoptosis [21]. First, we examined whether TGFBR2 and EXT1 enhanced apoptosis induced by IFN-α/5-FU. As reported, nuclear condensation of HepG2 cells was induced by 5-FU and IFN-α/5-FU (Figure 3A and S3). Adenovirus-mediated expression of TGFBR2 and EXT1 increased nuclear condensation compared with the expression of LacZ (Figure 3A and S3). Supporting this observation, caspase 3/7...
Figure 4. Effects of TGFBR2 on 5-FU- and IFN-α/S-FU-induced TGF-β signaling pathway. (A) 5-FU-induced TGFβ1 mRNA expression normalized to β-actin. Data are expressed as mean ± standard deviation (SD) (n=3). Statistical significance was determined using Student’s t-test. \( *P<0.05 \) and \( **P<0.001 \) compared to control. N.S., not significant. (B) Luciferase reporter assay for TGF-β signaling activation. Data are expressed as mean ± SD (n=3). Recombinant TGF-β was used as a positive control. The Y-axis is expressed as a logarithmic scale. (C) Western blot analysis of BAX, BCL-2, and BCL-xL in 5-FU and IFN-α/S-FU treatment with or without TGFBR2 overexpression. Each band was quantified by Image J software and normalized to actin.

doi:10.1371/journal.pone.0056197.g004
activity was increased by 5-FU and IFN-α/5-FU treatment in HepG2 and HuH7 cells, respectively (Figure 3B). Further enhancement of caspase 3/7 activation was indicated by overexpression of TGFBR2 and EXT1 in these cells. These results suggest that TGFBR2 and EXT1 enhanced IFN-α/5-FU effects by accelerating apoptosis.

Regulation of Apoptosis by TGFBR2

To explore the precise role of TGFBR2 in enhancing apoptosis by IFN-α/5-FU, we investigated possible mechanisms. First, we determined that TGFB1 mRNA levels after 5-FU and IFN-α/5-FU treatment were significantly increased, although neither TGFBR2 nor LacZ affected their expression (Figure 4A). The reporter assay using TGF-β-responsive plasmids showed that 5-FU and IFN-α/5-FU induced TGF-β-dependent transcriptional activity in HepG2 and HuH7 cells (Figure 4B). Additionally, TGFBR2 overexpression significantly enhanced TGF-β signaling activity compared with that in the controls. These findings suggest that the TGF-β signaling pathway is involved in the antitumor mechanisms of 5-FU alone or IFN-α/5-FU, and that TGFBR2 overexpression facilitates its autocrine stimulation. Next, we examined the protein levels of proapoptotic BAX and antiapoptotic BCL-2 and BCL-xL, which are targets of the TGF-β signaling pathway during apoptosis induction [22–24]. In HepG2 cells, TGFBR2 overexpression induced BAX expression in the presence of 5-FU and IFN-α/5-FU (Figure 4C). Moreover, 5-FU alone did not alter BCL-2 levels, whereas IFN-α/5-FU decreased BCL-2 levels, which were further decreased by TGFBR2 overexpression. However, only a slight change in BCL-xL levels was observed after 5-FU or IFN-α/5-FU treatment or with TGFBR2 expression. In HuH7 cells, 5-FU and IFN-α/5-FU induced BAX expression, which was further induced by exogenous TGFBR2 expression, whereas BCL-2 and BCL-xL were only slightly affected. Additionally, it was reported that TGF-β also induces apoptosis by...
activating the c-jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signaling pathway in a Smad-independent manner [25–29]. Therefore, we examined their phosphorylation by western blot analysis. 5-FU and IFN-α/5-FU treatment increased the phosphorylation of JNK, but not of p38, in HepG2 and HuH7 cells (Figure S4). Although, TGFBR2 overexpression increased phosphorylation of JNK in HepG2 cells in the absence of 5-FU or IFN-α/5-FU, the cell viability was not altered as indicated in Figure 2B. Additionally, TGFBR2 overexpression did not alter the phosphorylation of JNK and p38 in the presence of 5-FU or IFN-α/5-FU, indicating that the MAPK signaling pathway is unlikely involved in the enhancing mechanisms of TGFBR2 overexpression (Figure S4). Taken together, these results indicate that TGFBR2 overexpression activates the Smad-dependent TGF-β signaling pathway and modulates the expression of apoptosis-related genes, including BAX, BCL-2, and BCL-xl.

**Induction of Endoplasmic Reticulum (ER) Stress by EXT1**

EXT1 overexpression enhanced IFN-α/5-FU-induced apoptosis; however, the relationship between EXT1 and apoptosis remains unclear. EXT1 encodes an ER-resident type II transmembrane glycosyltransferase that is involved in the chain elongation step of heparan sulfate biosynthesis [29]. Because the link between ER stress and apoptosis has been established [30,31], and because EXT1 is predominately localized in the ER [29], we examined whether EXT1 induces ER stress. In HepG2 cells, EXT1 overexpression in the presence of 5-FU and IFN-α/5-FU induced significant upregulation of BiP/GRP78 mRNA (Figure 3A). Similarly, a significant elevation of CHOP, a hallmark of ER stress-induced apoptosis [30,31], was also observed (Figure 3B). ER stress triggers ATF4 translation [30]. As shown in Figure 3C, along with BiP/GRP78 and CHOP expression, EXT1 further enhanced 5-FU-induced ATF4 expression. Meanwhile, in HuH7 cells, these ER stress markers were undetectable by 5-FU, IFN-α/5-FU, or EXT1 overexpression (Figure 3C). Furthermore, ER stress is closely linked to autophagy, which is a physiological response similar to apoptosis [32]. As shown in Figure 5C, the conversion of LC3B-I to LC3B-II (markers of autophagy) was significantly increased by 5-FU and IFN-α/5-FU treatment and particularly enhanced by EXT1 overexpression in HepG2 and HuH7 cells. Similar findings were observed by immunofluorescence analysis with an LC3B antibody (Figure 5D).

To investigate whether the enhancing effect of EXT1 on IFN-α/5-FU treatment is involved in ER stress. To this end, we examined the effect of taurosodesoxycholate (TUDCA), which is a chemical chaperone that ameliorates ER stress [3–7], on the viability of the cells treated with 5-FU alone or IFN-α/5-FU [33–35]. As shown in Figure 5E, TUDCA treatment recovered the cell viability of EXT1-overexpressing HepG2 cells in the presence of 5-FU alone or IFN-α/5-FU, whereas it was not observed in LacZ-overexpressing HepG2 cells, indicating that increased ER stress is involved in the mechanisms by which EXT1 enhances the cytotoxic effect of 5-FU and IFN-α/5-FU. These data suggest that EXT1 overexpression enhanced the effects of IFN-α/5-FU through ER stress-induced autophagy and apoptosis.

**Association of PRKAG2 and TGFBR2 Expression with the Clinical Outcomes of IFN-α/5-FU Therapy in Advanced HCC Patients**

To gain further insight into the clinical significance of these genes, we examined the relationship between their mRNA expression levels in tumor tissues and clinical outcomes in 17 advanced HCC patients treated with IFN-α/5-FU therapy. The clinical parameters of the patients are summarized in Table 1. Responders to IFN-α/5-FU therapy survived longer than non-responders (P<0.05, Figure 6A), suggesting that patients sensitive to IFN-α/5-FU therapy can achieve longer overall survival. PRKAG2 expression tended to be higher in responders than in non-responders (Figure S5A) and was positively correlated with survival period (P<0.05, Figure 6B), indicating that PRKAG2 expression can serve as a prognostic marker for IFN-α/5-FU therapy. In sharp contrast to PRKAG2, TGFBR2 expression was significantly lower in responders than that in non-responders (Figure S5B) and negatively correlated with the survival period (P<0.05, Figure 6C). EXT1 expression showed no difference between responders and non-responders with no correlation between EXT1 expression and survival period (Figure S5C and S5D). The association between immunohistochemical analyses and survival in four representative cases are described as follows. A 51-year-old female patient (no. 07642160) with high PRKAG2 expression in tumor tissue survived for 1.519 years (Figure 6D, left panel), whereas a 32-year-old male patient (no.02585881) with low PRKAG2 expression survived for 0.331 years (Figure 6D, right panel). A 53-year-old male patient (no.08205481) with high TGFBR2 expression survived for 0.508 years (Figure 6E, left panel), whereas a 55-year-old male patient (no.08192686) with low TGFBR2 expression survived for 3.714 years (Figure 6E, right panel).

Additionally, univariate analysis revealed that factors associated with the survival of patients treated with IFN-α/5-FU therapy included hepatitis C virus (HCV) infection and PRKAG2 mRNA levels (Table S3), although the number of patients was small. HCC patients positive for anti-HCV antibodies survived longer than those testing negative for these antibodies (P<0.05, Figure S3E), as previously reported [36]. HCV-positive patients had lower TGFBR2 levels compared with HCC-negative patients (P<0.05, Figure S3F).

**Discussion**

Based on our hypothesis, we successfully identified genes that enhanced 5-FU- and IFN-α/5-FU-induced cytotoxicity of HCC cells when overexpressed in vitro. The identified genes, especially PRKAG2 and TGFBR2, can serve as prognostic markers for IFN-α/5-FU therapy. PRKAG2 encodes the gamma 2 non-catalytic but regulatory subunit of AMP-activated protein kinase (AMPK), an important cellular homeostasis sensor. Activated AMPK reserves cellular energy content and serves as the key determinant of cell survival in response to pathological energetic, oxidative, and ER stress [37]. Indeed, reports documenting that AMPK significantly enhances 5-FU antitumor effects via COX-2 suppression and inhibits the mTOR signaling pathway that regulates tumor growth may support our observations [38,39]. We found that siRNA-mediated knockdown of PRKAG2 strongly inhibited chemosensitivity to 5-FU; however, the effects of adenovirus-mediated overexpression of PRKAG2 on 5-FU were small compared with those of adenovirus-mediated overexpression of TGFBR2 or EXT1. These results can be explained as follows. PRKAG2 overexpression without the other subunits may be stoichiometrically insufficient to activate the AMPK complex, while its knockdown may be sufficient to suppress its kinase activity. In the present study, PRKAG2 expression was positively correlated with survival period in HCC patients treated with IFN-α/5-FU. Indeed, phosphorylated AMPK expression has been associated with survival period and disease-free survival in HCC and lung cancer patients [40,41]. Taken together, AMPK activation may inhibit tumorigenesis.
TGFBR2 is a type II TGF-β receptor that, upon binding to its ligand, triggers various responses, including proliferation, differentiation, and apoptosis [42]. In the present study, TGFB1 mRNA was not increased by the infection with Ad-TGFBR2. This result could be explained as follows: Because TGF-β is a pleiotropic cytokine, the drug-induced TGFB1 expression may provide an advantage for cancer cell survival, possibly in an endocrine fashion in vivo, such as immunosuppression and angiogenesis [43,44]. Therefore, our observation suggests that TGFBR2 expression level may be a critical factor to determine the fate of the role of TGF-β in cancer cells. However, the discrepancy between the increased chemosensitization to IFN-α/5-FU in vitro by TGFBR2 and the negative correlation of TGFBR2 with survival period in patients treated with IFN-α/5-FU remains unclear. The TGF-β signaling pathway reportedly exhibits paradoxical roles of tumor suppression and oncogenesis. It is well known that TGF-β signaling is a potent suppressor of HCC cells [45]. A previous report [46] showed that TGF-β enhanced the lethal effects of 5-FU in human lung cancer cells; this supports our in vitro data that IFN-α/5-FU-induced apoptosis was enhanced by TGFBR2 expression. On the contrary, altered TGF-β signaling reportedly plays an important role in HCC progression [47,48]. Indeed, the TGF-β signaling pathway promotes hepatocarcinogenesis in experimental p53-depleted mice [49]. Furthermore, epithelial-mesenchymal transition (EMT), the underlying molecular mechanisms of which include the TGF-β pathway, is increasingly being recognized to occur during HCC progression [50]. These reports and our findings suggest that TGF-β signaling may be associated with tumor progression or development in patients with advanced HCC, rather than enhancing the antitumor effect of IFN-α/5-FU. Additionally, downregulation of TGFBR2 mRNA has been reported in HepG2 cells transfected with an HCV clone [51], suggesting that a potential benefit of HCV infection may exist. In consistent with this notion, a significantly prolonged survival period during IFN-α/5-FU therapy was observed in patients infected with HCV. Further evaluations are required to determine these associations between HCV infection, TGFBR2 expression, and EMF.

EXT1 expression was reported to be epigenetically silenced in tumors, while the restoration of EXT1 expression in cancer cells induced tumor-suppressive effects [52]. However, to our knowledge, little is known about the relationship between EXT1 expression and HCC. In our present study, EXT1 overexpression induced ER stress in HepG2 cells in the presence of 5-FU and IFN-α/5-FU. Induction of ER stress by EXT1 overexpression through the reduction of heparin sulfate N-sulfation has been reported [53]. As per these observations, EXT1 may sensitize HCC cells to 5-FU through ER stress, which is induced by alternating heparin sulfate posttranslational modification.

In conclusion, we identified PRKAG2, TGFBR2, and EXT1 as chemosensitizing genes of HCC cells to 5-FU. Furthermore, TGFBR2 and EXT1 overexpression enhanced the anti-tumor effects of IFN-α/5-FU on HCC cells. These genes are promising candidates to enhance the therapeutic effects of IFN-α/5-FU.

Supporting Information

Figure S1 Scheme for construction of ribozyme library. A plasmid DNA (pDNA) library expressing random ribozyme genes with as large as about 6×10^6 target recognition sequences from synthesized oligonucleotides (Rz1–Rz6; the sequences were listed in Table S1) using PCR and the Gateway technologies.

(TIF)

Figure S2 Detection of adenovirus-mediated gene transfer. These protein expressions after adenovirus-mediated gene transfer were examined in HepG2 (A) and HuH7 (B) cells. After adenovirus-mediated overexpression of each gene, protein samples were prepared from cells at 0, 24, 48, 72, and 96 h, and were then subjected to western blotting using the indicated antibodies (left). Actin was used as an internal control. (C–H) Flow cytometry analysis of mCherry fluorescent protein, which was used as marker protein. Negative control cells (C) and adenovirus-infected cells, which were respectively infected with adenovirus carrying LacZ (D), PRKAG2 (E), TGFBR2 (F), and EXT1 (G), were used to analyze the ratio of mCherry positive cells. Gate was created by analysis of forward scatter (FS) and side scatter (SS) (each left panel). Percent histogram was determined by analysis of mCherry fluorescence intensity (each right panel). (H) Overlaid histogram shown in Figure S2C–S2H.

(TIF)

Figure S3 Increment of 5-FU- and IFN-α/5-FU-induced nuclear fragmentation by TGFBR2 and EXT1. Nuclear fragmentation shown in Figure 3A was counted and was normalized to total cell number.

(TIF)

Figure S4 Effect of TGFBR2 on Smad-independent pathway. JNK, p38 MAPK, and their phosphorylation were examined in HepG2 and HuH7 cells. After overexpression of each gene, cells were treated with indicated concentration of 5-FU and IFN-α for 48 h. Total JNK and p38 was used as an internal control.

(TIF)

Figure S5 Correlation between gene expressions and survival period of HCC patients with or without HCV antibody. (A–C) Gene expression levels in responders and non-responders to IFN-α/5-FU therapy. PRKAG2 (A), TGFBR2 (B), and EXT1 (C) expression levels in clinical HCC patients. mRNA

| Table 1. Clinical parameters of HCC patients. |
|---------------------------------------------|
| No of patients                              | 17 |
| Age (years)                                 | 56±12 |
| Gender                                      | Male/Female 16/1 |
| Child-pugh                                  | A/B/C 11/6/0 |
| Etiology                                    | HBV 9 |
|                                           | HCV 3 |
|                                           | HBV/MCV 4 |
|                                           | NonB/NonC 1 |
| HCV antibody                                | Negative/Positive 10/7 |
| Tumor size (cm)                             | 10.09±5.10 |
| PVTT                                        | Vp3/Vp4 4/13 |
| Survival period (year)                      | 1.73 (0.1–6.13) |
| Response (WHO)                              | CR/PR/NC/PD 3/3/1/0 |

Parenthetical values in survival period showed minimum and maximum survival days.

doi:10.1371/journal.pone.0056197.t001

IFN-α/5-FU-Sensitizing Genes in HCC Cells
expression levels were normalized to β-actin. Statistical significance was determined by the Mann-Whitney U test. *P<0.05. (D) Correlation of EXT1 expression levels with the survival periods. Data were analyzed by the Spearman’s rank correlation method. (E) Survival rates of the HCV-positive and HCV-negative patients treated with IFN-α/5-FU therapy. Data were analyzed by the Spearman’s rank correlation test. (F) TGFB2 mRNA expression levels in HCV-positive and HCV-negative patients. Statistical significance was determined by the Mann-Whitney U test. *P<0.05.

Table S1 Primers used in the experiments. All used in this study were obtained in purified form. Rz1 - Rz6 were used for the construction of random ribozyme library as described in Figure S1. N indicates any nucleotide (A, G, C and T). PRKAG2 BandII - atiiB2 reverse were used for the construction of adenovirus plasmid DNA carrying PRKAG2, TGFB2 and EXT1.

Table S2 Results of BLAST search of original ribozyme library and plasmid DNAs recovered after ten cycles of screening. The ribozyme target recognition sequences recovered from one hundred colonies of E. coli transformed by original ribozyme library, and plasmid DNAs recovered after ten cycles of screening were analyzed by using the BLAST. The numbers of colonies, whose sequences target the same gene, were shown.

References
1. Umemura T, Ichijo T, Yoshizawa K, Tanaka E, Kiyosawa K (2009) Epidemiology of hepatocellular carcinoma in Japan. J Gastroenterol 44: 102–107.
2. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, et al. (2008) Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 359: 378–390.
3. Cheung AL, Kang YK, Chen Z, Tsao CJ, Qin S, et al. (2009) Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol 10: 25–34.
4. Yau T, Chang P, Ng KK, Chow TK, Lau GW, et al. (2009) Phase 2 open-label study of single-agent sorafenib in treating advanced hepatocellular carcinoma in a hepatitis B–endemic Asian population: presence of lung metastasis predicts poor response. Cancer 115: 429–436.
5. Patt YZ, Hassan MM, Moczydlowski RK, Brown TD, Vauthey JN, et al. (2003) Phase II trial of systemic continuous fluorouracil and subcutaneous recombinant interferon Alfa-2b for treatment of hepatocellular carcinoma. J Clin Oncol 21: 421–427.
6. Ota H, Yoshida H, Toune R, Unuma T, Kanda M, et al. (2006) Combination therapy of intraarterial 5-fluorouracil and systemic interferon-alpha with portal venous invasion. Cancer 106: 1990–1997.
7. Ota H, Nagano H, Sakon M, Eguchi H, Kondo M, et al. (2003) Treatment of hepatocellular carcinoma with major portal vein thrombosis by combined therapy with subcutaneous interferon-alpha and intra-arterial 5-fluorouracil: role of type I interferon receptor expression. Br J Cancer 93: 557–564.
8. Sakon M, Nagano H, Dono K, Nakamori S, Unemizu K, et al. (2002) Combined intraarterial 5-fluorouracil and subcutaneous interferon-alpha therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches. Cancer 94: 435–442.
9. Daidjnsuren B, Nagano H, Hada W, Noda T, Nataj J, et al. (2007) Interferon alpha receptors are important for antiproliferative effect of interferon-alpha against human hepatocellular carcinoma cells. Hepatol Res 37: 77–83.
10. Noda T, Nagano H, Takemasa I, Yoshioka S, Murakami M, et al. (2009) Activation of Wnt/beta-catenin signalling pathway induces chemoresistance to interferon-alpha/5-fluorouracil combination therapy for hepatocellular carcino-
ma. Br J Cancer 100: 1647–1658.
11. Hagisawa S, Kudo M, Ushima K, Chug H, Yamaguchi M, et al. (2011) The cancer stem cell marker CD133 is a predictor of the effectiveness of S+ pregated interferon-92b therapy against advanced hepatocellular carcinoma. J Gastroenterol 46: 212–221.
12. Beger C, Pierce LN, Kruger M, Marcussen EG, Robbins JM, et al. (2001) Identification of ITG6 as a regulator of BCR/CA1 expression by using a ribozyme- library-based inverse gradient approaches. Proc Natl Acad Sci U S A 98: 130–
135.
13. Minasyan RT, Meregian TC, Carr A, Zack JA, Winters MA, et al. (2009) Phase 2 gene therapy trial of an anti-HIV ribozyme in autologous CD34+ cells. Nat Med 15: 203–202.
14. Wada H, Nagano H, Yamamoto H, Arai I, Ota H, et al. (2007) Combination therapy of interferon-alpha and 5-fluorouracil inhibits tumor angiogenesis in human hepatocellular carcinoma cells by regulating vascular endothelial growth factor and angiopoietins. Oncol Rep 16: 801–809.
15. Ishii K, Yoshida Y, Akechi Y, Sakabe T, Nishio R, et al. (2008) Hepatic differentiation of human bone marrow-derived mesenchymal stem cells by tetracycline-regulated hepatocyte nuclear factor 3beta. Hepatology 48: 597–606.
16. World Health Organization (1979) Handbook for reporting results of cancer treatment. Geneva: World Health Organization.
17. Miller AR, Hoogstraten B, Staquet M, Winkler A (1981) Reporting results of cancer treatment. Cancer 47: 207–214.
18. Kondo M, Nagano H, Hada W, Daidjnsuren B, Yamamoto H, et al. (2005) Combination of IFN-alpha and 5-fluorouracil induces apoptosis through IFN-alpha/beta receptor in human hepatocellular carcinoma cells. Clin Cancer Res 11: 1277–1286.
19. Eguchi H, Nagano H, Yamamoto H, Miyamoto A, Kondo M, et al. (2000) Augmentation of antitumor activity of 5-fluorouracil by interferon alpha is associated with up-regulation of p27Kip1 in human hepatocellular carcinoma cells. Clin Cancer Res 6: 2891–2896.
20. Nakamura M, Nagano H, Sakon M, Yamamoto T, Ota H, et al. (2007) Role of the Fas/FasL pathway in combination therapy with interferon-alpha and fluorouracil against hepatocellular carcinoma in vitro. J Hepatol 46: 77–82.
21. Yin H, Xie F, Zhang J, Yang Y, Deng B, et al. (2011) Combination of interferon-α and 5-fluorouracil induces apoptosis through mitochrondial pathway in hepatocellular carcinoma in vitro. Cancer Lett 306: 34–42.
22. Teramoto T, Kiso A, Thorogrinson SS (1998) Induction of p23 and Bax during TGF-beta1 induced apoptosis in rat liver epithelial cells. Biochem Biophys Res Commun 251: 56–60.
23. Biermann CL, Willy C, Buchmann A, Schmirich A, Schwarz M (2001) Transforming growth factor-beta-induced Smad signaling, cell-cycle arrest and apoptosis in hepatoma cells. Carcinogenesis 22: 447–452.
24. Tobin SW, Brown MK, Dowell K, Joyce DC, Eastman A, et al. (2001) Inhibition of transforming growth factor beta signaling in MCF-7 cells results in resistance to tumor necrosis factor alpha: a role for Bcl-2. Cell Growth Differ 12: 109–117.
25. Perlman R, Schieman WP, Brooks MW, Lodish HF, Weinberg RA (2001) TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. Nat Cell Biol 3: 704–714.
26. Liao JH, Chen JS, Chiu MQ, Zhao S, Song JG (2001) The involvement of p38 MAPK in transforming growth factor beta-induced apoptosis in murine hepatocytes. Cell Res 11: 89–94.
27. Yu L, Hebert MC, Zhang YE (2002) TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J 21: 3749–3759.
28. Zhu B, Zhai J, Zhu H, Kyprianou N (2010) Prohibitin regulates TGF-beta induced apoptosis as a downstream effector of Smad-dependent and - independent signaling. Prostate 2010 70: 17–26.
29. McQuinn D, Leduc G, McQuinn L, Matison K, Esford LE, et al. (1998) The putative tumour suppressor EXT1 alters the expression of cell-surface heparan sulfate. Nat Genet 19: 158–161.
30. Watanabe Y, Yotsuha Y, Sakabe T, Matsuoka S, Akechi Y, et al. (2008) CD45 induces apoptosis in ovarian adenocarcinoma cells via ER stress signaling. Biochem Biophys Res Commun 366: 840–847.
31. Oyadomari S, Mori M (2004) Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death Differ 11: 301–309.
32. Verfaille T, Salazar M, Velasco G, Agostinis P (2010) Linking ER stress to autophagy: Potential implications for cancer therapy. Int J Cell Biol 2010: 1–19.
33. Xie Q, Khaoustov VI, Chung CC, Sohn J, Krishman B, et al. (2002) Effect of tauroursodeoxycholic acid on endoplasmic reticulum stress-induced caspase-12 activation. Hepatology 36: 592–601.
34. Malo A, Krüger B, Seyhun E, Schäfer C, Hoffmann RT, et al. (2010) Tauroursodeoxycholic acid reduces endoplasmic reticulum stress, trypsin activation, and acinar cell apoptosis while increasing secretion in rat pancreatic acini. Am J Physiol Gastrointest Liver Physiol 2010 299: G877–896.
35. Lee YY, Hong SH, Lee YJ, Chung SS, Jung HS, et al. (2010) Tauroursodeoxycholic acid reduces endoplasmic reticulum stress, trypsin activation, and acinar cell apoptosis while increasing secretion in rat pancreatic acini. Am J Physiol Gastrointest Liver Physiol 2010 299: G877–896.
36. Lee YY, Hong SH, Lee YJ, Chung SS, Jung HS, et al. (2010) Tauroursodeoxycholic acid reduces endoplasmic reticulum stress, trypsin activation, and acinar cell apoptosis while increasing secretion in rat pancreatic acini. Am J Physiol Gastrointest Liver Physiol 2010 299: G877–896.
37. Lee YY, Hong SH, Lee YJ, Chung SS, Jung HS, et al. (2010) Tauroursodeoxycholic acid reduces endoplasmic reticulum stress, trypsin activation, and acinar cell apoptosis while increasing secretion in rat pancreatic acini. Am J Physiol Gastrointest Liver Physiol 2010 299: G877–896.
38. Lee YY, Hong SH, Lee YJ, Chung SS, Jung HS, et al. (2010) Tauroursodeoxycholic acid reduces endoplasmic reticulum stress, trypsin activation, and acinar cell apoptosis while increasing secretion in rat pancreatic acini. Am J Physiol Gastrointest Liver Physiol 2010 299: G877–896.
39. Lee YY, Hong SH, Lee YJ, Chung SS, Jung HS, et al. (2010) Tauroursodeoxycholic acid reduces endoplasmic reticulum stress, trypsin activation, and acinar cell apoptosis while increasing secretion in rat pancreatic acini. Am J Physiol Gastrointest Liver Physiol 2010 299: G877–896.
31. Li H, Han Y, Guo Q, Zhang M, Cao X (2009) Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. J Immunol 182: 240–249.
32. Balzarini P, Benetti A, Invernici G, Cristini S, Zicari S, et al. (2012) Transforming growth factor-beta1 induces microvascular abnormalities through a down-modulation of neural cell adhesion molecule in human hepatocellular carcinoma. Lab Invest 92: 1297–1309.
33. Sun CK, Chua MS, He J, So SK (2011) Suppression of glypican 3 inhibits growth of hepatocellular carcinoma cells through up-regulation of TGF-beta. Neoplasia 13: 735–747.
34. Raynal S, Noventini S, Croisy A, Lawerence DA, Jullien P (1997) Transforming growth factor-beta1 enhances the lethal effects of DNA-damaging agents in a human lung-cancer cell line. Int J Cancer 72: 396–396.
35. Musch A, Rabie C, Paik MD, Berna MJ, Schmitz V, et al. (2005) Altered expression of TGF-beta receptors in hepatocellular carcinoma—effects of a constitutively active TGF-beta type I receptor mutant. Digestion 71: 78–91.
36. Mamiya T, Yamazaki K, Masugi Y, Moet T, Eifendi K, et al. (2010) Reduced transforming growth factor-beta receptor II expression in hepatocellular carcinoma correlates with intrahepatic metastasis. Lab Invest 90: 1339–1145.
37. Morris SM, Bark JY, Konzark A, Kangnum S, Kroblaugh SE, et al. (2012) Transforming growth factor-beta signaling promotes hepatocarcinogenesis induced by p53 loss. Hepatology 55: 121–131.
38. Presto J, Thuveson M, Carlsson P, Basse M, Wilén M, et al. (2000) Heparan sulfate biosynthesis enzymes EXT1 and EXT2 affect NDST1 expression and heparan sulfate sulfation. Proc Natl Acad Sci U S A 105: 4731–4736.
31. Li H, Han Y, Guo Q, Zhang M, Cao X (2009) Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. J Immunol 182: 240–249.
32. Balzarini P, Benetti A, Invernici G, Cristini S, Zicari S, et al. (2012) Transforming growth factor-beta1 induces microvascular abnormalities through a down-modulation of neural cell adhesion molecule in human hepatocellular carcinoma. Lab Invest 92: 1297–1309.
33. Sun CK, Chua MS, He J, So SK (2011) Suppression of glypican 3 inhibits growth of hepatocellular carcinoma cells through up-regulation of TGF-beta. Neoplasia 13: 735–747.
34. Raynal S, Noventini S, Croisy A, Lawerence DA, Jullien P (1997) Transforming growth factor-beta1 enhances the lethal effects of DNA-damaging agents in a human lung-cancer cell line. Int J Cancer 72: 396–396.
35. Musch A, Rabie C, Paik MD, Berna MJ, Schmitz V, et al. (2005) Altered expression of TGF-beta receptors in hepatocellular carcinoma—effects of a constitutively active TGF-beta type I receptor mutant. Digestion 71: 78–91.
36. Mamiya T, Yamazaki K, Masugi Y, Moet T, Eifendi K, et al. (2010) Reduced transforming growth factor-beta receptor II expression in hepatocellular carcinoma correlates with intrahepatic metastasis. Lab Invest 90: 1339–1145.
37. Morris SM, Bark JY, Konzark A, Kangnum S, Kroblaugh SE, et al. (2012) Transforming growth factor-beta signaling promotes hepatocarcinogenesis induced by p53 loss. Hepatology 55: 121–131.
38. Presto J, Thuveson M, Carlsson P, Basse M, Wilén M, et al. (2000) Heparan sulfate biosynthesis enzymes EXT1 and EXT2 affect NDST1 expression and heparan sulfate sulfation. Proc Natl Acad Sci U S A 105: 4731–4736.