Biochemical and Biophysical Research Communications 422 (2012) 687–692

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Combined anti-tumor effects of IFN-α and sorafenib on hepatocellular carcinoma *in vitro* and *in vivo*

Lijing Wang [a], Dongwei Jia [a], Fangfang Duan [b], Zhichao Sun [a], Xiaojuan Liu [a], Lei Zhou [a], Linlin Sun [a], Shifang Ren [a,*], Yuanyuan Ruan [a,*], Jianxin Gu [a,b]

[a] *Gene Research Center, Shanghai Medical College, Fudan University, Shanghai 200032, PR China*
[b] *Institutes of Biomedical Sciences, Fudan University, Shanghai 200032, PR China*

ARTICLE INFO

Article history:
Received 4 May 2012
Available online 23 May 2012

Keywords:
Hepatocellular carcinoma
Interferon-α
Sorafenib
Combination therapy

ABSTRACT

Hepatocellular carcinoma (HCC) is among the most common and aggressive cancers worldwide, and novel therapeutic strategies are urgently required to improve clinical outcome. Interferon-alpha (IFN-α) and sorafenib are widely used as anti-tumor agents against various malignancies. In this study, we investigated the combined effects of IFN-α and sorafenib against HCC. We demonstrated that the combination therapy synergistically suppressed HCC cellular viability, arrested cell cycle propagation and induced apoptosis in HCC cells. Further research revealed that IFN-α and sorafenib collaboratively regulated the expression levels of cell cycle-related proteins Cyclin A and Cyclin B as well as the pro-survival Bcl-2 family proteins Mcl-1, Bcl-2 and Bcl-X_L. Moreover, sorafenib inhibited IFN-α induced oncogenic signaling of STAT3, AKT and ERK but not the activation of the tumor suppressor STAT1. Xenograft experiments also confirmed the combined effects of IFN-α and sorafenib on tumor growth inhibition and apoptosis induction *in vivo*. In conclusion, these results provide rationale for the clinical application of IFN-α and sorafenib combination therapy in HCC treatment.

1. Introduction

Hepatocellular carcinoma (HCC) is among the most common and lethal cancers, accounting for more than 748,300 new cases and 695,000 cancer deaths worldwide in 2008 [1]. Curative therapies such as surgical resection, liver transplantation and ablative therapies have led to improvement in the survival of patients with HCC [2,3]. Unfortunately, most patients are still diagnosed at advanced stages and could only receive palliative treatments [2,3]. Thus, novel therapeutic strategies are urgently required for patients with advanced HCC.

Interferon-α (IFN-α) belongs to type I interferon family of cytokines originally identified for their antiviral properties, and exerts its biological functions mainly through activation of the JAK–STAT signaling pathway. However, the MAPK and the PI3K–AKT pathways are also activated by IFN-α [4]. Further studies revealed the anti-tumor activity of IFN-α against various tumors via direct inhibitory effects on tumor cells, anti-angiogenesis, enhanced immunogenicity of tumors and immunomodulatory effects [5,6]. IFN-α has been used against malignancies including renal cell carcinoma [7], melanoma [8], chronic myelogenous leukemia [9], and there is increasing interest of the cytokine in the prevention and treatment of HCC. IFN-α has been reported to inhibit cell proliferation and induce apoptosis in HCC cells [10,11]. Though the clinical outcome of IFN-α monotherapy is lack of satisfaction [12], several studies have reported the strong anti-tumor activity and survival benefit of IFN-α-based combination therapy in HCC [13,14].

Sorafenib is an oral multikinase inhibitor that has shown anti-tumor activity against a wide variety of cancers [15]. Sorafenib blocks angiogenesis and tumor cell proliferation through inhibition of several tyrosine kinases (e.g. VEGFR2 and PEGFR) and serine/threonine kinases (e.g. b-Raf) [16,17]. The SHARP trial conducted in patients with advanced HCC treated with sorafenib has shown improvement in survival of about 3 months [18]. In addition to its monotherapy on tumor cells, sorafenib has been widely studied in combination therapy with other drugs such as chemotherapeutic agents for more efficient therapeutic strategies [19,20].

Though IFN-α and/or sorafenib have been widely used as anti-tumor agents against a variety of malignancies, whether IFN-α plus sorafenib treatment has clinical therapeutic potential for HCC patients remains unknown. Our results demonstrated the combined anti-tumor effects of IFN-α and sorafenib against HCC *in vitro* and *in vivo*, thus providing basis for clinical application of IFN-α and sorafenib combination therapy in HCC cases.

* Corresponding authors. Address: Gene Research Center, Shanghai Medical College, Fudan University, P.O. Box 103, Shanghai 200032, PR China. Fax: +86 21 64164489 (S. Ren), +86 21 64437703 (Y. Ruan).
E-mail addresses: renshifang@fudan.edu.cn (S. Ren), yuanyuanruan@fudan.edu.cn (Y. Ruan).

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http://dx.doi.org/10.1016/j.bbrc.2012.05.056

2. Materials and methods

2.1. Reagents and antibodies

Recombinant human interferon-alpha A (HuIFN-α 2a) was obtained from R&D system. Sorafenib (BAY 43-9006) was purchased from AdipoGen. Rabbit anti-phospho-STAT1, -STAT1, -phospho-STAT3 (Tyr705), -STAT3, -phospho-ERK1/2, -phospho-AKT, -AKT (pan), -phospho-Cdk2 (Thr160), and -Cdk2 antibodies, pro-survival Bcl-2 Family Antibody Sampler Kit and Cyclin Antibody Sampler Kit were purchased from Cell Signaling Technology. Rabbit anti-ERK1, and mouse anti-GAPDH, -Ki-67 antibodies were obtained from Santa Cruz Biotechnology. Mouse anti-ACTB was from Proteintech Group.

2.2. Cell culture and western blot assay

HCC cell lines (Huh-7 and Sk-Hep-1) were obtained from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 incubator. Protein extraction from cultured cells and western blot analysis were performed as previously described [21].

2.3. WST assay

Cell viability was determined using WST-8 dye (Beyotime, China) according to manufacturer's instructions. Briefly, cells were treated as indicated and stained with WST-8 dye for 1 h. The absorbance was finally determined at 450 nm using a microplate reader.

2.4. Apoptosis analysis

Cells were treated with IFN-α (4000 U/ml) and/or sorafenib (5 μM) for 48 h, and drug-induced apoptosis was assessed using Annexin V assay kit (BD Biosciences) according to manufacturer's instructions.

2.5. Cell cycle analysis

Briefly, 48 h after treatment, cells were harvested, fixed and incubated with PBS containing 1% RNase. Then cells were stained with PI at 50 mg/ml and analyzed by flow cytometer. The percentages of cells in G0/G1, S or G2/M phase were calculated using Modfit software.

2.6. Tumor xenograft experiments

All animal experiments were performed according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health, and approved by the Ethics Committee of Fudan University. 5-week-old male BALB/c nude mice were obtained from Shanghai Laboratory Animal Center of Chinese Academy Sciences and housed in a specific pathogen-free room. The experiments were carried out as previously described [22]. Briefly, 1×10^7 Huh-7 cells suspended in 100 μl of PBS were injected subcutaneously into the flank of mice ($n = 5$ in each group). 1 week later, IFN-α (7.5×10^6 U/kg) [23] was given by intratumoral injection every 3 days, and sorafenib (5 mg/kg) [17] was given once daily by gavage. Tumor volume was estimated by the formula [length (mm) × width $(mm)^2$]/2.

2.7. Immunohistochemistry

Tumor sections from subcutaneous tumor xenografted nude mice were analyzed as previously reported [22]. The tissue sections were viewed at 400× magnification. Three fields per section were analyzed and Ki-67 positive cells were calculated using Image-Pro Plus software.

2.8. TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed by using *in situ* cell death detection kit, *AP* (Roche Applied Science) according to manufacturer's instructions. Cells were visualized by fluorescence microscopy. Three fields per section were analyzed. TUNEL positive cells were counted and expressed as a percentage of total number of nucleus counted.

2.9. Statistical analysis

Results are presented as means ± SD. Differences between two groups were tested using Student's *t* test. 2-way ANOVA analysis was performed where indicated. Statistical significance was determined at the level of $P < 0.05$.

3. Results

3.1. Combined anti-tumor effects of IFN-α and sorafenib on HCC cell lines

IFN-α and sorafenib are widely used as anti-tumor agents against a variety of malignancies. To investigate the combined anti-tumor effects of IFN-α and sorafenib on HCC cell lines, we first examined the effects of both agents on cell viability. WST assay demonstrated that IFN-α administrated alone showed moderately inhibitory effect, and about 20% inhibition ratio was achieved at a high concentration of 4000 U/ml in both types of HCC cell lines. Administration of sorafenib induced about 40% inhibition ratio of cell viability at a high dose of 10 μM. Moreover, combined treatment of IFN-α and sorafenib prominently suppressed cell viability compared with either agent in dose-dependent manner (Fig. 1A).

To understand the synergistic anti-tumor effects of IFN-α and sorafenib, we next evaluated the combined effects of IFN-α and sorafenib on cell cycle propagation and cellular apoptosis in HCC cells. PI staining analysis by flow cytometry showed an increased accumulation of S phase and a remarkable decrease in G2/M phase in the combination group compared with the single agent group (Fig. 1B). Annexin V staining also revealed that the combined treatment of IFN-α and sorafenib induced significant increase in levels of apoptosis in both types of HCC cell lines (Fig. 1C). Taken together, these data imply the synergistic anti-tumor effects of IFN-α and sorafenib on HCC cells *in vitro*.

3.2. Effects of IFN-α and sorafenib on the expression levels of cell cycle- and apoptosis-related proteins

Since IFN-α and sorafenib cooperatively induced cell cycle arrest at S phase in HCC cells, we next assessed the effects of IFN-α and sorafenib on the expression levels of cell cycle-related proteins. Previous research reported that Cyclin A–Cdk2 complex is required for cells progression from S into G2/M, and Cyclin B functions importantly as G2/M checkpoint regulator [24]. As shown in Fig. 2A, IFN-α plus sorafenib synergistically attenuated the expression of Cyclin A and Cyclin B compared with either agent alone. Moreover, sorafenib, but not IFN-α, remarkably blocked Cdk2 phosphorylation in HCC cells. The combination therapy syn-

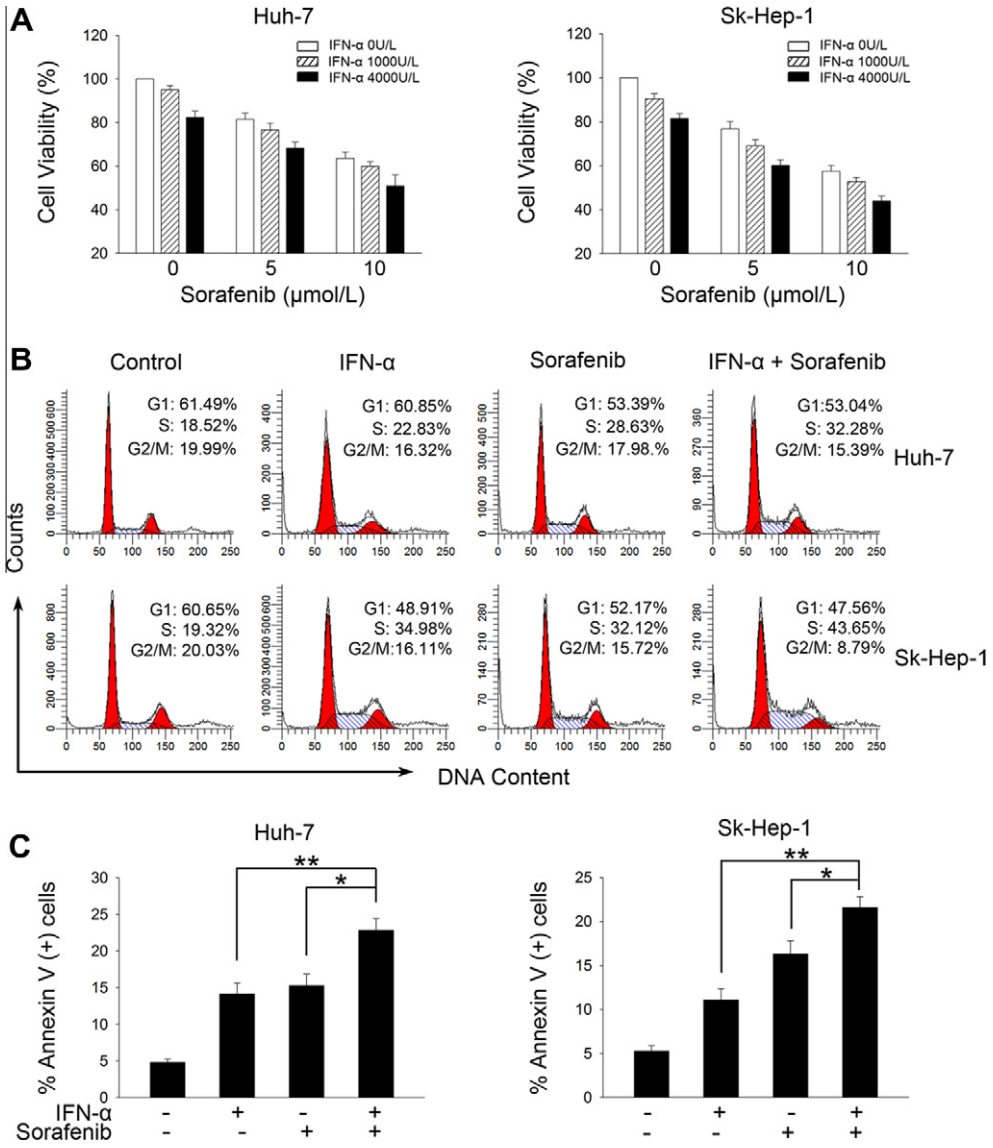

Fig. 1. Combined anti-tumor effects of IFN-α and sorafenib on HCC cells. (A) Cells were incubated with IFN-α and/or sorafenib for 24 h at varying concentrations, and cell viability was analyzed using the WST-8 method. The relative viability of cells in control group was defined as 100%. (B and C) Cells were treated with IFN-α (4000 U/ml) and/or sorafenib (5 μM) for 48 h. In (B), cells were stained with PI and cell cycle distribution was analyzed by flow cytometry. In (C), cells were harvested and double stained with FITC-Annexin V and PI. All assays were performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$.

ergistically down-regulated total Cdk2 expression in Sk-Hep-1 cells, while little change was observed in Huh-7 cells.

We also examined the effects of IFN-α and sorafenib on the expression levels of apoptosis-related proteins. Administration of IFN-α remarkably up-regulated the expression of TRAIL, which has been reported to be involved in IFN-α-induced apoptosis in HCC cells [25]; however, sorafenib showed little effect on basal or IFN-α-induced TRAIL expression. In addition, combined treatment of IFN-α and sorafenib evidently suppressed the expression levels of pro-survival Bcl-2 family proteins, including Mcl-1, Bcl-2 and Bcl-X_L, and the inhibitory effect on Mcl-1 was more prominent than that on Bcl-2 and Bcl-X_L (Fig. 2B). However, the expression of pro-apoptosis Bcl-2 family proteins Bax, Bim and Bad showed no meaningful changes upon IFN-α and/or sorafenib treatment (data not shown).

3.3. Effects of sorafenib on IFN-α-mediated signaling pathways

IFN-α activates multiple distinct signaling cascades including the canonical JAK–STAT pathway as well as the MAPK and the PI3K cascade, while sorafenib is a multikinase inhibitor targeting several tyrosine kinases (VEGFR2 and PEGFR) and serine/threonine kinase (b-Raf), etc. [4,16]. Therefore, we next examined the effects of sorafenib on IFN-α-mediated signaling pathways. As shown in Fig. 3, IFN-α strongly induced phosphorylation of STAT1, STAT3 and AKT, and marginally enhanced ERK phosphorylation in both types of HCC cell lines. However, sorafenib significantly inhibited IFN-α-induced STAT3, AKT and ERK activation, while showed little effect on STAT1 phosphorylation.

3.4. Effects of IFN-α and sorafenib combination therapy on Huh-7 xenografts in nude mice in vivo

To confirm whether the synergistic effects of IFN-α and sorafenib have potentially relevant clinical implications, we next assessed the anti-tumor effects of IFN-α plus sorafenib *in vivo*. As shown in Fig. 4A and B, treatment of IFN-α and sorafenib synergistically suppressed tumor growth and reduced tumor weight *in vivo*. Moreover, cooperation of IFN-α and sorafenib also induced remarkable increasing in cellular apoptosis by TUNEL staining

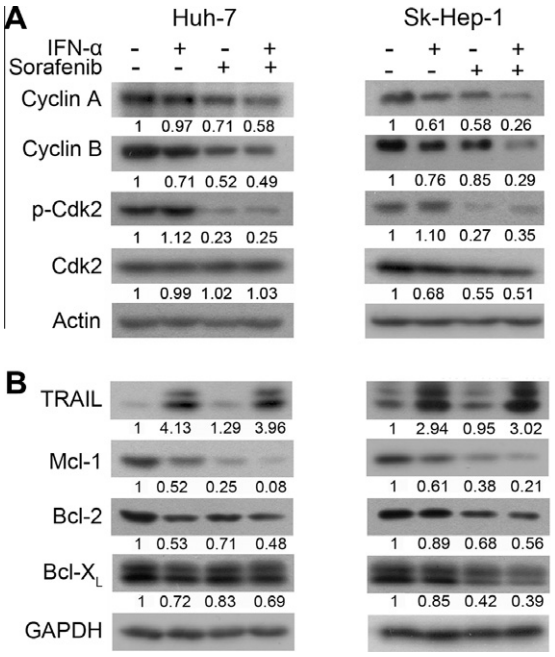

Fig. 2. Combined effects of IFN-α and sorafenib on the expression levels of cell cycle- and apoptosis-related proteins. Cells were incubated with IFN-α (4000 U/ml) and/or sorafenib (5 μM) for 48 h, and whole cell lysates were collected and subjected to western blot analysis. Quantifications were made by comparing with Actin or GAPDH, and relative expression of the control group was normalized to 1.

and reduction of proliferation marker Ki-67 expression (Fig. 4C–E). These results imply that treatment of IFN-α plus sorafenib inhibited tumor growth and induced cellular apoptosis of HCC *in vivo*.

4. Discussion

Hepatocellular Carcinoma (HCC) is a major health problem, being the fifth most common cancer and the third leading cause of cancer-related death worldwide [3]. IFN-α and sorafenib are extensively used against a variety of tumors. Moreover, combination therapies based on sorafenib and/or IFN are widely studied to develop more efficient therapeutic strategies [13,14,19]. In the present study, we demonstrated the synergistic anti-tumor effects of IFN-α and sorafenib against human HCC both *in vitro* and *in vivo*, thus providing basis for clinical application of IFN-α and sorafenib combination therapy in HCC cases.

Our results showed a combined effect of IFN-α and sorafenib on S phase accumulation and G2/M phase reduction in HCC cells (Fig. 1B). It is well established that Cyclin–Cdk complex is pivotal for cell cycle progression, and our results demonstrated that IFN-α and sorafenib collaboratively down-regulated the expression of Cyclin A, an S to G2 phase required protein and Cyclin B, a key regulator of G2/M checkpoint. Previous report indicated that the activation of IFN-responsive transcription factor, STAT1, induced the down-regulation of Cyclin A and Cyclin B, and the consequent inhibition of cell cycle progression [26]. Therefore, it is likely that IFN-α suppressed the expression of Cyclin A and Cyclin B through STAT1 activation. However, sorafenib showed little effect on basal or IFN-α-induced STAT1 phosphorylation (Fig. 3), suggesting that IFN-α and sorafenib may attenuate Cyclin A and Cylin B expression via different mechanisms. Our results also indicated that sorafenib, but not IFN-α, significantly blocked Cdk2 phosphorylation (Fig. 2A). It could possibly be explained by our previous report that RAF/MEK/ERK signaling acts upstream of and regulates the phosphorylation of Cdk2 [27], thus sorafenib may suppress Cdk2 phosphorylation through inactivating ERK. Moreover, total Cdk2 expression responded distinctly to IFN-α and/or sorafenib treatment in Huh-7 and Sk-Hep-1 cells (Fig. 2A), implying that the regulatory mechanism of IFN-α and/or sorafenib in cell cycle progression may vary in different HCC cell lines as well.

Our results also demonstrated that IFN-α and sorafenib synergistically induced apoptosis in HCC cells (Fig. 1C). Previous research reported that the increased expression of TRAIL and the consequent activation of the extrinsic pathway were involved in IFN-α-induced apoptosis of HCC cells [25]. Though sorafenib showed no effect on basal or IFN-α-induced expression of TRAIL (Fig. 2B), it has been reported that sorafenib sensitized tumor cells to TRAIL-induced apoptosis via inhibiting Mcl-1 expression [28,29]. Therefore, it is likely that down-regulation of Mcl-1 by sorafenib may also enhance IFN-induced extrinsic apoptosis. The mechanism how sorafenib induces apoptosis is not fully

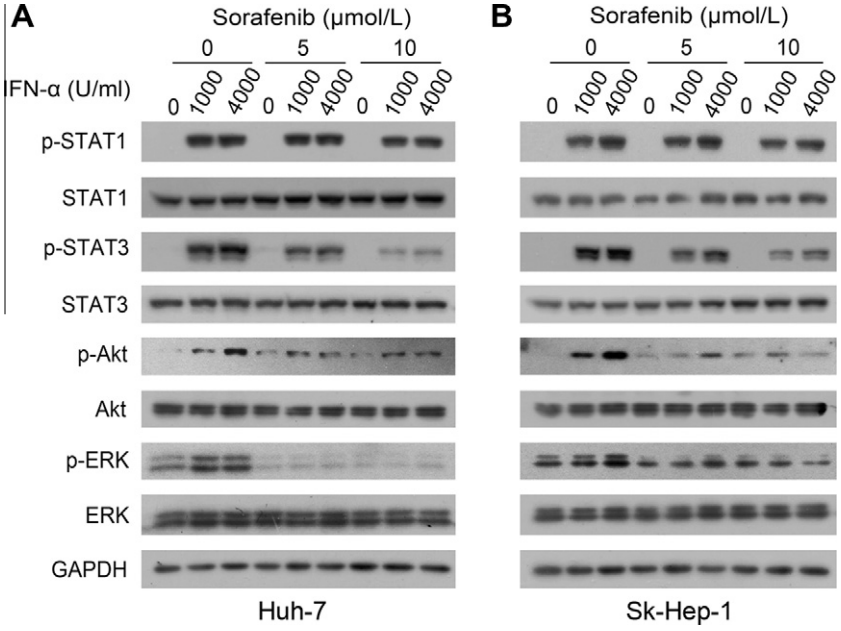

Fig. 3. Effects of sorafenib on IFN-α-induced signaling in HCC. Huh-7 (A) and Sk-Hep-1(B) were serum starved for 24 h, and incubated with sorafenib and IFN-α at varying concentrations for 1 h. Whole cell lysates were prepared and applied to western blot analysis.

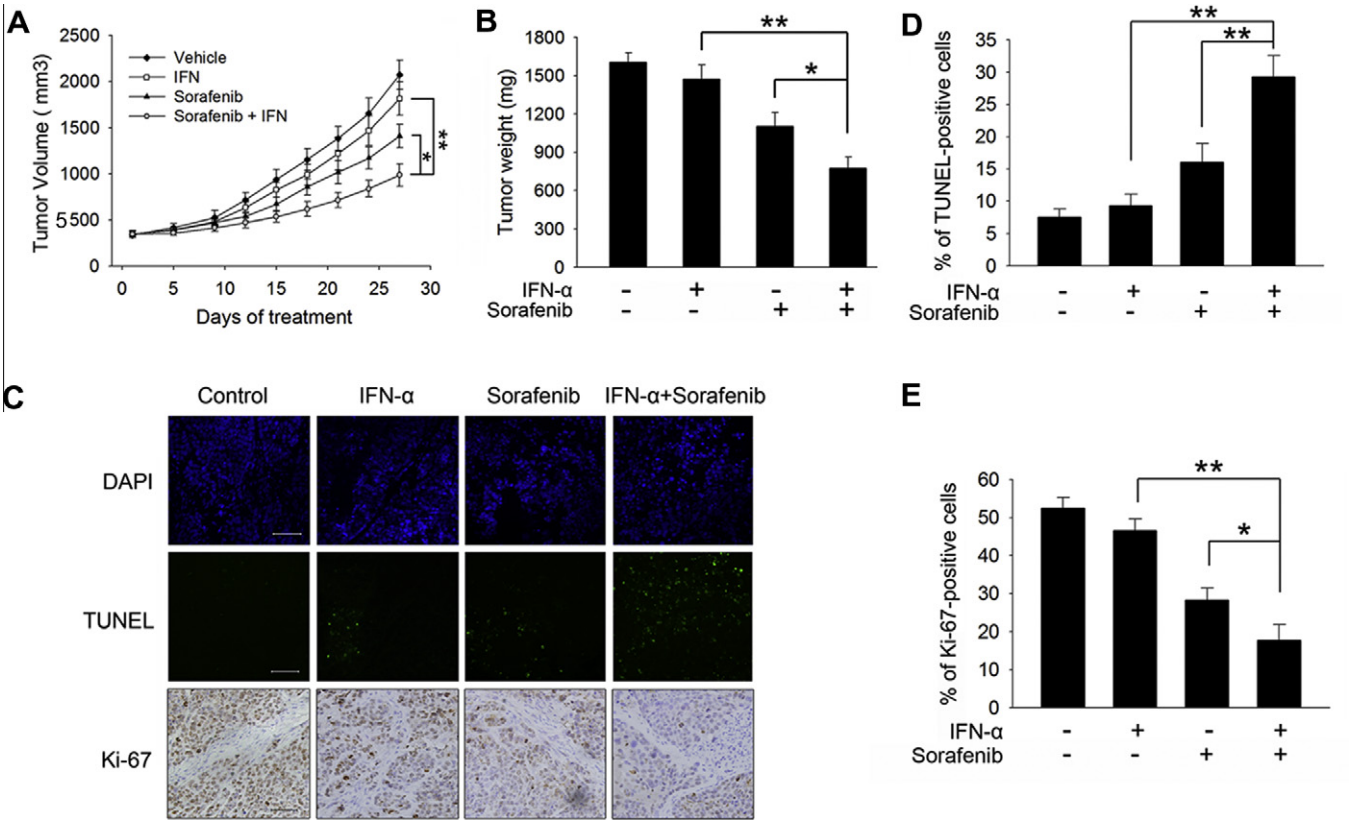

Fig. 4. Combined anti-tumor effects of IFN-α and sorafenib on HCC *in vivo*. Xenografts were generated as described in Section 2. (A) Tumor volume was measured at indicated time points, and the statistical significance was analyzed by two-way ANOVA. (B) Tumor weight was measured on the day of harvest, after excision of the tumor from the euthanized mouse. (C–E), TUNEL assay was performed to detect cellular apoptosis, and immunohistochemistry staining was performed to detect Ki-67 expression; Scale bar, 50 μm; $^{*}P < 0.05$, $^{**}P < 0.01$.

elucidated. A previous report indicated that sorafenib regulated mitochondria-related proteins and increased the release of cytochrome c into the cytosol [30], implying that the intrinsic pathway is involved in sorafenib-induced apoptosis. Interestingly, IFN-α cooperated with sorafenib to attenuate pro-suvival Bcl-2 family proteins (Fig. 2B), which play a critical role in the regulation of the intrinsic mitochondrial pathway. Therefore, we speculate that the activation of intrinsic and extrinsic pathways are both involved in the regulation of cellular apoptosis mediated by IFN-α and sorafenib combination therapy.

Although type I interferons have been used with varying effectiveness in the treatment of malignancies, several studies reported cases of IFN resistance in treating HCC [31]. Though the underlying mechanism is little defined, one possible explanation is that treatment of IFN-α induced the phosphorylation of STAT3, AKT and ERK, which have been reported to be constitutively activated in and recognized as poor prognostic factors of HCC [32–34]. Intriguingly, sorafenib treatment significantly inhibited IFN-α-induced STAT3, AKT and ERK phosphorylation, which was consistent with previous reports that sorafenib is a potent suppressor of STAT3, AKT and ERK signaling pathways [17,35]. However, additional administration of sorafenib showed little effect on IFN-α-induced STAT1 activation, suggesting the specific regulation of STAT3 phosphorylation by sorafenib. Previous research reported that sorafenib inhibits STAT3 phosphorylation via kinase-independent mechanism but SHP-1-dependent inactivation [36]. And this is also supported by our data that phosphorylation of JAK1 and JAK2 were unblocked by sorafenib (data not shown). Nevertheless, how sorafenib specifically regulates the activation of STAT3 remains to be further investigated.

Currently, there is no standard treatment for unresectable HCC. IFN-α has been reported to inhibit cell proliferation and induces apoptosis in HCC cells and tumor models. However, clinical trial results revealed minimal effects of IFN-α treatment on HCC patients. On the other side, sorafenib, which has shown about 3 months survival benefit, has been proved for FDA and EMEA for HCC treatment, and combination therapies based on sorafenib have been widely studied to develop more efficient therapeutic strategies. In this study, we demonstrated that IFN-α and sorafenib exhibited combined anti-tumor activity against HCC both *in vivo* and *in vitro*, which holds promise for clinical application of IFN-α and sorafenib combination therapy in patients with advanced HCC.

5. Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by grants from the National Basic Research Program of China 973 Program (2012CB8221004, 2010CB912104), the State Key Project Specialized for Infectious Diseases of China (2012ZX10002-008, 2012ZX10002-012), and National Natural Science Fund (31100586, 31100977, 31170766, 31010103906, 3100060, 31000348, 30900266, 30930025).

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