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Original article

Synergy with interferon-lambda 3 and sorafenib suppresses hepatocellular carcinoma proliferation

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ABSTRACT

Hepatocellular carcinoma (HCC) is a common and fatal malignancy of the liver. Sorafenib is a small molecule multikinase inhibitor that acts against different cancer cell lines and is used for the treatment of HCC. However, some advanced HCC patients fail to respond to sorafenib, and those who do lack a meaningful clinical benefit. Interferon-lambda 3 (IFN-λ3) is a type III interferon with antiviral, antiproliferative, and immunomodulatory functions. Here, we evaluated the use of IFN-λ3 as an adjuvant treatment with sorafenib in HCC. In the present study, CCK-8 and colony formation assay results showed that treatment with a combination of IFN-λ3 and sorafenib suppresses the viability of HepG2 and SMMC7721 liver cancer cell lines more than treatment with either alone. In addition, flow cytometry results confirmed that treatment with a combination of IFN-λ3 and sorafenib promotes the loss of mitochondrial membrane potential and induces the production of ROS more than treatment with either alone. Furthermore, using a subcutaneous SMMC7721 tumor model, treatment with a combination of IFN-λ3 and sorafenib significantly reduced the tumor growth/volume and induced apoptosis compared to treatment with sorafenib alone. These results show that combined treatment with IFN-λ3 and sorafenib facilitates a synergistic effect on suppressing HCC cancer growth and promoting cell apoptosis in *vitro* and in *vivo*. Thus, IFN-λ3 in combination with sorafenib might prove to be a useful adjunctive strategy for the clinical treatment of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is a common and fatal malignant cancer of the liver, and is the third highest cause of cancer-related deaths in the world [1]. More than 600,000 new cases of HCC are diagnosed each year, and approximately 80% of patients with HCC are infected with chronic hepatitis B virus (HBV) and/or hepatitis C virus (HCV) [2,3]. Although a variety of therapies and drugs have been used for the treatment of HCC, the 5-year survival rate of patients with HCC remains below 12% [4]. More than 70% of patients with HCC are not diagnosed until their cancer is at an advanced and/or inoperable stage, resulting in a poor prognosis for these patients [4].

Numerous deregulated signaling pathways that lead to elevated hepatocellular carcinoma cell proliferation and angiogenesis have been identified in patients with HCC, including the RAF-MEK-ERK pathway, the insulin-like growth (IGF) pathway, and the PI3K/AKT-mTOR pathway [5]. The United States Food and Drug Administration (FDA) has approved an oral drug, sorafenib, for inoperable HCC patients [6]. Sorafenib is a small molecule multikinase inhibitor that is active in several cancer cell lines through its ability to inhibit cell proliferation, induce cell apoptosis, and suppress angiogenesis [7–11]. The activity of sorafenib is mediated by its suppression of several kinases, including vascular endothelial growth factor receptor 2 (VEGFR2), vascular endothelial growth factor receptor 3 (VEGFR3), B-Raf proto-oncogene serine/threonine kinase (B-Raf), and fibroblast growth factor receptor 1 (FGFR1) [8,12]. The RAF-MEK-ERK signaling pathway has been found to be overexpressed in

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more than 90% of patients with HCC and in the abundant vasculature of hepatomas [13], providing rationale for the use of sorafenib in the treatment of HCC.

Sorafenib has rapidly gained acceptance as a first-line treatment for advanced and metastatic HCC, and treatment with the drug has afforded patients with almost 3.0 months of increased survival compared to placebo-treated patients [6,14]. However, some advanced HCC patients fail to respond to sorafenib, and those who do respond experience limited benefits. Antiviral therapy can inhibit viral DNA replication and viral oncoprotein expression, potentially improving the anticarcinogenic microenvironment of cells. Recently, interferon-lambda3 (IFN-λ3) has shown strong activity against HBV and HCV *in vitro* [15,16]. We hypothesized that treatment with sorafenib in combination with IFN-λ3 may be a useful combination therapy for improving the survival of HCC patients. In the present study, we demonstrate that combined treatment with IFN-λ3 and sorafenib suppresses hepatocellular carcinoma cell proliferation and induces cell apoptosis *in vitro*. Furthermore, *in vivo* data show that this combination treatment reduces the tumor volume of SMMC7721 subcutaneous xenografts, and results in increased apoptosis in the neoplasm. Therefore, we propose that adding IFN-λ3 to traditional sorafenib therapy might provide increased benefit to patients with HCC.

2. Materials and methods

2.1. Drug preparations

Sorafenib (Nexavar®, BAY 43-9006), the chemical name is *N*-(3-trifluoromethyl-4-chlorophenyl)-*N*′-(4-(2-methylcarbamoyl pyridin-4-yl)oxyphenyl)urea (Fig. 1), kindly provided by Bayer HealthCare LLC. (Tarrytown, NY, USA), was dissolved in dimethyl sulfoxide (DMSO) to create a 10 mM stock solution and stored at −20 °C for *in vitro* studies. For the *in vivo* study, Sorafenib was dissolved in Cremophor EL (Sigma-Aldrich) and 95% ethanol in 1:1 ratio to make a 4 × concentration, which was stored in the dark at room temperature [17]. Recombinant human IFN-λ3 (catalog no: CYT-105) was purchased from ProSpec-Tany TechnoGene Ltd. (Ness-Ziona, Israel) and was diluted to appropriate concentrations with culture medium immediately at time of use.

2.2. Clinical tissue samples

Human hepatocellular carcinoma (HCC) tissues (n = 21) and matched adjacent normal tissues (n = 21) were obtained from HCC patients at the Department of General Surgery, Lanzhou University Second Hospital in 2016. All patients had an accurate histological diagnosis according to the clinicopathological criteria of the International Union for Cancer Control (UICC). All patients provided the consent for using their specimens in the present study, and this use was approved by the Institute Research Ethics Committee of the Lanzhou University Second Hospital.

2.3. Cell lines and culture conditions

Human hepatocellular carcinoma cells (HepG2, SMMC7721, SK-Hep-1, and BEL-7402) and normal human liver cell line L-02 were obtained from Shanghai Institute of Cell Biology (Shanghai, China) and the Cell Culture Center at Peking Union Medical College (Beijing, China). Four cancer cell lines were cultured in Ham's F12 Dulbecco's Modified Eagle's Medium (ATCC, Manassas, VA, USA) and L-02 cells were cultured in RPMI 1640 medium (ATCC, Manassas, VA, USA). All cells were cultured in the medium with 10% fetal bovine serum (FBS; Gibco, CA, USA), 100 UI/mL penicillin, and 100 UI/mL streptomycin and were incubated in a humidified incubator at 37 °C and 5% CO_2.

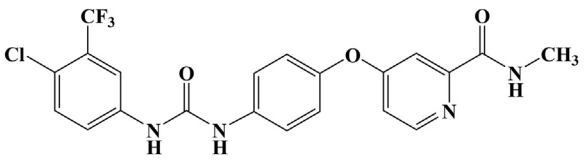

Fig. 1. Chemical structure of sorafenib.

2.4. Cell proliferation assays

Cell proliferation was assayed by Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) and colony formation assays. For the CCK-8 assay, HCC cells or L-02 cells were incubated with different concentrations of sorafenib, IFN-λ3, or a combination of sorafenib and IFN-λ3 for 24 or 48 h. Then, 100 μL CCK-8 solution was added to each well, and the plates were incubated at 37 °C for 60 min. Finally, the absorbance was measured at 450 nm using a microplate reader (Rayto Life and Analytical Science C. Ltd, Germany).

For the colony formation assay, HepG2 and SMMC7721 cells were seeded in 6-well plates and maintained in medium containing 10% FBS and 3 μM sorafenib, 10 ng/mL IFN-λ3, or a combination of 3 μM sorafenib and 10 ng/mL IFN-λ3 for 2 weeks. Then, colonies were fixed with 4% paraformaldehyde in phosphate-buffered solution (PBS), stained with 0.5% crystal violet (Sigma, St. Louis, MO, USA) for 15 min, and manually counted.

2.5. Quantitative real-time PCR

Total RNA was isolated using TRIZOL (Invitrogen Inc., CA, USA), and its concentration was measured by determining the absorbance at 260 nm using GeneQuant II (Pharmacia, Uppsala, Sweden). RNA (2 μg) was used in the reverse transcription reaction for cDNA synthesis using M-MLV Reverse Transcriptase (Promega, WI, USA). PCR analysis was performed on an Applied Biosystems 7500 Sequence Detection system (ABI, CA, USA) using SYBR Green Real-Time PCR Master Mixes (ThermoFisher, MA, USA). The primers used include the following: for *IL-28R1*, 5′-ACCTATTTTGTGGCCTATCAGAGCT-3′ (forward) and 5′-CGGCTCCACTTCAAAAAGGTAAT-3′ (reverse); for *IL-10R2*, 5′-TATTG-GACCCCCTGGAAT-3′ (forward) and 5′-GTAAACGCACCACAGCAA-3′ (reverse); and for *GAPDH*, 5′-ATCGTGCGTGACATTAAGGAGAAG-3′ (forward) and 5′-AGGAAGGAAGGCTGGAAGAGTG-3′ (reverse). Gene expression of *IL-28R1* and *IL-10R2* was normalized relative to the level of *GAPDH* within each sample using the relative $2^{-\Delta\Delta CT}$ method.

2.6. Protein extraction and western blot

After treatment with sorafenib, IFN-λ3, or a combination of sorafenib and IFN-λ3 for 48 h, 5×10^6 HCC cells were harvested and resuspended with SDS lysis buffer (Beyotime, Shanghai, China) on ice for 10 min. Then, cells were centrifuged at 12000 rpm for 20 min at 4 °C, and the supernatant was collected. The total concentration of extracted protein was determined using a BCA Protein Assay kit (Pierce, MA, USA). An equal concentration of total protein was loaded per lane on a 12% SDS polyacrylamide gel (SDS-PAGE), separated at 120 V for 90 min, and transferred to polyvinylidene difluoride membrane (PVDF) (Millipore, Billerica, MA, USA) at 50 V for 3 h at 4 °C. Membranes were blocked in Tris-buffered saline (TBS) containing 5% nonfat milk at 4 °C overnight and washed with TBS containing 0.05% (v/v) Tween 20 (TBST) three times. Membranes were then incubated with primary antibody: Cyclin D1 (Abcam, ab134174, Cambridge, MA, USA, 1:800), p21 (Abcam, ab109520, Cambridge, MA, USA, 1:1000), or cleaved