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ORIGINAL ARTICLE

Effects of sorafenib and regorafenib on the expression of hypoxia-inducible factors in hepatocellular carcinoma-transplanted nude mice

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Abstract

Objective The objective of this study was to investigate the inhibitory effects of sorafenib and regorafenib on the growth of hepatocellular carcinoma (HCC) using a subcutaneous transplantation tumor model in nude mice and exploring the effects of sorafenib and regorafenib on the expression of hypoxia-inducible factor (HIF)-1α, HIF-2α, and HIF-1β in HCC tissues collected from HCC-transplanted nude mice.

Methods HepG2 cells were inoculated intradermally into nude mice. The mice were randomly assigned to either sorafenib treatment (100 mg/kg), regorafenib treatment (20 mg/kg), or solvent control group (dimethylsulfoxide) (n = 8 per group) and received once-daily treatment for 14 days. The tumor volumes were recorded every 3 days after the initiation of treatment. The expression levels of HIF-1α, HIF-1β, HIF-2α, and SART1 in the HCC tissues were examined via quantitative real-time PCR (qRT-PCR) analysis and Western blotting.

Results The tumors in the sorafenib and regorafenib treatment groups grew slower and smaller than did the tumors in the solvent control group. qPCR analysis and western blotting demonstrated that the mRNA and protein expressions of HIF-1α and HIF-1β were down-regulated. The expression of HIF-2α and SART1 was up-regulated in the sorafenib treatment group ($P < 0.05$); meanwhile, the expression of HIF-1α and HIF-1β was up-regulated, and that of HIF-2α and SART1 was down-regulated in the regorafenib treatment group ($P < 0.05$).

Conclusion The expression of hypoxia-associated factor is up-regulated by sorafenib and down-regulated by regorafenib, which may induce the different effects of sorafenib on the expression of HIFs.

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In 2018, liver cancer became the sixth most common cancer and the fourth leading cause of cancer-related deaths worldwide [1]. Hepatocellular carcinoma (HCC) is one of the most common cancers, accounting for more than 90% of primary liver cancers, with approximately 850,000 new cases per year globally [2, 3]. The main curative treatment is surgical resection and liver transplantation. Most patients with HCC show intrahepatic or extrahepatic metastasis at the time of diagnosis. Therefore, the recurrence and mortality rates of HCC are high, and the prognosis remains poor [4]. In recent years, new biotargeted drugs have become available [5], providing new hope for the treatment of advanced HCC.

Studies have shown that sorafenib (BAY 43-9006), a novel multi-target drug, has broad anti-tumor and anti-angiogenic effects [6] and is a standard first-line therapeutic drug for HCC. Clinical studies have confirmed that in patients with advanced HCC and Child–Pugh A liver function, sorafenib is the only therapeutic drug that has been shown to improve overall survival in randomized

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studies [7]. Regorafenib (BAY 73-4506) is a diphenylurea multikinase inhibitor that is clinically effective and well tolerated according to phase I–III clinical trials [8, 9]. On April 27, 2017, the US Food and Drug Administration approved regorafenib treatment for patients with HCC with disease progression after sorafenib treatment. Thus, regorafenib has started to be used as a second-line therapeutic drug in patients with HCC.

The rapid growth of HCC cells results in insufficient blood and oxygen supply to certain tumor tissues. The hypoxic environment accelerates tumor angiogenesis and metastasis, contributing to the development of multidrug resistance [10]. Hypoxia-inducible factor (HIF)-1 and HIF-2 are important transcription factors associated with hypoxic conditions and closely involved in the development of solid tumors [11]. Their structural domains and regulatory mechanisms are shown in Fig. 1. HIF-1 comprises a functional subunit (HIF-1α) and a constitutive subunit (HIF-1β) [12]. Our previous studies have found that an increased HIF-1α expression is a beneficial predictive factor for a poor prognosis in patients with HCC. HIF-2α may exert an anti-tumor activity by inducing apoptosis in HCC cells [13], suggesting that HIF-1α and HIF-2α may play different roles in HCC.

To date, the effects of sorafenib and regorafenib on HIF expression have not yet been studied. In the present study, we utilized a nude mouse model inoculated with human HCC cells to investigate the effects of sorafenib and regorafenib on tumor growth and HIF-1α, HIF-2α, and HIF-1β expression in HCC tumors.

Materials and methods

Reagents

Sorafenib tosylate and regorafenib tablets were purchased from Bayer AG (Leverkusen, Germany) and dissolved in dimethylsulfoxide (DMSO; MP Biomedicals, Santa Ana, CA, USA) stored at –20°C. The stock solution was diluted to a working concentration using a cell culture medium (Dulbecco's modified eagle medium [DMEM]; Gibco BRL, Grand Island, NJ, USA), and the final DMSO concentration was < 0.1%.

Cell lines and culture conditions

Human hepatoma HepG2 cells were purchased from the China Center for Type Culture Collection (Wuhan University, Wuhan, China). The cells were cultured in DMEM (Gibco BRL) containing 10% fetal bovine serum (Logan, UT, USA) and 1% penicillin–streptomycin (Mediatech, Inc., Herndon, VA, USA). The medium was changed every 2 days.

Animals and xenotransplantation

The BALB/c nude mice included in this study were half male and half female, were 4–6 weeks old, and weighed 14–20 g. Xenografts were transplanted according to the method published by Yang *et al.* [13]. The tumor diameters were measured every 3 days after the initiation of dosing until Day 15. The largest (a) and smallest (b) diameters were measured, and the tumor volume was calculated using the following formula: V (mm^3) = $ab^2/2$. The

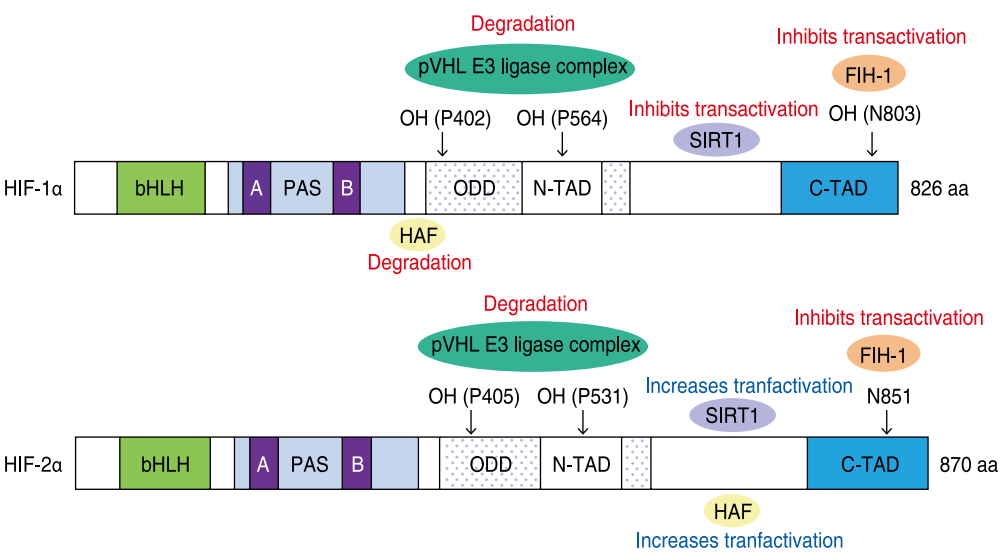

Fig. 1 Structural domains of hypoxia-inducible factor (HIF)-1/2α and their post-translational protein modifications. The von Hippel–Lindau protein (pVHL) E3 ligase complex regulates the oxygen-dependent degradation of HIF-1α and HIF-2α. Hypoxia-associated factor (HAF) causes HIF-1α ubiquitylation and degradation but promotes HIF-2α transactivation under prolonged hypoxia. Sirtuin 1 (SIRT1) selectively binds to HIF-1α and HIF-2α, mediating degradation and transactivation, respectively

xenografted mice were randomly assigned to either of the following three groups (n = 8 per group): sorafenib treatment (100 mg/kg/day), regorafenib treatment (20 mg/kg/day), and solvent control groups (DMSO). The animals were dosed via oral gavage once daily for 14 days (the first day of treatment was considered as Day 1). The tumor growth rates in the sorafenib and regorafenib treatment groups were compared with that in the solvent control group over time. The mice were sacrificed 24 h after the last administration, and their tumors were separated. Representative tumor tissues were frozen immediately in liquid nitrogen for quantitative real-time PCR (qPCR) analysis and western blotting.

qPCR analysis and western blotting

qPCR analysis and western blotting were performed as previously described [14]. The following PCR primers synthesized by Google Biotechnology Co., Ltd. (Wuhan, China) were used in this study: HIF-1α forward: 5'-ACTTCTGGATGCTGGTGATTTG-3', reverse: 5'-GCTTCGCTGTGTGTTTTGTTCT-3'; HIF-2α forward: 5'-TCATGCGACTGGCAATCAGC-3', reverse: 5'-GTCACCACGGCAATGAAACC-3'; HIF-1β forward: 5'-TCGCGTCCTTCTTCATCCGTTAGC-3', reverse: 5'-TTTCGAGCCAGGGCACTACAGG-3'; SART1 forward: 5'-AAGTACAGCCGGAGGGAGGAATAC-3', reverse: 5'-TTCATCTTGCCTGAGCCCTTG-3'; and GAPDH forward: 5'-TCGACAGTCAGCCGCATCTTCTTT-3', reverse: 5'-GCCCAATACGACCAAATCCGTTGA-3'. GAPDH was used as an internal control for both qPCR analysis and Western blotting. Anti-HIF-1α, anti-HIF-2α, anti-HIF-1β, and anti-SART1 antibodies were purchased from Proteintech Group, Inc. (Chicago, IL, USA).

Statistical analysis

SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Data were expressed as means ± standard deviations ($\chi \pm s$). Two-group comparisons were performed using an independent-samples t-test and multiple-group comparisons using the least significant difference test. P values of < 0.05 were considered statistically significant.

Results

Growth of transplanted HCC in the nude mice

All nude mice (n = 24) across the treatment groups survived during the dosing period, with a tumor formation rate of 100%. The tumor growth curve (Fig. 2a) revealed that the tumor growth rates of the sorafenib and regorafenib treatment groups were significantly lower than those of the solvent control group. After the last dose administration on Day 15, the HCC tumors were separated. As shown in Fig. 2b, the tumors of the sorafenib and regorafenib treatment groups were markedly smaller than those of the solvent control group.

Expression of HIF-1α, HIF-2α, HIF-1β, and SART1 in the HCC cells

At the mRNA level, there were significant decreases in the HIF-1α and HIF-1β expression and significant increases in the HIF-2α and SART1 expression following treatment in the sorafenib treatment group compared with those in the solvent control group (P < 0.05) (Fig. 3a). In contrast, the HIF-1α and HIF-1β expression was up-regulated, and the HIF-2α and SART1 expression was down-regulated in the regorafenib treatment group (P < 0.05). These changes in expression at the mRNA level were confirmed at the protein level by western blotting (Fig. 3b).

Discussion

HCC is a malignancy with high morbidity and mortality rates. Hepatitis B virus (HBV) infection is one of the leading causes of HCC. Globally, approximately 54.4% of HCC cases are attributed to chronic infection with HBV, and the proportion can reach as high as 80% in Chinese and Black African populations [15]. HIFs are the master regulators of gene expression in hypoxic conditions and play a central role in the regulation of human metabolism. The expression of hepatitis B virus X protein (HBx)

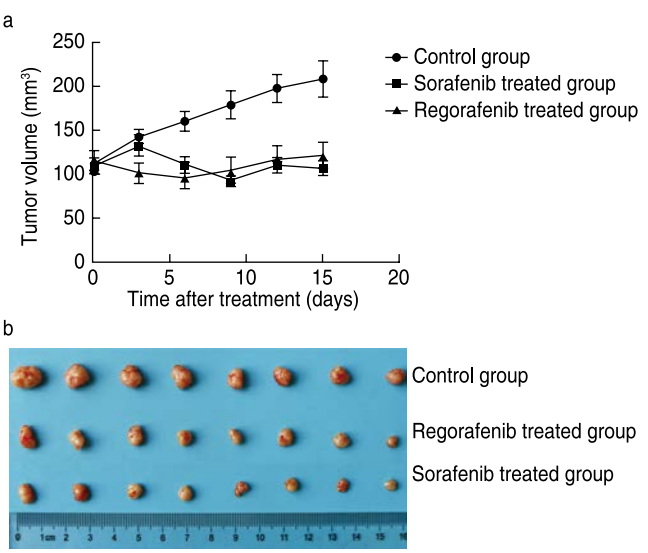

Fig. 2 (a) Tumor growth curves of BALB/c nude mice in the sorafenib treatment (square symbol), regorafenib treatment (triangle symbol), and solvent control groups (circle symbol). The tumor growth in the sorafenib and regorafenib treatment groups significantly slowed down; (b) Sizes of stripped tumor tissues from the sorafenib treatment, regorafenib treatment, and solvent control groups after 15 days of treatment. At the end of drug administration, the tumors in the sorafenib and regorafenib treatment groups were smaller than those in the solvent control group

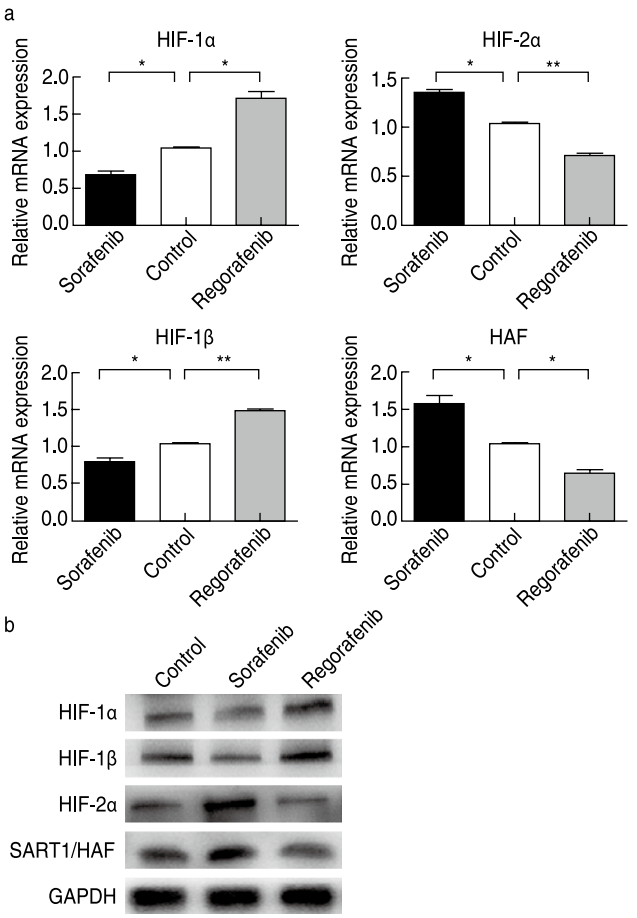

Fig. 3 (a) mRNA expression levels of hypoxia-inducible factor (HIF)-1α, HIF-2α, HIF-1β, and SART1 analyzed via quantitative real-time PCR testing. The mRNA expression levels of HIF-1α and HIF-1β significantly decreased, while those of HIF-2α and SART1 significantly increased in the sorafenib treatment group compared with those in the solvent control group. In contrast, the mRNA expression of HIF-1α and HIF-1β was up-regulated, while that of HIF-2α and SART1 was down-regulated in the regorafenib treatment group. *P < 0.01, **P < 0.001; (b) Protein expression of HIF-1α, HIF-2α, HIF-1β, and HAF examined via western blotting. The expression of these genes at the protein level coincided with their expression at the mRNA level. GAPDH was used as a loading control

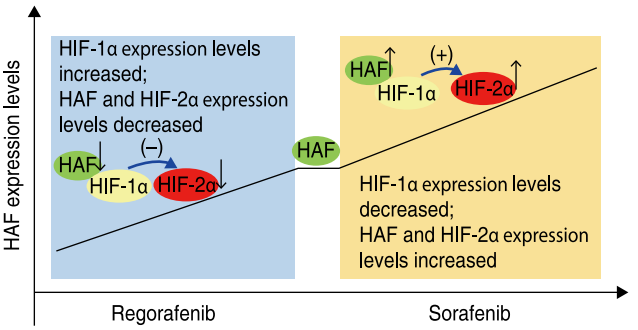

Fig. 4 Different effects between sorafenib and regorafenib on the expression of HIFs. The HAF and HIF-2α expression levels decreased, while the HIF-1α expression level increased after regorafenib treatment. Meanwhile, the HAF expression level increased after sorafenib treatment, providing a mechanism for the switch from HIF-1α to HIF-2α. This is a possible reason for the decrease in the HIF-1α expression level and increase in the HIF-2α expression level after sorafenib administration

has been reported to be positively correlated with the expression of HIF-α in patients with HBV-related HCC [16]. HBx can modulate chemoresistance by activating HIF-1α and increasing the HIF-2α expression level via inhibition of HIF-2α degradation [17]. The novel anticancer drugs sorafenib and regorafenib have been widely used in the systemic treatment of patients with HCC; however, it is unclear whether these two drugs affect the expression of HIF-1 and HIF-2.

In this study, we confirmed the inhibitory effects of sorafenib and regorafenib on the growth of HCC tumors using a nude mouse model, which is consistent with previous reports. Through qPCR analysis and western blotting, we found that sorafenib down-regulated the expression of HIF-1α and HIF-1β and up-regulated that of HIF-2α. In contrast, regorafenib up-regulated the expression of HIF-1α and HIF-1β and down-regulated that of HIF-2α. These results demonstrate the opposite effects of these two drugs on the expression of HIF-1 and HIF-2. In addition, qPCR analysis and western blotting showed that the expression of SART1 was significantly up-regulated by sorafenib but down-regulated by regorafenib.

Hypoxia-associated factor (HAF, encoded by SART1) is expressed in both normal and malignant tissues [18]. It triggers HIF-1α ubiquitylation and degradation and promotes HIF-2α transactivation under prolonged hypoxia. Studies have shown that overexpression of HAF decreases the HIF-1α expression level, whereas knockdown of HAF increases the HIF-1α expression level independent of the presence of oxygen [19, 20]. These findings have also been demonstrated in other mouse HCC models [21]. In addition, a study on bladder cancer found that HIF-1α can switch to HIF-2α owing to HAF-mediated activation of the NF-κB pathway [22]. The present study showed that the expression of HAF was up-regulated by sorafenib and down-regulated by regorafenib, which may explain the opposing effects of sorafenib and regorafenib on the expression of HIFs (Fig. 4).

We have previously demonstrated that a higher expression of HIF-1α is correlated with a poor prognosis in patients with HCC. Although the role of HIF-2α remains controversial, most studies have shown that patients with HCC with a high expression of HIF-2α had a better prognosis. In this study, we found that sorafenib inhibited the expression of HIF-1α and activated the expression of HIF-2α via up-regulation of the expression of HAF, which may provide an explanation for the clinical effectiveness of sorafenib. In the future, we will

further investigate how HAF regulates HIFs and explore the option of combination therapy for HCC.

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Conflicts of interest
All authors have completed the ICMJE uniform disclosure form. The authors have no conflicts of interest to declare.

Author contributions
All authors collected and interpreted the data and reviewed and approved the final version of the manuscript.

Data availability statement
All data generated or analyzed during this study are included in this published article (and the accompanying supplementary information files).

Ethical approval
Not applicable.

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