Original article

Sorafenib suppresses the rapid progress of hepatocellular carcinoma after insufficient radiofrequency ablation therapy: an experiment *in vivo*

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Abstract

Background: Radiofrequency ablation (RFA) is a widely applied treatment for hepatocellular carcinoma (HCC), but insufficient RFA can promote rapid progression of the residual tumor through the hypoxia inducible factor-1α (HIF-1α)/vascular endothelial growth factor A (VEGFA) pathway. Although sorafenib has been successfully applied to advanced HCC, the use of sorafenib in residual tumor cells after RFA has rarely been tested.

Purpose: To evaluate the potential role of sorafenib as an adjunct to RFA to reduce the recurrence rate after insufficient RFA.

Material and Methods: Xenograft tumors of SMMC 7721 were created by subcutaneously inoculating nude mice with hepatoma cells (5×10^6 cells per mouse). Fourteen days after inoculation, all mice were divided into three groups (control group [sham puncture], RFA group, and RFA combined with sorafenib treatment group) with six mice in each group. Each group was given a different treatment procedure. After treatment, the volume of the tumors was calculated from the resected specimens. The mRNA and protein expression of HIF-1α and VEGFA was quantified by real-time PCR and immunohistochemistry analysis. The micro-vessel density (MVD) was determined by CD34 immunohistochemistry.

Results: Real-time PCR and immunohistochemistry analysis showed that, compared to the RFA group, HIF-1α and VEGFA expression were significantly decreased in the group that received RFA combined with sorafenib treatment ($P < 0.05$). By comparing the control group with the RFA group, we found that insufficient RFA promoted HIF-1α and VEGFA expression ($P < 0.05$). Similar results were obtained for MVD expression. Additionally, the combination of RFA with sorafenib therapy resulted in a synergistic reduction in tumor growth compared to insufficient RFA and sham puncture ($P < 0.05$).

Conclusion: Sorafenib was able to inhibit the expression of HIF-1α and VEGFA, and sorafenib was able to increase time to recurrence when used as an adjunct to RFA.

Keywords: Radiofrequency ablation, hepatocellular carcinoma, sorafenib

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Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most common cause of cancer-related death globally (1). Although hepatic resection and transplantation have been considered being the main curative therapies for HCC, the vast majority of patients are not eligible for treatment when the tumor is detected. Surgical therapy are applied only in 10–20% of the patients (2, 3). Currently, various local ablative therapies, such as radiofrequency ablation (RFA), have been accepted as an alternative treatment option with several advantages, such as definitive therapeutic effect, minimal invasiveness, repeatability, safety, and shorter hospitalization (4). However, one of the major problems with RFA is that it is difficult to achieve complete tumor destruction. Both local and

systemic recurrences after RFA treatment have limited the application of RFA. Local recurrency is reported in up to 60% of the cases (5, 6). Accumulated evidence have shown that both hypoxia and hypoxia-driven angiogenesis are a consequence of insufficient RFA, and both of these factors play important roles in tumor growth (7, 8). HIF-1α has been shown to be over-expressed in more than 70% of solid tumors. It is recognized as the key activator response to hypoxia in the transcription of multiple genes related to angiogenesis, energy metabolism, invasion, and metastasis (9). RFA may similarly affect the local growth of residual tumor located on the periphery of the lesion through the induction of hypoxia and neovascularization. Therefore, additional treatment strategies are needed.

As a multikinase inhibitor, sorafenib demonstrates activity against several tyrosine (VEGFR2, PDGFR, c-Kit receptors) and serine/threonine (b-Raf, p38) kinases. As a chemotherapeutic agent, sorafenib, which is currently used as a standard treatment for patients with advanced HCC (10), is the first systemic treatment drug to prolong significantly the survival of advanced-stage patients. However, while sorafenib has been successfully used in patients with advanced, non-resectable HCC, its use in residual tumor cells after RFA has rarely been tested.

Based on the aforementioned risk factors, we hypothesize that insufficient RFA, which leads to incomplete ablation, might play an important role in facilitating rapid progression of residual tumor. The present study is designed to test this hypothesis and to evaluate the use of sorafenib as an adjunct to RFA to reduce the recurrence rate by inhibiting the underlying mechanism of recurrence.

Material and Methods

Cell lines and subcutaneously transplanted tumor models

SMMC 7721 human hepatoma cells were obtained from the Cell Bank of the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences, Shanghai, China) and cultured in RPMI 1640 (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) in 5% CO_2 at 37°C.

Male athymic BALB/c nu/nu mice, weighing 18–20 g at 4 weeks of age, were obtained from the Beijing Laboratory Animal Research Center (Beijing, China). All mice were handled according to the recommendations of the National Institutes of Health Guidelines for Care and Use of Laboratory Animals. The experimental protocol was approved by the Sun Yan-sen University Medical Experimental Animal Care Committee. The SMMC 7721 cells were inoculated subcutaneously (s.c) into the right flank of the mice (5×10^6 cells per mouse). Tumor growth was measured every 3 days using vernier calipers and tumor volume was calculated using the formula: length (mm) × width^2 (mm) × π/6. When the length of the tumors reached 12–15 mm (on day 14 after vaccination of tumor cells), the mice were divided into three groups: control group (sham puncture), RFA group, and RFA with concomitant administration of sorafenib group. Each group contained six mice.

Radiofrequency ablation of tumor

RFA was performed using a bipolar RFA device (MedSphere International, Shanghai, China) which is a micro RFA probe with an active tip length of 10 mm. To simulate the clinical setting of tumor recurrence after RFA, a "partial RFA" strategy using 180 s of RFA at 1 watt power was employed on the 14th day of tumor growth. This procedure could result in partial ablation of tumors. Sham punctures were performed using an identical probe without applying energy. All athymic BALB/c nu/nu mice were anesthetized with 30 mg/kg pentobarbital.

Compounds

Sorafenib (N-(3-trifluoromethyl-4-chlorophenyl)-N′-(4-(2-methylcarbamoyl pyridin-4 -yl)oxyphenyl)urea) was synthesized at Bayer Corporation (West Haven, CT, USA). Sorafenib was dissolved in distilled water to the desired concentration. Sorafenib (30 mg/kg/day, according to references (11, 12)) was administered daily by oral gavage for 3 days prior to RFA. On the day of RFA no sorafenib was applied. After RFA, mice again received sorafenib by daily perorally until the day before sacrifice.

Tissue preparation

In all groups, the tumors were harvested 7 days after RFA treatment. Prior to sacrifice by cervical dislocation, the mice were anesthetized with pentobarbital. Samples from each area were snap-frozen in liquid nitrogen for RNA preparation. Corresponding tissue samples were fixed in 4% formalin for paraffin-embedded sections.

RNA extraction, reverse transcription, and real-time PCR analysis

Total RNA was isolated from tissues using Trizol (Takara Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2.0 μg of RNA using oligo (dT)18 (0.5 μg/μL). The samples were incubated at 70°C for 10 min, chilled on ice, and then reverse-transcribed into cDNA in a cDNA synthesis mixture containing 5 × RT buffer (4 μL), RNasin (0.5 μL), 10 mM dNTPs (2 μL) and M-MLV-RTase (1 μL) in a total volume of 11 μL. The mixture was incubated at 42°C for 60 min and at 70°C for 10 min to deactivate reverse transcription. PCR was performed in a 20 μL final volume containing the following: H_2O up to 20 μL; 1 μL cDNA diluted in RNase-free water; 10 μL SYBR premix ex-Taq; the antisense and sense primer (0.5 μL each). After an initial denaturation step at 95°C for 15 s, temperature cycling was initiated. Each cycle consisted of denaturation at 95°C for 5 s, hybridization at 60°C for 30 s, and elongation at 60°C for 30 s. The fluorescent signal was acquired at the end of the elongation step. The forward primer for HIF-1α is: 5′-TGA-AGT-GTA-CCC-TAA-CTA-GCCG-3′, and the reverse is: 5′-GTT-CAC-AAA-TCA-GCA-CCAAGC-3′. The forward primer for VEGFA is 5′-AGA-AGG-AGG-AGG-GCA-GAA-TC-3′, and the reverse primer is 5′-ACA-CAG-GAT-GGC-TTG-AAG-ATG-3′. The forward primer for the

internal control gene GAPDH is 5′-CGA-CAC-CCA-CTC-CTC-CAC-CTTT-3′, and the reverse is 5′-CCA-CCA-CCC-TGT-TGC-TGT-AGCC-3′. A total of 45 cycles were performed. After the PCR reaction, the melting curve from 55°C to 95°C was read every 0.5°C and held for 4 s. The samples were then incubated at 95°C for 60 s. Cycling conditions for GAPDH were the same as above. The comparative Ct (threshold cycle) method was used to calculate the relative changes in gene expression using the real-time PCR system.

Immunohistochemistry analysis

Histological sections were obtained from tumor tissues resected from the mice. Slides were routinely dewaxed and hydrated. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min and non-specific binding was blocked with goat serum in PBS for 20 min, then the sections were incubated with anti-HIF-1α (Novus Biologicals, Littleton, CO, USA, diluted 1:100), anti-VEGFA (Santa Cruz Biotechnology, Santa Cruz, CA, USA, diluted 1:50), and anti-CD34 (Santa Cruz Biotechnology, CA, USA, diluted 1:50) antibody for 18 h at 4°C in 2% bovine serum albumin in PBS. The secondary antibody was added and incubated for 1 h at 37°C. Positive reactions were detected by incubating the slides with 3,3′-diami-nobenzidine tetrahydrochloride for 3–5 min. Sections were counterstained with hematoxylin for 3–5 min. A negative control staining without primary antibody was performed.

The brown-yellow staining of the cytoplasm was considered positive. For each case, the entire stained tissue section was scanned. Five visual fields at 400 × magnification were randomly chosen and 1000 cells in each field were counted. The immunohistochemical results for HIF-1α and VEGFA are classified as follows: –, no expression; +, weak expression; ++, strong expression. All brown-stained endothelial cells or endothelial cell clusters, that were clearly separate from connective tissue elements, were considered a microvessel. And the mean counts for each specimen were recorded as the micro-vessel density (MVD).

Statistical analysis

All statistical analyses were performed by using SPSS13.0 (Version 13.0; SPSS Inc., Chicago, IL, USA). Comparisons among all groups were performed with the one-way analysis of variance (ANOVA) test. If statistical significance was found, the Tukey post hoc test was used. Values of $P < 0.05$ were considered statistically significant.

Results

Sorafenib inhibits tumor growth after RFA treatment in mice

To test the efficacy of sorafenib combined with RFA, mice bearing SMMC 7721 tumors were treated with partial RFA (Fig. 1). As shown in Fig. 2, when the treatments ended, RFA treatment alone resulted in a moderate antitumor effect compared to no treatment ($P = 0.000$). Combination RFA with sorafenib therapy resulted in a synergistic reduction in tumor growth compared with RFA treatment alone or with no treatment ($P = 0.000$ for combination therapy *vs.* RFA treatment and $P = 0.001$ for combination therapy *vs.* no treatment).

HIF-1α and VEGFA mRNA expression in tumor tissues

To explore sorafenib-induced changes in tumor growth, HIF-1α and VEGFA mRNA expression levels were analyzed by real-time PCR. As shown in Fig. 3, HIF-1α and VEGFA were significantly over-expressed in the partial RFA group compared with controls ($P = 0.000$). However, in the combination sorafenib and RFA therapy group, HIF-1α and VEGFA mRNA expression levels were decreased compared with RFA treatment alone ($P = 0.002$). Changes in growth factor expression should be further examined to investigate whether they are a potential modulator of tumor recurrence and tumorigenesis after RFA.

Sorafenib decreases HIF-1α and VEGFA expression and micro-vessel density in the residual tumor after RFA

To investigate the underlying mechanisms of sorafenib inhibition of the growth of residual tumor, immunohistochemistry for HIF-1α, VEGFA and MVD was performed. Immunostaining for HIF-1α and VEGFA revealed a marked increase of expression in the partial RFA group. In the group that received RFA combined with sorafenib treatment, HIF-1α and VEGFA expression were markedly decreased compared to the group which received RFA treatment alone (Fig. 4a, b).

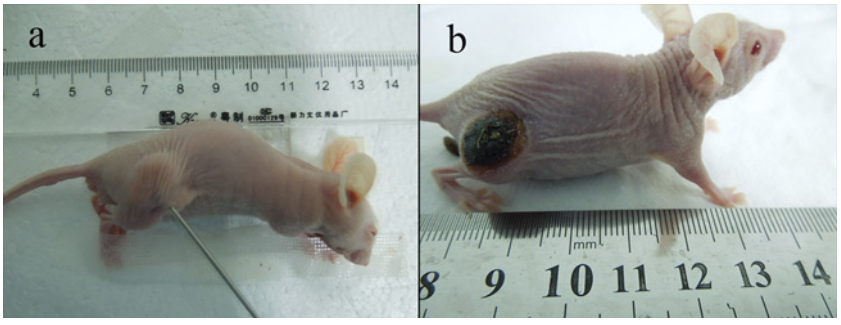

Fig. 1 The picture shows the process of radiofrequency ablation (RFA). (a) After the subcutaneously transplanted tumor had formed, the RFA probe was inserted into the tumor tissue. (b) The mice that had received RFA showed burned skin and smaller tumor volume after treatment

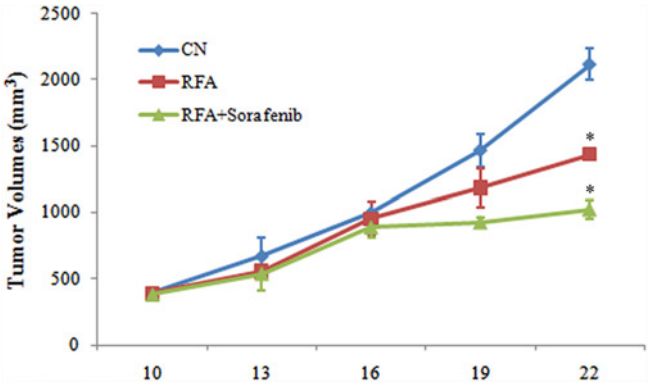

Fig. 2 After subcutaneously transplanted tumors had formed, we measured the tumor volume of the mice every 3 days. On the 14th day, we performed treatments according to the previously designed scheme. Following treatment, the tumor volume was significantly different among the three groups ($P < 0.05$). When the treatments ended, the RFA + Sorafenib group had smaller tumor volume than the RFA-alone and control groups ($P < 0.05$), and the RFA-alone treatment group had smaller tumor volume than the control group ($P = 0.000$), also, RFA + Sorafenib group had smaller tumor volume than the RFA-alone group ($P = 0.000$). $^{*}P < 0.05$

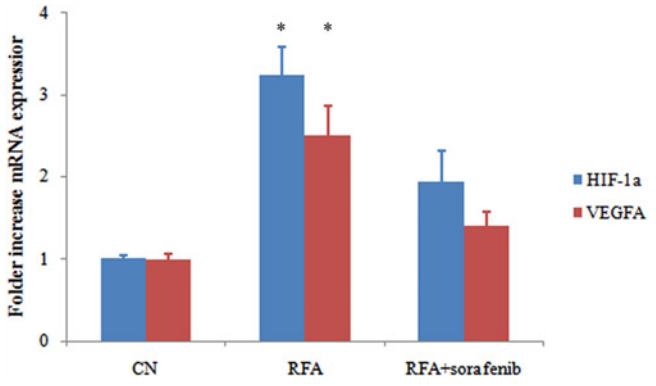

Fig. 3 Hypoxia inducible factor-1α (HIF-1α) and vascular endothelial growth factor A (VEGFA) mRNA expression were measured by real-time PCR. Compared to RFA treatment, RFA + Sorafenib significantly decreased HIF-1α and VEGFA expression ($P = 0.002$). Compared to control, RFA treatment induced significantly increased HIF-1α and VEGFA expression ($P = 0.000$). This phenomenon may explain why RFA treatment induced residual tumor cell metastasis, and when RFA treatment is combined with sorafenib, this metastasis may be inhibited. $^{*}P < 0.05$

Parallel to HIF-1α and VEGFA expression, immunostaining for CD34 positive endothelial cells showed a reduction of MVD in the combined treatment group compared to the RFA alone group (Fig. 4c). Table 1 presents the MVD for these three groups.

Discussion

More than 80% of patients with HCC could not receive surgical treatment due to advanced tumor stage and poor hepatic function (2). Local ablation is considered the first-line treatment option for patients with early stage disease that is not suitable for surgical therapy (1). In selected patients, ablative treatments could achieve good results, similar to those of surgical resection in small HCCs (13). As a therapeutic method for HCC, RFA is of high value because it can induce tumor necrosis while causing minimal damage to non-neoplastic liver tissue. RFA is also easily repeated (14). Therefore, RFA is an important option among treatment strategies against HCC.

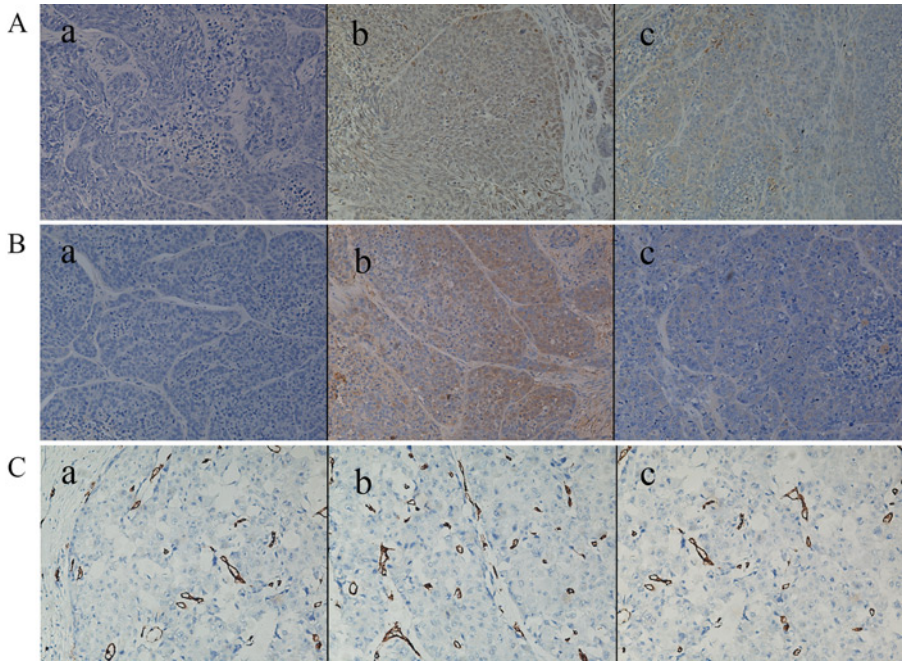

Fig. 4 The immunohistochemistry analysis of hypoxia inducible factor-1α (HIF-1α) (A), vascular endothelial growth factor A (VEGFA) (B), and micro-vessel density (MVD) (C). Compared to the RFA-alone group, RFA + Sorafenib markedly decreased HIF-1α and VEGFA protein expression. MVD was also significantly decreased ($P < 0.05$). Additionally, compared to control, RFA-alone treatment induced HIF-1α and VEGFA protein expression and significantly increased MVD ($P < 0.05$). These results were consistent with the real-time PCR results, which demonstrated that RFA treatment induced residual tumor cell metastasis, and sorafenib inhibited the metastasis. (a) represents the control group, (b) represents the RFA-alone treatment group, (c) represents RFA + Sorafenib treatment group

Table 1 MVD for the three treatment groups

	CN	RFA	RFA + Sorafenib
MVD	15.1 ± 5.1	25.4 ± 5.4*	20.3 ± 0.3

The MVD was significantly different when CN group compared with RFA group and RFA + Sorafenib group compared with RFA group (*$P < 0.05$). But it had no difference between CN and RFA + Sorafenib

Unfortunately, local recurrence with rapid progression of HCC after RFA have been reported (15–17). Recent research has revealed that the local recurrence rate of small HCC was approximately 2–60% (6).

The high local recurrence rates following ablative treatment are usually related to the large diameters of tumors (6). In large tumors, it is difficult to reach sufficiently high temperatures far away from the heat source. Because of the complicated anatomical location of tumors or accidental operator error, local recurrences may develop from residual viable tumor cells that are located at the periphery of the lesion. Such measures will cause ischemia/reperfusion (I/R) in the residual tumors. The biologic behavior of residual tumors determines the time to develop a recurrence, which eventually influences survival. Accumulated evidence has demonstrated that both hypoxia and hypoxia-driven angiogenesis are a consequence of insufficient RFA and both these phenomena play an important role in tumor growth (7, 8). The expression of related genes in response to decreased oxygen availability is largely regulated by hypoxia inducible factor-1 (HIF-1), a heterodimeric transcription factor (18). There are two trans-activating isoforms, HIF-1α and HIF-2α, whose expression and activity are tightly regulated by oxygen and which appears to have complementary functions (18, 19). In patients, HIF-1α levels have been positively correlated with tumor aggressiveness and poor prognosis (20). Given that HIF-1-mediated VEGFA expression plays a pivotal role in tumor angiogenesis, factors that modulate HIF-1 activity are potential targets for anti-cancer therapy (21, 22). Yang *et al.* (23) found that after hypoxia and chemotherapy exposure, tumor progression and angiogenesis might occur through HIF-1-dependent activation of pro-angiogenic factors. Suppression of HIF-1 activity by high-dose sodium salicylate could block angiogenic processes and thus improve survival rates. Yeo *et al.* (24) reported that up-regulation of HIF-1 activity may promote cell survival during hypoxia or ischemia, and may increase angiogenesis in oxygen-deficient tissues. Therefore, it can be inferred that the modulation of HIF-1 activity may be a good strategy for the treatment of a wide range of hypoxia or ischemia-related pathologies.

Multi-tyrosine kinase inhibitor sorafenib, which has become the standard treatment for advanced HCC, is currently under investigation in phase III trials as an adjuvant treatment with transarterial chemoembolization (25). Nilsson *et al.* (26) have reported that sorafenib could abrogate hypoxia-induced expression of HIF-1α and HIF-2α and decrease VEGF production in neuroblastoma cell lines. In this study, we found that the combination of RFA with sorafenib resulted in reduced residual tumor volume due to reduced HIF-1α and VEGFA expression compared to RFA alone. Given the known mode of sorafenib activity and its inhibitory effects on the VEGFR and PDGFR signal pathways as well as the Ras-Raf-Erk pathway, this reduction of HIF-1α and VEGFA expression may be the mechanism that leads to the decreased tumor volume and reduced proliferation of HCC cells. This hypothesis was confirmed by immunohistochemistry analysis which clearly showed a reduction in micro-vessel density in the sorafenib treated group.

Our results demonstrated that the expression of HIF-1α and VEGFA was increased in residual tumors and that sorafenib could abrogate hypoxia-induced expression of HIF-1α and decrease VEGF production in the tumors. We think sorafenib may impair tumor angiogenesis by directly acting on endothelial cells and indirectly decreasing the secretion of pro-angiogenic molecules from the tumor cells. Mertens *et al.* (27) concluded that sorafenib promotes necrosis volume after RFA due to decreased tissue repair. The conclusion was similar to our research. They demonstrated that there was transient compensatory over-expression of growth signals up to day 7. It is of interest to examine the specific changes during this time.

A limitation of this study was that we only used one type of hepatoma cell for the *in-vivo* experiment. In the future, we might use other hepatoma cells to test our hypotheses. Despite this limitation, our results could partially explain the clinical performance of sorafenib.

In conclusion, up-regulation of HIF-1α activity in the residual tumor after RFA may promote cell survival during hypoxia or ischemia by increasing angiogenesis in oxygen-deficient tissues. Sorafenib can inhibit the expression of HIF-1α and VEGFA, and sorafenib used as an adjunct to RFA can prolong time to recurrence.

Conflict of interest: None.

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