Effect of Rapamycin Alone and in Combination with Sorafenib in an Orthotopic Model of Human Hepatocellular Carcinoma

Zheng Wang, Jian Zhou, Jia Fan, Shuang-Jian Qiu, Yao Yu, Xiao-Wu Huang, and Zhao-You Tang

Abstract

Purpose: Novel therapeutic strategies are needed to prevent the tumor recurrence or metastasis after liver transplantation for hepatocellular carcinoma (HCC). This study was to investigate the effect of rapamycin, alone and in combination with sorafenib, on HCC *in vivo*.

Experimental Design: Xenograft of a highly metastatic human HCC tumor (LCI-D20) was used to evaluate primary tumor growth and lung metastasis after treatment with rapamycin alone or in combination with sorafenib. Tumor cell proliferation was determined by Ki-67 immunostaining. To detect tumor cell apoptosis, the terminal deoxynucleotidyl-transferase–mediated dUTP nick-end labeling assay was used. Tumor angiogenesis was investigated by using a monoclonal anti-CD31 antibody. A vascular endothelial growth factor ELISA kit was used to measure vascular endothelial growth factor protein levels in the mice serum.

Results: Rapamycin, alone and in combination with sorafenib, strongly inhibited primary tumor growth and lung metastases in LCI-D20 model. Furthermore, the combination therapy significantly enhanced the effect of antitumor on primary tumor growth compared with single treatment with either rapamycin ($P < 0.001$) or sorafenib ($P < 0.001$). Rapamycin alone inhibited HCC cell proliferation, induced apoptosis, and decreased tumor angiogenesis. Nevertheless, the combination therapy showed a significant inhibition of tumor cell proliferation ($P < 0.05$). Additionally, the combination therapy also further enhanced suppression of tumor cell angiogenesis compared with rapamycin treatment ($P < 0.01$). However, the induction of apoptosis in combination therapy group was not significantly higher than in the rapamycin-treated group ($P > 0.05$).

Conclusions: The combination therapy of rapamycin and sorafenib could be a new and promising therapeutic approach to the treatment of HCC and prevention of HCC recurrence after liver transplantation.

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third cause of cancer-related death globally. There are many methods in the treatment of HCC such as chemotherapy by using new antitumor drugs, operation, intervene therapy, liver transplantation (LT), and so on. Liver resection is still the mainstay of treatment for HCC and provides the consistent long-term survival. However, the resectability is limited by tumor extent, location, or underlying liver dysfunction, with only a minority of HCC being potentially resectable. All these leave LT rather than liver resection as the only potentially curative option, which increase the possibilities of HCC resection for patients with nonresectable tumor or severe hepatic failure. It is reported that the 5-year survival for HCC patients undergoing LT has been steadily improved from 25.3% in 1987 to 61.1% during the most recent period studied (1). Despite the total hepatectomy and liver replace, recurrence and metastasis remained the major obstacles to more prolonged survival after LT for HCC (2). Thereby, novel therapeutic strategies to prevent recurrence after LT are needed.

Conventional cytotoxic chemotherapy did not significantly prolong survival of patients transplanted for HCC. Due to increasingly understanding with intense research of the molecular biology of carcinogenesis and tumor progression of HCC in recent years, effective agents targeting these molecular abnormalities have been developed and widely tested in preclinical studies of HCC cell lines or xenograft models. Thus, it is necessary to investigate whether molecular targeting drugs as adjuvant therapy could prevent tumor recurrence after LT for HCC.

The growth of HCC is known to depend on stimulatory effects of various growth factors, which bind to tyrosine kinase receptors to activate various intracellular signaling pathways that contribute to tumor cell proliferation, survival, migration, and metastasis. One of the best-characterized intracellular signaling pathways is the phosphatidylinositol-3-OH kinase/Akt/ mammalian target of rapamycin (mTOR) pathway, the activation of which could induce cell proliferation and prolong

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cell survival. The most promising target in this pathway is mTOR. Rapamycin, an antibiotic that inhibits mTOR, is clinically used as an immunosuppressive drug to prevent graft rejection after LT (3). It has been reported that phosphorylation of mTOR and the expression of p70s6k is up-regulated in 45% of human HCCs, and that rapamycin as a single agent as well as in combination with other chemicals inhibits *in vitro* HCC cell line proliferation, *in vivo* HCC growth in animal model, and tumor angiogenesis in HCC (4, 5). Thus, it seems reasonable to speculate that rapamycin could simultaneously contribute to inhibition of recurrence and preventing of rejection in LT for patients with HCC.

The mitogen-activated protein kinase pathway is another crucial pathway in HCC, which includes cascade of phosphorylation involving at least four kinases: ras, raf, mitogen-activated protein extracellular kinase, and extracellular signal-regulated kinase (ERK). The Ras/Raf/mitogen-activated protein extracellular kinase/ERK pathway in HCC is a focal point for cell proliferation and differentiation signal transduction (6). Sorafenib (BAY 43-9006), an oral multikinase inhibitor, blocks tumor cell proliferation by targeting Raf/mitogen-activated protein extracellular kinase/ERK signaling at the level of Raf kinase, and exerts an antiangiogenic effect by targeting vascular endothelial growth factor (VEGF) receptor-2/-3 and platelet-derived growth factor receptor β tyrosine kinase. Sorafenib is currently the most promising molecular targeting drug for HCC that has shown an improved overall survival benefit in advanced HCC and sets the new standard for first-line treatment of advanced HCC. Due to its favorable safety profile on a continuous administration schedule, sorafenib seems to be easily combined with other anticancer therapies (7).

Given the complexity of the interactions between tumor cells and their environment, and the variety of proangiogenic and/or growth-promoting (autocrine) factors that tumors can produce, there is a strong rationale to combine agents with different mechanisms of action. Furthermore, combining drugs that have different mechanisms of action may enhance antitumor activity by overcoming mechanisms of drug resistance. In this study, we describe experiments indicating that immunosuppressive doses of rapamycin inhibit primary and metastatic tumor growth in HCC *in vivo*. Furthermore, we show that a combination therapy of rapamycin, administered in conjunction with sorafenib, improves the results achieved in the single-treatment groups.

Materials and Methods

Drugs. Rapamycin was purchased from Wyeth. Sorafenib was purchased from Bayer Pharmaceutical Corporation, and dissolved in sterile DMSO and stored frozen under light-protected conditions at -20°C.

Animals. BALB/ca male nude mice (Shanghai Institute of Materia Medica, Chinese Academy of Science) of ~20 g at ages 4 to 6 wk were used in this study, which were kept in laminar-flow cabinets under specific pathogen-free conditions, cared, and handled according to the recommendations of the NIH guidelines for care and use of laboratory animals. Experimental protocol was approved by Shanghai Medical Experimental Animal Care Committee.

Orthotopic tumor model. At the authors' institution, by using orthotopic implantation of histologically preserved metastatic tumor tissues of 30 surgical specimens, a highly metastatic model of human HCC in nude mice (named as LCI-D20) has been established. All mice with transplanted LCI-D20 tumors in the liver exhibited 100% transplantability and metastatic ability, as well as various manifestations of tumor behavior in HCC patients. These included local growth, regional invasion, and spontaneous metastasis to liver, lungs, lymph nodes, and peritoneal seeding (8). Abnormal serum α-fetoprotein level of >20 μg/L and hepatitis B surface antigen were found in this model.

The experiments composed of 28 nude mice, which were randomly assigned to four experimental groups. The four experimental groups were as follows: untreated control group, rapamycin treatment group, sorafenib treatment group, and rapamycin combined sorafenib treatment group. Rapamycin (2 mg/kg) was given (orally, once daily) in 100 μL by gavage on day 3 after tumor implantation. The dosage of rapamycin is based on dosages commonly used in murine models of allograft transplantation (9). The mice randomized to sorafenib treatment group were given 30 mg/kg in 100 μL by gavage (orally, once daily) on day 3 after tumor implantation. The mice treated with both rapamycin and sorafenib was administered by using the same schedule for each drug as described for the single treatment. All control mice received an equal volume of carrier solution by gavage.

The mice were sacrificed 5 wk after tumor implantation. At necropsy, tumor volume was measured for largest (a) and smallest (b), and the tumor volume was calculated as $V = a \times b^2/2$. Tumors were either homogenized in tumor lysis buffer for Western blot analysis or fixed in paraformaldehyde for 24 h, and paraffin sections were used for immunohistochemical staining. Paraffin blocks of 10% buffered formalin-fixed samples of lung were prepared and serial sections were cut at 4 μm and stained with H&E to determine the presence of lung metastases. The blood of those killed mice was collected for the examination of serum VEGF level by ELISA.

Western blot analysis. To further determine the changes in downstream effectors that were altered by rapamycin and sorafenib, we did Western blotting analysis. For Western blotting analysis, extracts were prepared from randomly selected three tissue samples of each group with SDS sample buffer. Equal amounts of protein extract were separated on polyacrylamide SDS gels, transferred, and probed with rabbit monoclonal against phospho-p70/S6 Kinase (Thr389), total p70/S6 kinase, phospho-ERK1/2 (phospho-p44/42 mitogen-activated protein kinase, Thr202/Tyr204; Cell Signaling Technology), or total ERK1/2 (Santa Cruz Biotechnology). Detection of the primary antibody was done with an anti-rabbit horseradish peroxidase antibody (DAKO A/S) using the enhanced chemiluminescence (Amersham) Western blotting system. Glyceraldehyde-3-phosphate dehydrogenase (Kang Chen) was detected in the same way as internal control.

Immunohistochemical Ki-67 staining for tumor cell proliferation. Paraffin-embedded tissue sections of all tumors were prepared and were labeled first with a Ki-67-specific monoclonal rat antibody (DAKO A/S), followed by staining with a biotinylated antirat immunoglobulin antibody (DAKO A/S). The biotinylated antibody was then detected histochemically using the DAKO StrepABComplex staining kit. The color reaction was visualized with diaminobenzidine, and tissues were counterstained with Mayer's hematoxylin. The Proliferation Index was determined by Ki-67 immunostaining and calculating the number of 3,3′-diaminobenzidine–positive cells per total number of cells (hematoxylin-positive plus 3,3′-diaminobenzidine–positive cells) in 5 randomly selected fields at ×200.

Detection and quantitation of apoptosis. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method was based on the specific binding of terminal deoxynucleotidyl transferase to the 3′-OH ends of DNA, ensuring the synthesis of a polydeoxynucleotide polymer. For this purpose, the ApopTagPlus *In Situ* Apoptosis Detection kit-Peroxidase (Oncor) was used. Briefly, after routine deparaffinization, rehydration, and blocking of endogenous peroxidase with 0.3% H_2O_2 in methanol for 30 min at room temperature, the tissue sections of all tumors were digested with 20 μg/mL proteinase K for 15 min at room temperature. After they were washed in distilled water, Equilibration Buffer was applied to the sections for 60 s at room temperature, followed by incubation with Working

Strength Terminal Deoxynucleotidyl Transferase Enzyme, and then they were covered with a coverslip in a humidified chamber for 60 min at 37°C. The reaction was terminated in prewarmed Working Strength Stop/Wash Buffer for 30 min at 37°C. After being washed in PBS, the sections were covered with Anti–Digoxigenin-Peroxidase for 30 min at room temperature, followed by color development with 3, 3′-diaminobenzidine-H_2O_2 solution. The sections were counterstained with hematoxylin. To confirm the staining specificity, the TUNEL procedure was modified as follows: for the positive control, control slides (ApopTag kit) were stained as described above; and the negative control sections were obtained by substituting distilled water for terminal deoxynucleotidyl transferase. The stained sections of tumors of each group were reviewed, and the Apoptosis Index, determined by TUNEL staining, was determined by counting at least 1,000 cells in 5 randomly selected high-power fields (magnification, ×200).

Quantitation of blood vessels in solid tumors. The sections of all tumors from LCI-D20 were stained with biotinylated anti-mouse CD31 monoclonal antibody (BD PharMingen) followed by streptavidin-peroxidase and a diaminobenzidine-reaction system for immunohistochemical assessment of tumor microvessels in 5 randomly selected fields at ×200. To stop the staining reaction, slides were placed in 4% formaldehyde-acetate buffer (pH 5) for 10 min. Counterstaining was done with hematoxylin.

ELISA. Blood specimens were obtained from mice of each group after termination of *in vivo* experiments, and the serum was separated and stored at -20°C until measurement of cytokine levels by ELISA. A VEGF ELISA kit (Oncogene TM) was used to measure VEGF protein levels in the mice serum.

Statistics. Tumor volumes and histologic quantitations were analyzed using Student's *t* test. Categorical variables were tested with the use of Fisher's exact test. Two-tailed *P* values of <0.05 were considered statistically significant.

Results

***Effect of rapamycin alone and in combination with sorafenib on primary tumor growth in HCC* in vivo.** Changes in the phosphorylation levels of key proteins in phosphatidylinositol-3-OH kinase/AKT/mTOR and Ras/Raf/mitogen-activated protein extracellular kinase/ERK pathway were determined by Western blot analysis to evaluate the selective inhibition of the targets of rapamycin and sorafenib. Western blotting analysis showed that the dosage of rapamycin and sorafenib used in this study was sufficient to inhibit the phosphorylation of p70/S6 Kinase, and phosphorylation of ERK1/2 in the tumors, which were the key downstream effectors of phosphatidylinositol-3-OH kinase/AKT/mTOR and Ras/Raf/mitogen-activated protein extracellular kinase/ERK pathway, respectively (Fig. 1). Total ERK and p70/S6 Kinase levels were unchanged.

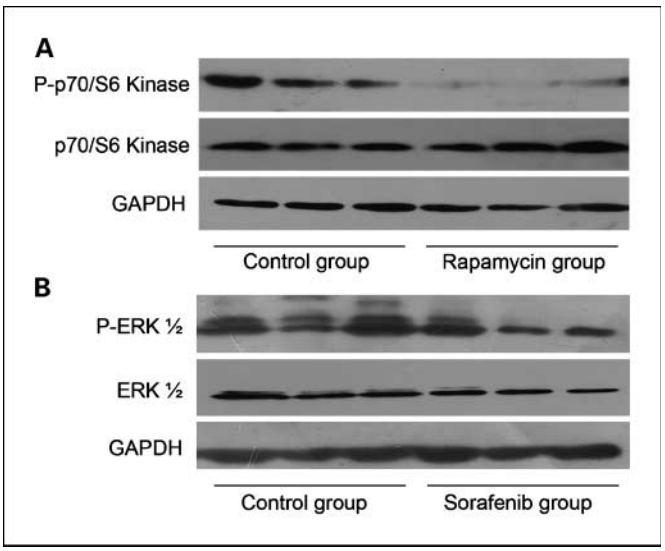

Fig. 1. Rapamycin and sorafenib inhibit downstream targets. Extracts were prepared from randomly selected three tissue samples of each group with SDS sample buffer, and immunoblotted with phosphospecific antibodies. Membranes were stripped and reprobed with antibodies to measure total levels of each protein. glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) was used as a loading control. *A,* rapamycin reduces the phosphorylation level of p70/S6 Kinase in LCI-D20 tumors in mice. *B,* sorafenib reduces the phosphorylation level of ERK1/2 in LCI-D20 tumors in mice.

In the treatment group, as shown in Fig. 2, statistical differences were found among the groups of control, rapamycin, and sorafenib in LCI-D20 model regarding tumor volume ($P < 0.05$). The average tumor size was 3.06 ± 0.77cm^3 in the control group, 0.99 ± 0.41 cm^3 in the rapamycin-treated group ($P < 0.001$, Mann-Whitney test versus control group), and 1.30 ± 0.59 cm^3 in the sorafenib-treated group ($P < 0.001$, Mann-Whitney test versus control group). The highest suppression of primary tumor growth was found in mice treated with the combination therapy. The average tumor size was 0.37 ± 0.24 cm^3 ($P < 0.001$, Mann-Whitney test versus control group). The combination therapy significantly improved the results achieved in the rapamycin-treated group and the sorafenib-treated group ($P < 0.001$, Mann-Whitney test for comparison between combination therapy–treated group and rapamycin-treated group or the sorafenib-treated group).

Effect of rapamycin alone and in combination with sorafenib on lung metastases. The animals treated with single-agent rapamycin therapy showed a significant suppression of metastatic tumor growth in comparison with the control group ($P < 0.05$, Fisher's Exact test versus control group; Fig. 3; Table 1). Although single-agent sorafenib treatment caused a reduction of the lung metastases of 28.6%, this difference did not reach statistical significance ($P > 0.05$, Fisher's Exact test versus control group). The highest suppression of lung metastases was observed in mice receiving the combination therapy ($P < 0.01$, Fisher's Exact test versus control group).

Effect of rapamycin alone and in combination with sorafenib on tumor cell proliferation in HCC. To determine whether the observed tumor growth suppression was caused by inhibition of cell proliferation, we investigated the effect of rapamycin alone and in combination with sorafenib on tumor cell proliferation as measured by Ki-67 staining. As shown in Fig. 4, the average amount of proliferation index in 5 randomly selected microscopic fields was 62.26 ± 8.94 observed in the control group. Rapamycin and sorafenib caused a substantial decrease in proliferation rate. The average amount of proliferation index was 46.66 ± 11.68 ($P < 0.01$, Student's *t* test versus control group) in rapamycin group, and 50.44 ± 9.65 ($P < 0.01$, Student's *t* test versus control group) in sorafenib group. The combination therapy showed a significantly stronger suppression of tumor cell proliferation in comparison with both single agent–treated groups (proliferation index, 33.36 ± 8.33; $P < 0.01$, Student's *t* test for comparison between combination therapy–treated group and sorafenib-treated group; $P < 0.05$, Student's *t* test for comparison between combination therapy–treated group and rapamycin-treated group).

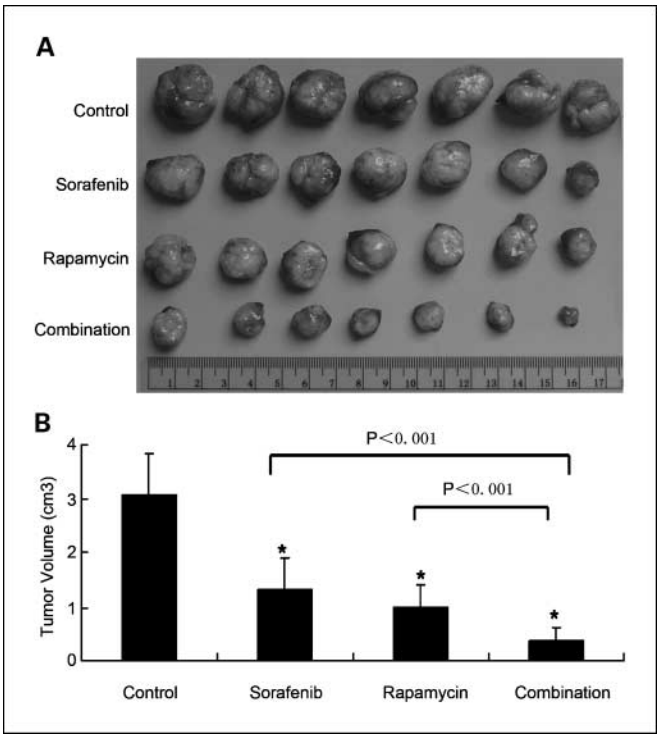

Fig. 2. Effect of rapamycin alone and in combination with sorafenib on primary tumor growth in LCI-D20 xenograft model. Columns, mean (seven samples per group); bars, SD. All statistical tests were two sided. *A,* tumors of mice from each group on 5 wk after tumor implantation. *B,* average tumor volume of each group on 5 wk after tumor implantation. *, $P < 0.001$, Student's *t* test versus control group.

Effect of rapamycin alone and in combination with sorafenib on apoptosis in HCC. To further investigate the mechanism of the observed tumor-suppressive activities, we examined the effect of rapamycin alone and in combination with sorafenib on tumor cell apoptosis by TUNEL (Fig. 4). The average number of apoptosis index measured in 5 randomly selected microscopic fields in the control group was 0.68 ± 0.37. The rapamycin-treated group showed a significant increase in the number of apoptosis index. The calculated average was 2.87 ± 0.86 ($P < 0.001$, Student's *t* test versus control group). A significant increase of apoptosis index was also observed in the combination therapy–treated group with 3.00 ± 0.89 ($P < 0.001$, Student's *t* test versus control group). No significant difference was observed between the sorafenib-treated group (apoptosis index, 0.73 ± 0.50) and the control group ($P > 0.05$, Student's *t* test). The combination therapy did not improve the results achieved in the rapamycin-treated group ($P > 0.05$, Student's *t* test for comparison between the rapamycin-treated group and the combination therapy–treated group).

Effect of rapamycin alone and in combination with sorafenib on tumor angiogenesis in HCC. Because recent investigations suggest that rapamycin may have antiangiogenic activities linked to a decrease in production of VEGF and to a markedly inhibited response of vascular endothelial cells to stimulation by VEGF (10), we were interested in determining the effect of rapamycin alone and in combination with sorafenib on tumor angiogenesis. Therefore, we stained tumor sections with a rat antimouse monoclonal CD31 antibody (Fig. 4). The average number of CD31-positive vessels measured in the control group was 614.1 ± 122.2. We found a significant decrease of tumor vessels measured in the rapamycin-treated group. The average number of stained vessels was 407.7 ± 60.7 ($P < 0.001$, Student's *t* test versus control group). As expected, we also found a significant inhibition of tumor angiogenesis in the group treated with only sorafenib. The average number of stained vessels was 357.3 ± 84.3 ($P < 0.001$, Student's *t* test versus control group). An additional effect on tumor angiogenesis was observed in the group that received both drugs. The average number of stained blood vessels was 248.7 ± 55.3 ($P < 0.001$, Student's *t* test versus control group). The combination therapy–treated group showed significantly fewer tumor vessels than the group treated with rapamycin alone ($P < 0.01$, Student's *t* test for comparison between the combination therapy–treated group and the rapamycin-treated group), and the group treated with sorafenib alone ($P < 0.05$, Student's *t* test for comparison between the combination therapy–treated group and the sorafenib-treated group).

The effects of rapamycin alone and in combination with sorafenib on VEGF production were also investigated by measurement of the circulating levels of VEGF in tumor-bearing mice. Treatment with rapamycin alone and in combination with sorafenib resulted in a significant reduction in serum VEGF levels. The mean serum VEGF levels, measured 5 weeks after tumor implantation, was 162.4 ± 63.1 pg/mL in the untreated control mice, and 68.8 ± 28.1 pg/mL in rapamycin treated in mice ($P < 0.01$, Student's *t* test versus control group), and 87.8 ± 21.9 pg/mL in sorafenib treated in mice ($P < 0.05$, Student's *t* test versus control group). An additional effect on circulating levels of VEGF was observed in the group that received both drugs (39.9 ± 14.5 pg/mL; $P < 0.05$, Student's *t* test for comparison between the combination therapy–treated group and the two monotherapy group).

Discussion

Our data indicated that rapamycin, alone and in combination with sorafenib, strongly inhibited primary and metastatic tumor growth in HCC *in vivo*. Furthermore, we could show that the combination therapy inhibited primary tumor volume of mice more assertively than treatment with a single agent.

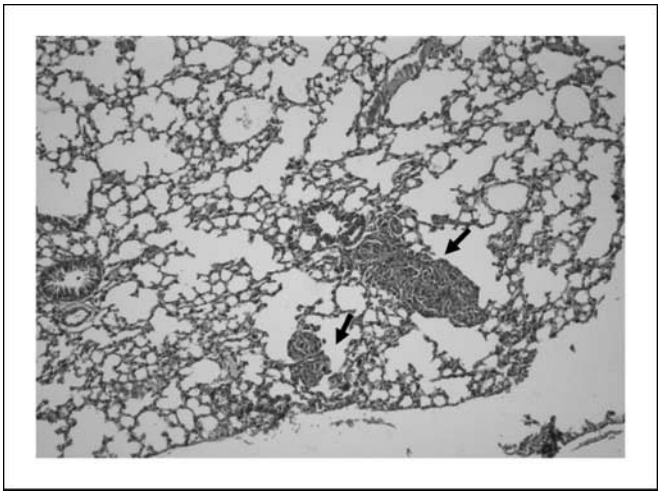

Fig. 3. The representative pulmonary metastasis (*arrows*) in LCI-D20 xenograft model (magnification, ×40).

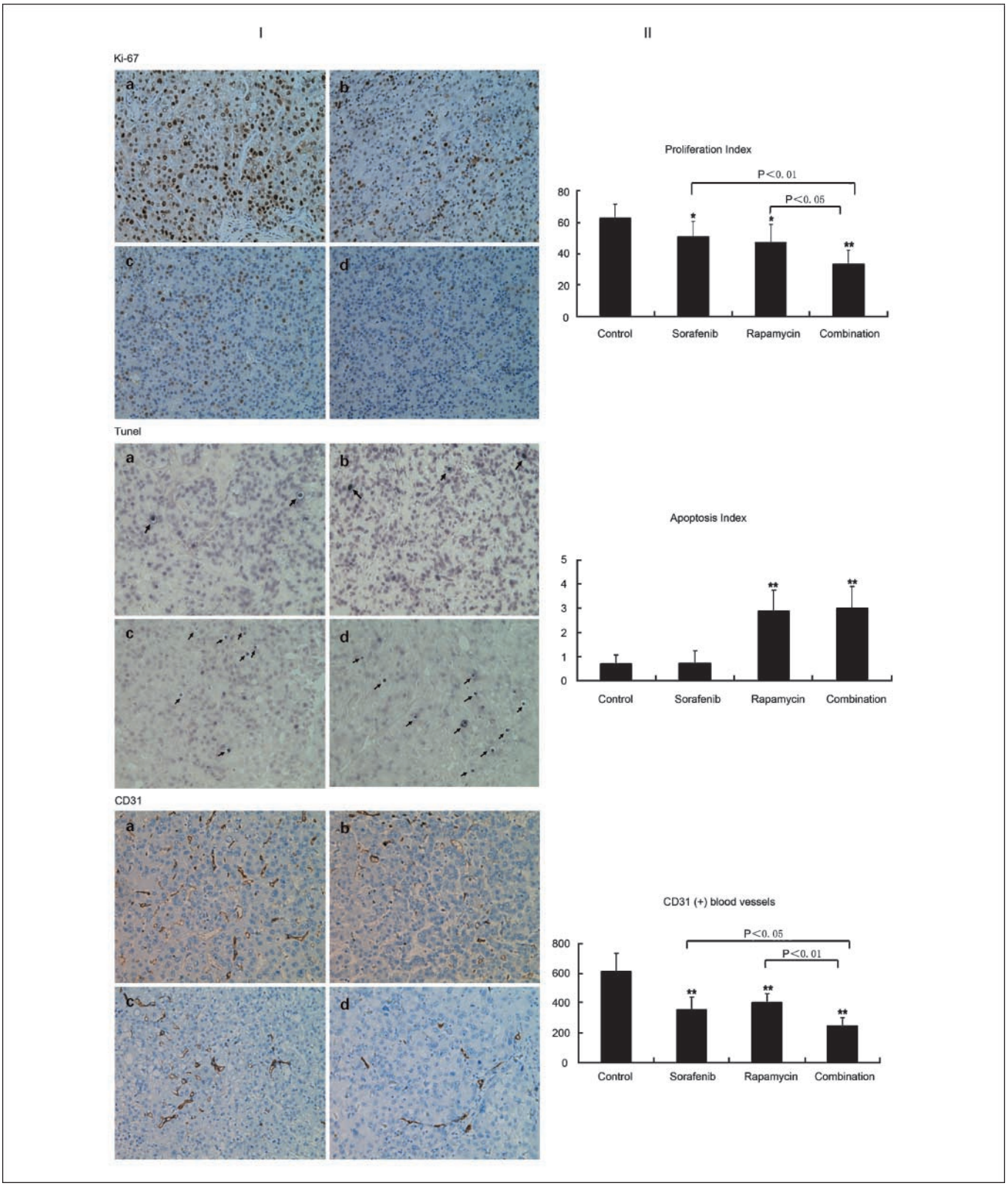

Fig. 4. *In vivo* mechanism of action of rapamycin alone and in combination with sorafenib in xenograft model. Tumors were collected 5 wk after tumor implantation, formalin fixed for 24 h, and then analyzed by immunohistochemistry. *I,* histologic examination of LCI-D20 tumor xenografts (5 wk) stained with anti – Ki-67 nuclear antigen (*brown*), apoptosis by TUNEL assay (*arrows,* hyacinthine), and anti-CD31 vessel staining (*brown*). Magnification, ×200. Tumors from mice were randomly assigned to treat with vehicle (100 μL, orally by gavage, once daily; *a*), sorafenib (30 mg/kg, orally by gavage, once daily; *b*), rapamycin (2 mg/kg, orally by gavage, once daily; *c*), or rapamycin (2 mg/kg, orally by gavage, once daily) plus sorafenib (30 mg/kg, orally by gavage, once daily; *d*). *II,* quantitation of Ki-67 staining Proliferation Index, Apoptosis Index, and CD31 vessel staining from immunohistochemical analysis. Columns, mean (seven samples per group); bars, SD. All statistical tests were two sided. *, $P < 0.01$, Student's *t* test versus control group; **, $P < 0.001$, Student's *t* test versus control group.

Table 1. Effect of rapamycin alone and in combination with sorafenib on lung metastasis in LCI-D20 xenograft model

Treatment group	Mice-bearing pulmonary metastasis/ total mice	Percentage
Control	7/7	100%
Sorafenib	5/7	71.4%
Rapamycin	2/7	42.9%*
Combination	1/7	14.3%*

*$P < 0.05$, Fisher's Exact test versus control group. All statistical tests were two-sided.

To explain the potential antitumor effect of rapamycin alone and in combination with sorafenib, we considered three basic theories.

First, rapamycin may directly inhibit tumor cell proliferation. Rapamycin inhibits basal p70s6K activity and induces dephosphorylation of p70s6K and 4E-BP-1, and thereby inhibits cap structure–dependent protein synthesis and cell growth (11). Sorafenib can also inhibit the growth of some types of tumor by inhibition of mitogen-activated protein kinase pathway. Our results *in vivo* provided supportive evidences for above hypotheses. We found an inhibition of tumor cell proliferation of ~25.1% after single-agent rapamycin treatment, and 19.0% after single-agent sorafenib treatment, respectively. Combination treatment with rapamycin and sorafenib significantly enhanced the antiproliferative effect on HCC *in vivo*. A potential explanation for this sensitization is that multiple proliferation and survival pathways are typically involved in driving the proliferation of HCC cells and, conversely, that inhibition of several of relevant pathways in combination may achieve a synergistic effect on inhibition of cell growth.

Second, the observed antitumor effect of rapamycin alone and in combination with sorafenib may be a result of drug-induced apoptosis. Rapamycin has been shown to induce apoptosis via activation of caspase-3 and disruption of mitochondrial membrane potential, as well as by down-regulation of antiapoptotic protein Bcl-2 and up-regulation of proapoptotic protein Bcl-xl on HCC cells (12). In accordance with these hypotheses, our studies also showed that rapamycin might have an effect on regulation of programed cell death, supported by the fact that a nearly 4-fold induction of tumor cell apoptosis was observed in mice treated by single-agent rapamycin treatment. Sorafenib was recently found to induce apoptosis in several human cancer lines, including HepG2 HCC, by down-regulation the levels of the antiapoptotic protein myeloid cell leukemia sequence 1 (7). Our evaluation, with the use of TUNEL as a marker of apoptotic cells *in vivo*, failed to implicate apoptosis of HCC as a mechanism for the sorafenib-associated reduction in the tumor progression. Further investigations will be necessary to elucidate the relationship of sorafenib and apoptosis.

Angiogenesis is another essential mechanism for HCC growth and metastasis, and its inhibition is an attractive target for investigators. VEGF and its tyrosine kinase receptor are the principal molecules involved in endothelial cell proliferation, survival, formation of new blood vessels, and vascular permeability (13). mTOR proteins regulate the phosphorylation of p70/S6 kinase and the translational repressor protein PHAS-1/4E-BP. Both proteins regulate the translation of proliferation- and angiogenesis-relevant proteins, such as c-*myc*, cyclin-D1, ornithine decarboxylase, HIF-α, and are indirectly involved in the expression of VEGF (14). Rapamycin has showed antiangiogenic activities *in vivo* not only by decreasing the production of VEGF but also by inhibiting the response of vascular endothelial cells on stimulation by VEGF via inhibiting of mTOR (15). Our findings strongly supported this theory because we found a significant inhibition of tumor angiogenesis after single-agent rapamycin treatment. Due to its inhibition of VEGF receptor, sorafenib has also been classified as an antiangiogeneic drug. The inhibitory effect of sorafenib on tumor angiogenesis was shown by a significant reduction in microvessel density and circulating levels of VEGF in the LCI-D20 xenograft model. Approaches to suppress HIF-a by blocking its translation (rapamycin) in combination with inhibition of downstream HIF-a–induced gene products and their functions (sorafenib) are a good example of "vertical blockade." In vertical blockade, the same pathway is targeted at two or more different levels by two or more different agents such as VEGF and its receptor tyrosine kinase. Vertical blockade could also overcome an aspect of resistance that may develop through feedback mechanisms (16). In this study, the combination of rapamycin and sorafenib inhibited targeting of angiogenesis at multiple levels, and amplified the inhibition of tumor angiogenesis from 33.6% after single-agent rapamycin treatment to 59.5%, compared with the control.

Accordingly, the combination therapy has significant advantages compared with the single-agent treatment. Both rapamycin and sorafenib are important for inhibiting tumor cell proliferation and angiogenesis. On the other hand, rapamycin plays a major role in regulating programmed cell death in the combination therapy–treated group.

Although rapamycin is not approved by the Food and Drug Administration for LT recipients, many centers have reported the safety and tolerance of rapamycin as monotherapy or in combination with other drugs in LT recipients (17). The immunosuppressive and antitumor effects of rapamycin share a common mechanism of action. And the most important is that the antitumor activity of rapamycin has been shown at the same concentrations as maintenance target levels in posttransplant patients (10, 18). These effects are reinforced by clinical results with rapamycin in organ transplantation. Complete remission could be achieved in the patients with lung or bulky ovarian metastasis of HCC after LT by conversion to rapamycin-based immunosuppression (19, 20). Our previous clinical studies also showed immunosuppressive regimen with rapamycin could provide apparent clinical benefits to the LT recipient at high risk of HCC recurrence (3). In this study, the significant suppression of primary tumor volume and the reduction of metastasis in the combination therapy–treated group were caused by a combinatorial effect of the immunosuppressive doses of rapamycin and sorafenib.

In addition, there are two other advantages of rapamycin in combination with sorafenib therapy compared with conventional cytotoxic chemotherapy. First, these two agents are orally active and can be taken by patients on outpatient basis. Second, the toxicities of these agents are tolerable and easy to mange. These two advantages could translate into significantly

improved quality of life, which is an important aspect in management of patients with HCC after LT. Actually, clinical trials with CCI-779 or RAD001 (rapamycin analogue), in combination with sorafenib, are now under way in renal cell carcinoma (16).

Taken together, the combination therapy of rapamycin and sorafenib is an attractive approach for combined therapeutic strategies to the treatment and prevention of HCC recurrence after LT. These warrants further investigation in clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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