

Sorafenib inhibits growth of hepatoma with hypoxia and hypoxia-driven angiogenesis in nude mice

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The study aimed to investigate the effects of sorafenib on growth of hepatoma with hypoxia and angiogenesis. Green fluorescent protein (GFP)-expressing SMMC-7721 hepatoma cells were established via transfection with a lentiviral vector carrying GFP. GFP-labelled cells were treated with CoCl₂ for 24 h with or without pretreatment of sorafenib for 30 min, and then injected subcutaneously into nude mice to induce hepatoma xenografts. This study used 16 BALB/c nude mice, which were divided into 4 groups: control group (GFP-labelled cells), CoCl₂ group (GFP-labelled cells treated with CoCl₂), CoCl₂ plus sorafenib group (GFP-labelled cells pretreated with sorafenib and then treated with CoCl₂) and CoCl₂ plus sorafenib (cell+i.g.) group (GFP-labelled cells pretreated with sorafenib and then treated with CoCl₂). After injection, CoCl₂ plus sorafenib (cell+i.g.) group received intragastrical administration of sorafenib daily for 40 days. Tumor volume and weight were measured for each mouse. Cy5.5-annexinV and *in vivo* bioluminescence imaging were used for *in vivo* detection of cell apoptosis in tumor. Vascular endothelial growth factor (VEGF) and CD 34 were detected by immunohistochemistry. The results showed that, under the hypoxia condition induced by CoCl₂, sorafenib pretreatment combined with intragastric administration of sorafenib more obviously suppressed tumor growth and decreased VEGF expression and MVD compared to sorafenib pretreatment alone, and induced cell apoptosis as well. Sorafenib pretreatment combined with intragastric administration is more effective than sorafenib pretreatment alone in the therapy of hepatoma.

Key words: VEGF, cobalt chloride, CD 34, tumor growth, in vivo bioluminescence imaging

Liver cancer is one of the most common cancers worldwide with the mortality rate ranking third among all cancers [1]. Hepatocellular carcinoma (HCC) is the primary type of liver cancer. Surgery removal is the standard therapy for hepatoma. Nevertheless, it is often not feasible because hepatoma is detected at late stage. Due to limited donors livers, option of liver transplantation is impossible for many patients. Therefore, investigation of the tumorigenesis of hepatoma is necessary for developing novel and effective medicines against hepatoma.

Hypoxia caused by uncontrollable cell proliferation is a common feature of hepatoma and strongly associated with aggressiveness of hepatoma [2]. Hypoxic microenvironment and angiogenesis have been intensively studied in molecular mechanisms. Cobalt chloride (CoCl₂) is a hypoxia-mimetic agent used to establish hypoxia model [3]. Dai Z J. *et al.* have reported that hypoxia stimulated by CoCl₂ could effectively potentiate apoptosis of pancreatic cancer cells [4]. In addition, Zhang B O *et al.* have found that CoCl₂ treatment inhibits

proliferation of osteosarcoma cells [5]. However, there is evidence that hypoxia can decrease apoptotic cell death [6] and promote cell HCC cellular growth [7]. These evidences suggest that there remain controversies concerning the exact effect of hypoxia on cancer development. Furthermore, it has been reported that hypoxia caused by cobalt chloride modulates the expression of vascular endothelial growth factor (VEGF) by regulating hypoxia inducible factor-1 α (HIF-1 α) and insulin-like growth factor I (IGF-1) [8].

Sorafenib is an approved drug for treatment of advanced hepatocellular carcinoma (HCC) [9, 10]. It inhibits various tyrosine protein kinases, such as VEGF receptors and platelet-derived growth (PDGF) receptors [11]. Moreover, Satoshi S *et al.* have found that sorafenib administration induces autophagy contributing to the antitumor activity of sorafenib in HCC [12, 13]. Sorafenib may restrain proliferation of HCC cells via downregulating PI₃K/AKT pathway [14]. Besides, it has been proved that sorafenib inhibits angiogenesis through

regulating extracellular signal-regulated kinase (ERK) pathway in HCC [15]. However, there are few studies focused on the impact of sorafenib on hepatoma cells treated with CoCl_2 mimicking hypoxia. Although Ming X *et al.* have provided *in vitro* evidence that sorafenib suppresses CoCl_2 -induced tumor cell invasiveness and induce cell apoptosis in hepatoma cells [16]. *In vivo* studies are needed to unravel the effect of Sorafenib on hypoxia-treated hepatoma growth and angiogenesis.

In this study, we constructed green fluorescent protein (GFP)-expressing SMMC-7721 hepatoma cells, which were treated with CoCl_2 to establish hypoxia model. In order to investigate the impact of sorafenib and CoCl_2 on tumor growth, GFP-expressing SMMC-7721 cells treated with or without CoCl_2 or sorafenib were injected subcutaneously into nude mice to form tumors. *In vivo* bioluminescence imaging was used for *in vivo* detection of tumor cell apoptosis. VEGF and cluster of differentiation (CD)34 expression (microvessel density) were detected immuno-histologically to reflect the effect of sorafenib and CoCl_2 on tumor angiogenesis.

Materials and methods

Cell culture. SMMC-7721 hepatoma cells were purchased from Shanghai cell bank of Chinese academy of sciences and cultured in Dulbecco's modification of Eagle's medium Dulbecco (DMEM, GIBCO, NY, USA) medium supplemented with 10% fetal bovine serum (FBS, GIBCO, NY, USA) in a 5% CO_2 incubator at 37°C. The study was approved by animal care committee of our hospital.

Lentivirus infection. SMMC-7721 cells were seeded in a six-well plate (2×10^5 cells/plate). The cells were cultured in complete medium and transfected with a lentiviral vector (LV) carrying green fluorescent protein (GFP) of our lab for 24 h. The cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan). Subsequently, the cells underwent selection with 4 $\mu\text{g}/\text{mL}$ puromycin (Sigma, CA, USA). After 3 passages, the GFP-expressing cells were used for further experiments.

Cell treatment. GFP-labelled SMMC-7721 cells were randomly divided into 3 groups: control group, CoCl_2 group and CoCl_2 +sorafenib group. Cells in the CoCl_2 +sorafenib group were pretreated with 4 $\mu\text{m}/\text{L}$ sorafenib for 30 min and then treated with 150 $\mu\text{m}/\text{L}$ CoCl_2 (Sigma, CA, USA) for 24 h. The CoCl_2 group was pretreated with DMSO (vehicle of sorafenib, Sigma, CA, USA) for 30 min, and then incubated with 150 $\mu\text{m}/\text{L}$ CoCl_2 (Sigma, CA, USA) for 24 h. The control group was incubated with complete medium for the same duration. These cells were then used for animal experiments.

Animal experiments. A total of 16 male SPF (specific pathogen free) BALB/c nude mice (4-week old) were provided by the experimental animal center of Shanghai Jiao Tong University. These mice were divided into 4 groups ($n = 4$): control group, CoCl_2 group, CoCl_2 plus sorafenib group and CoCl_2 plus sorafenib (cell+i.g.) group. Mice in the control group received a subcutaneous injection of control GFP-labelled

SMMC-7721 cells ($2 \times 10^7/\text{ml}$) in 0.2 ml serum-free medium. Mice in the CoCl_2 group were subcutaneously injected with 0.2 ml cell suspension made of CoCl_2 treated-GFP-labelled SMMC-7721 cells. Mice both in the CoCl_2 plus sorafenib group and CoCl_2 plus sorafenib (cell+i.g.) group received a subcutaneous injection of GFP-labelled SMMC-7721 cells ($2 \times 10^7/\text{ml}$) treated with sorafenib and CoCl_2 in 0.2 ml serum-free medium. Moreover, the mice in the CoCl_2 plus sorafenib (cell+i.g.) group then were administrated intragastrically with sorafenib (60 mg/kg) daily for 40 days. The growth of subcutaneous tumors and body weight were evaluated by measurement of the length and width of tumors using vernier gauges at 2 days, 9 days, 16 days, 23 days, 30 days and 40 days after injection. Tumor size was calculated as follow: length \times width² \times 0.4. At the end of the experiments (40 days), the mice were sacrificed with carbon dioxide and the tumor weight of each mouse was weighed.

In vivo bioluminescence imaging. After anaesthetized by isoflurane inhalation, the mice bearing subcutaneous GFP-labelled SMMC-7721 cells tumors in different groups were placed supine in the IVIS[®] chamber and visualized by *in vivo* bioluminescence imaging (IVIS Lumina II; Caliper Life Sciences, Hopkinton, MA; λ_{exc} : 480 nm, λ_{em} : 520 nm). Light emission was measured using Living Image software. The resulting pseudo-color images stood for the spatial distribution of photon counts within the tumor. Identical illumination settings were applied in obtaining all images.

Annexin V [17] (Sigma, St. Louis, Missouri, USA) were conjugated to Cy5.5 in 0.1 M sodium carbonate/bicarbonate buffer (pH 9.3). Briefly, 2 μg Cy5.5-annexinV were injected into each mice intravenously via a tail vein. After 24 h, the mice were anesthetized with isoflurane and visualized by *in vivo* bioluminescence imaging (λ_{exc} : 660 nm). Fluorescence images were obtained and average fluorescent value in select regions of interest (ROI) were quantified with IVIS[®] software.

IHC for detection of VEGF and CD34. VEGF and CD34 in tumor tissues were detected by immunohistochemistry. Subcutaneous tumor were removed from mice and washed with pre-cooled PBS buffer and fixed in 10 % paraformaldehyde for 24 h. The tissue samples were dehydrated via gradient ethanol immersion, clarified with dimethylbenzene and embedded in paraffin. The paraffin-embedded tissue samples were cut into sections at 6 μm . After dewaxing using a xylene-ethanol series and antigen retrieval, the tissue samples were incubated with 3% H_2O_2 for 10 min, blocked with goat serum for 1 h. Subsequently, the tissue sections were incubated with primaries antibodies (Santa cruz, Argentina; mouse monoclonal antibody; VEGF, sc-53462; CD 34, sc-74499) over night at 4°C, and incubated with secondary antibody (peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L) 111-035-003, Jackson ImmunoResearch Laboratories, West Grove, PA) at 37°C for 1 h. Finally, the tissue sections were stained using diaminobenzidine tetrahydrochloride (Golden Bridge Biological Technology, China). Brown staining was considered positive. Images were analyzed using Image-Pro

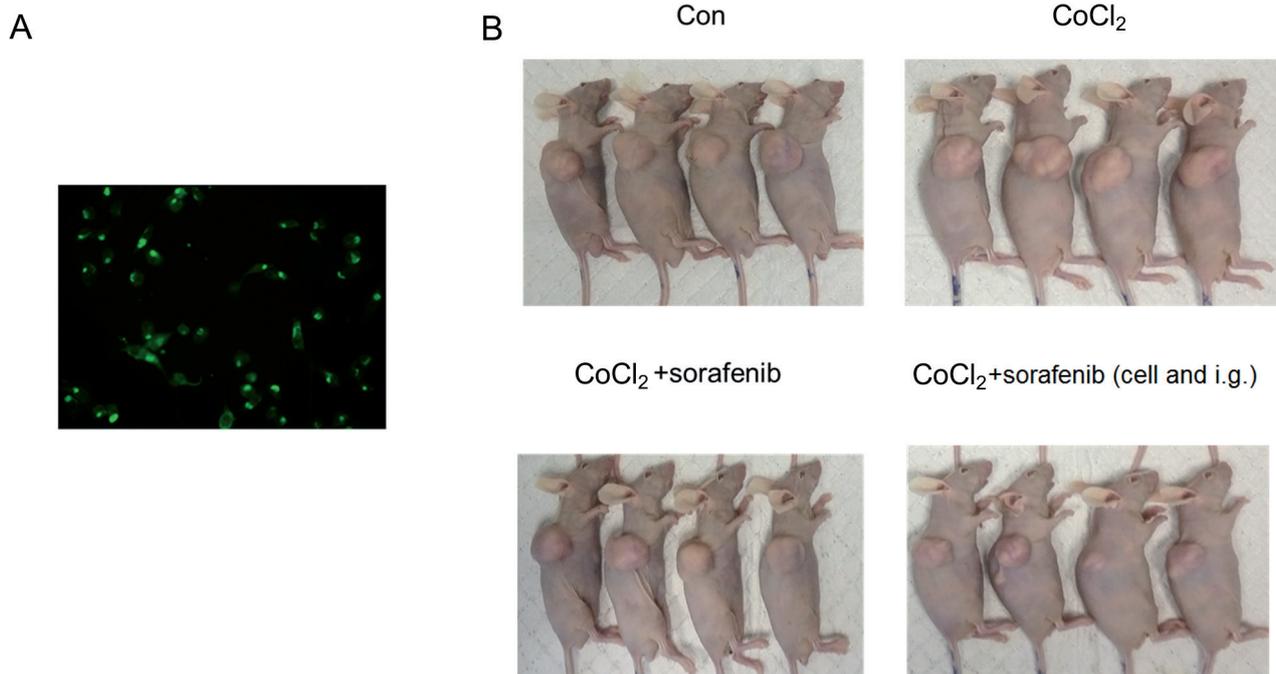


Figure 1. GFP-expressing SMMC-7721 cells after 3 passages (A) and the tumor size of mice in different groups after the mice were sacrificed at the end of the experiments (40 days)(B).

plus 6.0 image analysis software. Five view fields of each slice was randomly selected and observed under a light microscope (CKX31, Olympus, Center Valley, PA, USA).

Statistical analysis. Data were expressed as mean \pm SEM. Statistical analysis were performed using SPSS software (SPSS Inc., Chicago, USA). The differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by the post hoc least significant difference test. P -value < 0.05 was considered significant.

Results

GFP-expressing SMMC-7721 hepatoma cells were established and sorafenib suppressed growth of hepatoma with hypoxia in nude mice. As shown in Figure 1A, GFP-expressing SMMC-7721 cells were successfully established. GFP-expressing SMMC-7721 cells were injected subcutaneously into nude mice for establishment of a hepatoma transplantation model. These mice were divided into 4 groups ($n = 4$): control group, CoCl₂ group, CoCl₂ plus sorafenib group and CoCl₂ plus sorafenib (cell+i.g.) group. Figure 1B displayed the tumor size of mice in different groups after the mice were sacrificed at the end of the experiments (40 days). In addition, tumor volume, tumor weight and body weight were measured at 2 days, 9 days, 16 days, 23 days, 30 days and 40 days. As shown in Figure 2 A, tumor volume were significantly decreased in the CoCl₂ plus sorafenib group and CoCl₂ plus sorafenib (cell+i.g.) group relative to the control group

($p < 0.05$). The CoCl₂ plus sorafenib (cell+i.g.) group had further smaller tumor than the CoCl₂ plus sorafenib group ($p < 0.05$). The difference between the control group and the CoCl₂ group was not significant ($p > 0.05$). With regard to tumor weight (Figure 2B), only the CoCl₂ plus sorafenib (cell+i.g.) group had markedly decreased tumor weight compared to the control group ($p < 0.01$). Tumor weight did not significantly differ between control group, CoCl₂ group and CoCl₂ plus sorafenib group. Additionally, there was no significant difference in body weight between the mice in 4 groups (Figure 2C, $p > 0.05$).

Sorafenib pretreatment and intragastric administration induced cell apoptosis in tumor under hypoxia condition. *In vivo* imaging revealed that fluorescence intensity of mice in difference groups (Figure 3A). Moreover, fluorescently labeled annexin V (Cy5.5-annexinV) was used as a marker to detect apoptotic cells in tumor *in vivo*. As shown in Figure 3B-C, fluorescence intensity in the CoCl₂ plus sorafenib (cell+i.g.) group were obviously higher than that in the control group, CoCl₂ group and CoCl₂ plus sorafenib group ($p < 0.05$). The differences between control group, CoCl₂ group and CoCl₂ plus sorafenib group did not achieve significance ($p > 0.05$). These observations suggest that treatment of CoCl₂ plus sorafenib (cell+i.g.) promotes cell apoptosis in tumor *in vivo*.

Sorafenib pretreatment and intragastric administration inhibited angiogenesis under hypoxia condition. Microvessel density (MVD) is regarded as a surrogate marker for tumor angiogenesis and is detected by immunohistochemically by

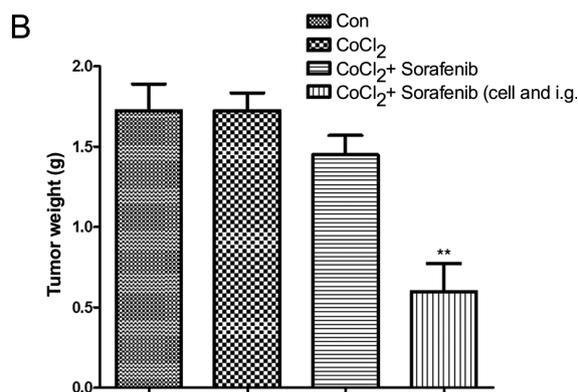
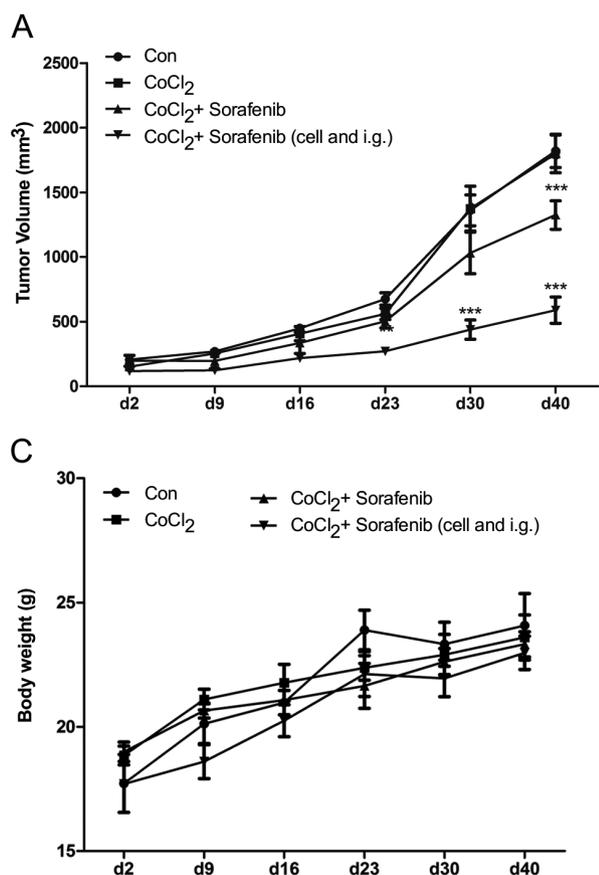


Figure 2. Tumor volume, tumor weight and body weight of mice in different groups. A, tumor volume of mice in different groups at 2 days, 9 days, 16 days, 23 days, 30 days and 40 days after injection of SMMC-7721 cells. B, tumor weight of mice in different groups at 40 days after injection of SMMC-7721 cells. C, body weight of mice in different groups at 2 days, 9 days, 16 days, 23 days, 30 days and 40 days after injection of SMMC-7721 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$ compared with the control group.

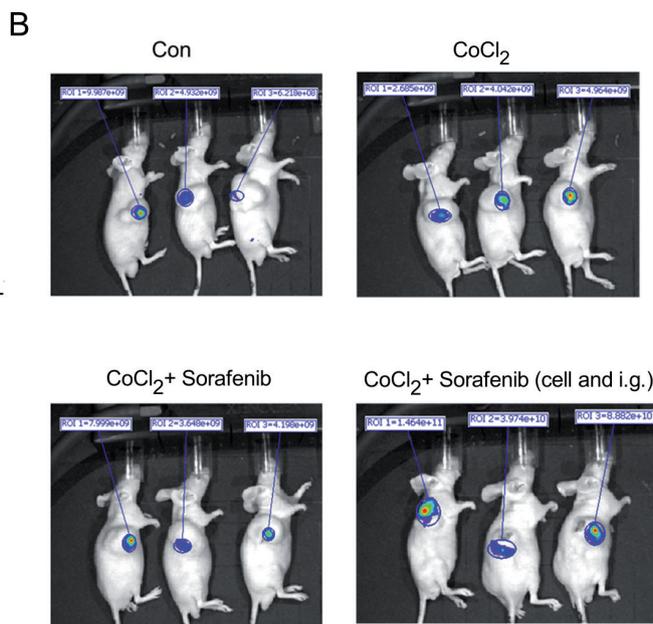
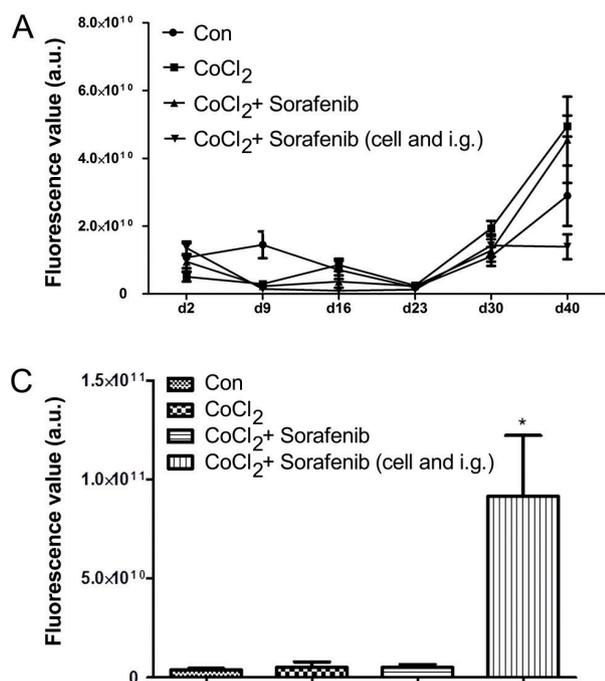


Figure 3. Whole Animal *In Vivo* Imaging. A, fluorescence intensity of mice in difference groups. GFP-expressing SMMC-7721 cells after different treatments are injected into nude mice of different groups to induce hepatoma xenografts. B-C Fluorescence value of Cy5.5-annexinV detected by *in vivo* bioluminescence imaging of mice in different groups. * $p < 0.05$ compared with the control group.

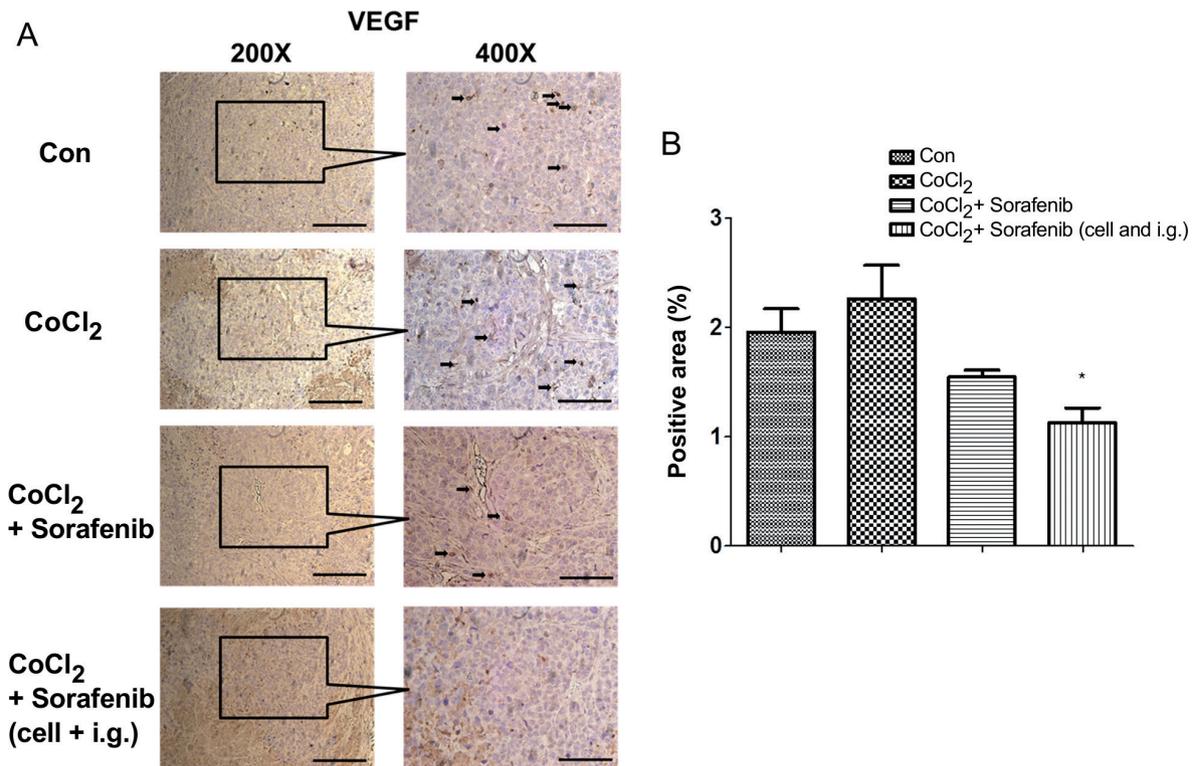


Figure 4. VEGF expression detected by immunohistochemistry. A, immunohistochemical staining images of VEGF in different groups. B, quantitative analysis of VEGF immunohistochemical staining in different groups. * $p < 0.05$ compared with the control group.

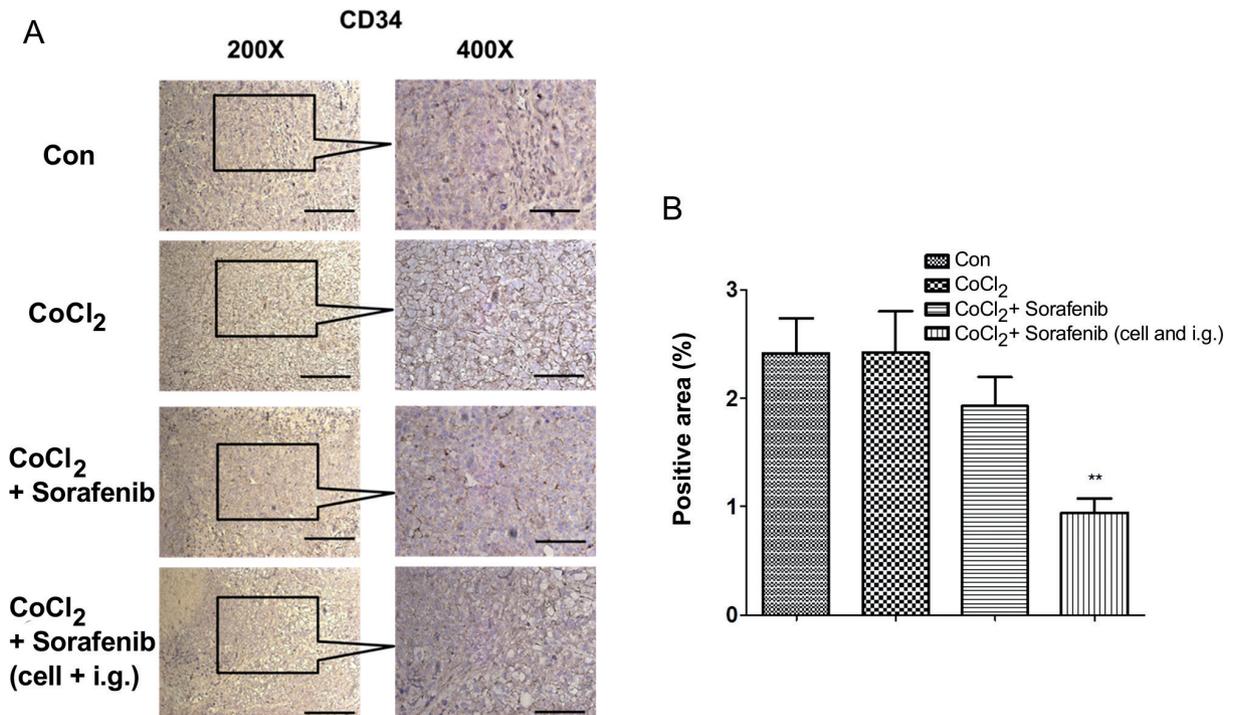


Figure 5. CD 34 expression detected by immunohistochemistry. A, immuno- histochemical staining images of CD34 in different groups. B, quantitative analysis of CD34 immunohistochemical staining in different groups. ** $p < 0.01$ compared with the control group.

antibody against CD 34 [18, 19]. VEGF is a angiogenesis stimulating signal in HCC [20]. Expression of VEGF and CD34 was measured by IHC to evaluate the impact of CoCl₂ and sorafenib on tumor-related angiogenesis. Compared with control group, positive staining area of VEGF was remarkably decreased in the CoCl₂ plus sorafenib (cell+i.g.) group ($p < 0.05$, Figure 4). However, the differences between the control group, CoCl₂ group and CoCl₂ plus sorafenib group did not reach significance ($p > 0.05$).

Similarly, CoCl₂ plus sorafenib (cell+i.g.) group had obviously decreased positive staining area of CD34 than the control group ($p > 0.05$, Figure 5). Nonetheless, positive staining area of CD34 was not significantly different between the control group, CoCl₂ group and CoCl₂ plus sorafenib group ($p > 0.05$).

Discussion

Hepatoma remains a major threat to the lives of people. Hypoxia caused by uncontrollable proliferation of tumor cells results in hypoxia, which induces angiogenesis. The study was centered on the influence of sorafenib on hepatoma xenografts induced by hepatoma cells treated with CoCl₂ in nude mice. One feature of the study was that GFP-expressing SMMC-7721 hepatoma cells were established to induce hepatoma xenografts in nude mice for *in vivo* bioluminescence imaging. Moreover, fluorescently labeled annexin V (Cy5.5-annexinV) was used to detect cell apoptosis *in vivo*. The study delivers *in vivo* evidence that sorafenib suppresses growth of hepatoma with hypoxia and hypoxia-driven angiogenesis. Furthermore, sorafenib pretreatment combined with continued intragastric administration exerted a stronger inhibitory effect on growth of hepatoma xenografts in nude mice than sorafenib pretreatment alone and potentiated cell apoptosis.

Sorafenib is safe and efficacious for patients with advanced HCC [21]. Recently, Ming X *et al.* have reported that sorafenib treatment inhibits cell proliferation and strengthens apoptosis in hepatoma cells *in vitro* [16]. In consistence with the finding, the present study found that sorafenib pretreatment suppressed growth of tumor induced by hypoxia-treated SMMC-7721 cells in nude mice. Moreover, compared with sorafenib pretreatment alone, sorafenib pretreatment combined with intragastric administration of sorafenib appeared to exert a stronger inhibitory effect on tumor growth and induced apoptotic death of tumor cells. These findings indicate that sorafenib pretreatment in conjunction with intragastric administration of sorafenib is more effective than sorafenib pretreatment alone. Continued administration of sorafenib is important for anticancer therapy of hepatoma cells in a hypoxic state.

It has been established that cobalt chloride-mimicking hypoxia leads to increased expression of VEGF [22]. Moreover, cobalt chloride treatment stimulates HIF-1 α /VEGFA pathway [16], which is inhibited by sorafenib [8]. Similarly, the study also found that cobalt chloride treatment resulted in increased VEGF expression and MVD. Sorafenib pretreatment alone

inhibited expression of VEGF and MVD induced by cobalt chloride. Moreover, a combination of sorafenib pretreatment and intragastric administration of sorafenib further decreased VEGF expression and MVD to a lower level in comparison with sorafenib pretreatment alone. These results reveal that sorafenib inhibits hypoxia-driven angiogenesis in hepatoma. Sorafenib pretreatment and intragastric administration of sorafenib appears to exert a stronger inhibitory impact on angiogenesis. Sorafenib may inhibit tumor growth via disturbing hypoxia-driven angiogenesis.

It is a preliminary study. We did not provide data about whether sorafenib treatment alone could lead to tumor growth suppression, which is a limitation of our study. Performing sorafenib treatment alone will provide a strong evidence to support the inhibitory effect of sorafenib on hepatoma cells under hypoxic condition. Moreover, considering the findings obtained from previous studies [12-15], more experiments are needed to uncover the molecular mechanisms behind the inhibitory effect of sorafenib on hepatoma cells under hypoxic condition. In addition, only 4 mice/group were used for *in vivo* study. The small sample size will influence the convince of our results. Thus, at least 6 mice/ group should be used for *in vivo* study in the future. Besides, there was no effect of CoCl₂-induced hypoxia on hepatoma in our study. The exact effect of hypoxia on cancer development remains elusive and need to be further investigated.

Conclusion

The study suggests that sorafenib inhibits growth of hepatoma with hypoxia and hypoxia-driven angiogenesis. Sorafenib pretreatment combined with intragastric administration induces hepatoma cell apoptosis and is more effective to suppress tumor growth and hypoxia-induced angiogenesis than sorafenib pretreatment alone. The study provides added insights into the effect of sorafenib on hepatoma with hypoxia. Further studies are warranted to verify and expand the findings of this study.

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