Sorafenib Down-regulates Expression of HTATIP2 to Promote Invasiveness and Metastasis of Orthotopic Hepatocellular Carcinoma Tumors in Mice

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BACKGROUND & AIMS: Antiangiogenic agents can sometimes promote tumor invasiveness and metastasis, but little is known about the effects of the antiangiogenic drug sorafenib on progression of hepatocellular carcinoma (HCC). **METHODS:** Sorafenib was administered orally (30 mg · kg^{-1} · day^{-1}) to mice with orthotopic tumors grown from HCC-LM3, SMMC7721, or HepG2 cells. We analyzed survival times of mice, along with tumor growth, metastasis within liver and to lung, and induction of the epithelial-mesenchymal transition. Polymerase chain reaction arrays were used to determine the effects of sorafenib on gene expression patterns in HCC cells. We analyzed regulation of HIV-1 Tat interactive protein 2 (*HTATIP2*) by sorafenib and compared levels of this protein in tumor samples from 75 patients with HCC (21 who received sorafenib after resection and 54 who did not). **RESULTS:** Sorafenib promoted invasiveness and the metastatic potential of orthotopic tumors grown from SMMC7721 and HCC-LM3 cells but not from HepG2 cells. In gene expression analysis, *HTATIP2* was down-regulated by sorafenib. HCC-LM3 cells that expressed small hairpin RNAs against *HTATIP2* (knockdown) formed less invasive tumors in mice following administration of sorafenib than HCC-LM3 without *HTATIP2* knockdown. Alternatively, HepG2 cells that expressed transgenic HTATIP2 formed more invasive tumors in mice following administration of sorafenib. Sorafenib induced the epithelial-mesenchymal transition in HCC cell lines, which was associated with expression of HTATIP2. Sorafenib regulated expression of HTATIP2 via Jun-activated kinase (JAK) and signal transducer and activator of transcription (STAT)3 signaling. Sorafenib therapy prolonged recurrence-free survival in patients who expressed lower levels of HTATIP2 compared with higher levels. **CONCLUSIONS: Sorafenib promotes invasiveness and the metastatic potential of orthotopic tumors from HCC cells in mice, down-regulating expression of *HTATIP2* via JAK-STAT3 signaling.**

Keywords: Tumor Suppressor; Gene Regulation; Tumor Progression; Mouse Model of Liver Cancer.

Liver cancer (mostly hepatocellular carcinoma [HCC]) in men is the fifth most frequently diagnosed cancer worldwide but the second most frequent cause of death from cancer.[1] Despite endeavors to improve prognosis, the overall survival rate is still rather dismal.[2] Angiogenesis inhibitors have been hailed as the beginning of a new era in cancer therapy. The SHARP and ORIENTAL trials showed survival benefits of sorafenib, making it the new standard therapy for patients with advanced HCC.[3,4] However, the survival benefit was only a few months. In addition, many patients required a dosage reduction or cessation of treatment because of the adverse effects of the drug, and some patients with renal cancer had tumor rebound after discontinuing the drug.[5] Furthermore, the tumor may progress during sorafenib treatment, as shown when progression-free survival curves from controls and sorafenib-treated patients are merged.[6]

Numerous animal studies have also suggested that antiangiogenesis drugs may, in certain situations, actually accelerate the spread of cancer,[7,8] which is recognized as a new form of adaptive resistance. Investigations of the main mechanism of the proinvasive or prometastatic effects of antiangiogenic agents have focused on the tumor microenvironment as well as the host environment.[9–13] For example, treatment-induced hypoxia caused increased tumor invasiveness[14–17] and was recognized as a mode of resistance to angiogenesis inhibitors.[9] We have reported that tumor-associated macrophages can be recruited when sorafenib is administered, and these have an important role in the progression of HCC managed with sorafenib, which is also associated with hypoxia.[18] However, sorafenib targets elements in both tumor and endothelial cells, and little is known about the effect of

Abbreviations used in this paper: EMT, epithelial-mesenchymal transition; IHM, intrahepatic metastasis; LV, lentiviral vector; PCR, polymerase chain reaction; RFP, red fluorescent protein.

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sorafenib on the invasiveness and metastatic potential of tumor cells.

In the present study, we found a direct proinvasive effect of sorafenib on tumor cells and explored the underlying mechanism.

Materials and Methods

Cell Culture and Transfection

Eight cell lines were used, including HCC-LM3, SMMC7721, HepG2, and cell lines derived from HCC-LM3[19] and HepG2. LM3-RFP cells were HCC-LM3 cells transfected with red fluorescence protein.[20] The LM3-LV-shHTATIP2 and LM3-LV-shNon cells were obtained by infecting HCC-LM3 cells with lentiviral vectors (LVs) encoding small hairpin RNA for HTATIP2 (LV-shHTATIP2) to eliminate its expression; LV-shNon (transfected with a vector) was a control. HepG2-LV-HTATIP2 and HepG2-LV-Non cells were constructed by LV-HTATIP2 or LV-GFP infection of HepG2-wt (HepG2 without modification) cells, which were gifts from Guo and Zhao's laboratory.[21,22] All of the cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Mouse hepatoma cell lines H22 and hepa1-6 were obtained from American Type Culture Collection (Manassas, VA).

Orthotopic Growth of HCC Tumors in Animal Models

LM3-RFP, LM3-wt (HCC-LM3 without modification), LM3-LV-shNon, LM3-LV-shHTATIP2, HepG2-wt, HepG2-LV-HTATIP2, HepG2-LV-Non, and SMMC7721 cells (1×10^7) were subcutaneously inoculated into the right flanks of 6-week-old BALB/c nu/nu male mice. After 3–4 weeks, non-necrotic tumor tissue was cut into 1-mm^3 pieces and orthotopically implanted into the liver. In the surviving observation group, treatment was started 2 weeks after orthotopic implantation of the tumor, and mice were randomly assigned to receive either a daily oral dose of vehicle solution (control group, n = 9) or a daily oral dose of 30 mg/kg sorafenib (n = 9) until they died. Another group of mice was divided into 2 subsets of 6 or 7 mice each and was treated with vehicle or 30 mg/kg sorafenib for 4 weeks. Tumor samples were then extracted for further analysis.

Orthotopic HCC models derived from H22 and hepa1-6 were established using the same methods in male C57BL/6 and BALB/c mice, respectively. The mice were treated with either 30 mg/kg sorafenib daily or vehicle for 2 weeks (H22/BALB/c model, n = 6) or 4 weeks (Hepa1-6/C57 model, n = 5). Tumor samples were obtained when treatment was stopped.

Drugs and Treatment

Sorafenib (Bayer Healthcare, Leverkusen, Germany) was prepared as previously described.[23] The concentration used in in vitro studies was 1–10 μmol/L, because the concentration of sorafenib in human plasma was between 5 and 7 mg/L, which is 7.8–10.9 μmol/L in humans.[24] U0126 (inhibitor of both MEK1 and MEK2), LY294002 (inhibitor of the phosphoinositide 3-kinase), BAY11-7082 (inhibitor of cytokine-induced IκB-α phosphorylation), and AG490 (inhibitor of Jak-2 protein tyrosine kinase) were purchased from Sigma (St. Louis, MO) and dissolved in dimethyl sulfoxide for further experiments.

Detection of Metastasis by Fluorescent Stereomicroscopy and H&E Staining

Tumors were excised and their largest (a) and smallest (b) diameters were measured to calculate tumor volume as follows: $V = ab^2 \div 2$. The lungs were excised and images of red fluorescent protein (RFP)-positive metastatic foci were obtained (stereomicroscope; Leica, Wetzlar, Germany) (MZ6; illumination, [Leica] L5FL; C-mount, 0.63/1.25; charge-coupled device, DFC 300FX). Integrated optical density was quantified by Image-Pro Plus software (Media Cybernetics, Bethesda, MD).[20,25] Intrahepatic metastasis (IHMs) were observed by fluorescent imaging and quantified as number per liver and were validated by immunohistochemistry and H&E staining.

Flow Cytometry Quantification of Number of Circulating Tumor Cells

Whole blood was mixed with heparin, centrifuged, and stored at −80°C. Red blood cells were lysed, and the rest of the cells were fixed with 4% paraformaldehyde solution. RFP-labeled cells were enumerated with a FACSCalibur Cytometer (BD Biosciences, San Jose, CA), and cell counts were expressed as percentage of total cells (percentage of RFP-positive tumor cells to peripheral blood mononuclear cells).

Polymerase Chain Reaction Microarray Analysis of Gene Expression in Tumors From Control and Treated Mice

Six tumors from each group were pooled to extract total RNA and studied using the Human Cancer PathwayFinder RT^2 Profiler PCR Array according to the manufacturer's instructions (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-033A.html).

Quantitative Real-Time Polymerase Chain Reaction Analysis

Polymerase chain reaction (PCR) procedures are described elsewhere.[18] The following primers for amplification of human genes were used: HTATIP2, forward 5′-TCACCTTCGACG AGGAAGCT-3′ and reverse 5′-GCTCTGCAGACTTCAGA CCA-3′; β-actin, forward 5′-CACCATGAAGATCAAGATCATTG C-3′ and reverse 5′-GGCCGGACTCATCGTACTCCTGC-3′; E-cadherin, forward 5′-TGCCCAGAAAATGAAAAAGG-3′ and reverse 5′-GTGTATGTGGCAATGCGTTC-3′; N-cadherin, forward 5′-CCGGAGAACAGTCTCCAACTC-3′ and reverse 5′-CCC ACAAAGAGCAGCAGTC-3′; and vimentin, forward 5′-ACGCTG CCCTCGGACAAG-3′ and reverse 5′-CCCTCCATCCTCCAGA CGG-3′.

Western Blotting Analysis

Procedures are described elsewhere.[18] Primary antibodies included anti–E-cadherin (Cell Signaling Technology, Danvers, MA); anti–N-cadherin (Abcam, Cambridge, MA); anti-vimentin (Santa Cruz Biotechnology, Santa Cruz, CA); anti-HTATIP2, anti-STAT3, and anti-pSTAT3 (Abcam, Hong Kong); and anti–β-actin (Kangcheng Technology, Shanghai, China).

Cell Proliferation, Migration, and Invasion Assay

LM3-wt, LM3-LV-shHTAITP, LM3-LV-shNon, HepG2-wt, HepG2-LV-HTATIP2, HepG2-LV-Non, and SMMC7721 cells were incubated in 96-well plates (5×10^3 cells/well) for 24, 48, or 72 hours. The procedure for cell proliferation was described previously.[18] Cell migration was assessed by Transwell assay