Circulating Tumor Cells are Correlated with Disease Progression and Treatment Response in an Orthotopic Hepatocellular Carcinoma Model

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Abstract

Hepatocellular carcinoma (HCC) is a highly malignant tumor characterized by rapid progression, poor prognosis, and frequent hematogenous metastasis. A minimally invasive diagnostic biomarker that can predict disease progression and treatment response would be of extraordinary benefit. Therefore, we have investigated whether the number of circulating tumor cells (CTCs) is correlated with disease progression and treatment response in HCC. Here we report that the number of CTCs, monitored by in vivo flow cytometry (IVFC), is strongly correlated with disease progression and treatment response in a highly metastatic orthotopic nude mouse model of green fluorescent protein (GFP)-labeled HCC. Sorafenib treatment reduces the number of CTCs significantly. The decreased number of CTCs is consistent with low lung metastasis. This study has demonstrated a considerable clinical value of CTCs as a biomarker in predicting disease progression and monitoring therapeutic efficacy in patients with HCC.

Key terms
circulating tumor cells; in vivo flow cytometry; hepatocellular carcinoma; disease progression; treatment response

Currently, liver cancer is the fifth most frequently diagnosed cancer worldwide and the second leading cause of cancer-related death in men. In women, it is the seventh most frequently diagnosed cancer and the sixth leading cause of cancer-related death (1). Hepatocellular carcinoma (HCC), the most common type of primary liver cancer, is a highly malignant tumor characterized by rapid progression, poor prognosis, and frequent intra-hepatic and extra-hepatic metastasis (2). The hematogenous spreading of circulating tumor cells (CTCs) from primary HCC is a crucial step in the metastatic cascade and is a step that leads ultimately to the formation of overt metastases (3). Recent studies have shown that the number of CTCs is an independent predictor of progression-free survival and overall survival in metastatic breast cancer and metastatic colorectal cancer (4,5). However, it remains unclear whether the number of CTCs is correlated with disease progression and treatment response in HCC.

The recent development of in vivo flow cytometry (IVFC) has provided a powerful tool for real-time, noninvasive detection and quantification of fluorescently labeled CTCs in cancer hematogenous metastasis (6–11). The IVFC can overcome the disadvantages of conventional CTC detection methods, such as invasiveness, low sensitivity caused by a limited blood sample volume, and the difficulty in recording
the dynamics of CTCs. Here, we have used IVFC to quantitatively detect CTCs during disease progression in a highly metastatic orthotopic nude mouse model of green fluorescent protein (GFP)-labeled HCC.

To date, chemotherapy, chemoembolization, surgery, immunotherapy, and radiotherapy remain disappointing in advanced HCC treatment (12,13). Sorafenib, a multi-kinase inhibitor that blocks the vascular endothelial growth factor (VEGF) and RAF/MEK/ERK pathways and has been approved for the treatment of advanced HCC by the US Food and Drug Administration since 2007, represents a milestone in HCC treatment (14–16). In this study, we have investigated whether the number of CTCs is correlated with the response in sorafenib treatment.

**Materials and Methods**

**Experimental Animals and Drugs**

Male Balb/c nude mice, 5- to 6-week-old and weighing 20–22 g, were purchased from Shanghai SLAC Laboratory Animal (Chinese Academy of Science, Shanghai, China) and maintained in laminar flow cages in a specific-pathogen-free animal facility at the Zhongshan Hospital of Fudan University and given a standard diet and water. All procedures involving rats and mice experiments were performed according to the recommendations of the NIH Guidelines for Care and Use of Laboratory Animals. Sorafenib was purchased from Bayer Pharmaceutical Corporation and was formulated as previously described (17). For the test group, 30 mg/kg body weight of sorafenib was administered intragastrically once daily as specified in a previous study (14). This study was approved by the Animal Care and Use Committee of Fudan University and Institutional Review Board of Zhongshan Hospital.

**Metastatic Orthotopic Models of HCC in Nude Mouse**

At the authors’ institution, a highly metastatic human HCC cell line - HCCLM3 was established as previously described (9–11,18). A linearized pEGFP-C1 (BD Clontech, Palo Alto, CA) plasmid was transferred to HCCLM3 cells using the Lipofectin reagent (Invitrogen, Carlsbad, CA). GFP-positive cells were isolated through fluorescence-activated cell sorting (FACS) to establish stably transfected HCCLM3-GFP multi-clone cells. HCCLM3-GFP cells were cultured at 37°C and 5% CO₂ in high-glucose DMEM medium (GIBCO, Rockville, MD, USA) containing 10% fetal bovine serum (HyClone, Rockville, MD). This GFP-labeled human HCC cell line with a highly metastatic potential was used throughout this study. Orthotopic implantation with GFP-labeled HCCLM3 tumor was performed as described by Yang et al. (19). The mice began to develop lung metastases during the fifth week and died during the ninth week after orthotopic implantation. All mice with transplanted GFP-labeled HCCLM3 tumors in the liver exhibited 100% transplantability and metastatic capacity.

**IVFC**

The IVFC method has been previously described (7). A diagram of the technique is shown in Figure 1A. Briefly, major veins and arteries of the mouse ear were visualized via transillumination with a 520-nm light emitting diode. An artery with ~50 μm in diameter was selected for measurement by IVFC. Light from a 488-nm semiconductor laser was shaped into a slit and imaged across the selected ear artery, where the laser power is about 300 μW. (Fig. 1B). The fluorescently labeled cells were excited as they flowed through the laser slit on the selected blood vessel, resulting in bursts of fluorescence (Fig. 1C). To ensure the detection specificity of GFP-labeled CTCs, the blood sample was extracted. GFP-positive cells were sorted by traditional flow cytometry and were confirmed as CTCs using cytopathological analysis methods (Fig. 1D). Therefore, this IVFC method enabled real-time noninvasive detection and quantification of GFP-labeled CTCs in nude mice. Each nude mouse underwent a one-hour measurement each week by IVFC, during which the dynamics and quantity of CTCs in vivo were recorded.

**Ultrasound Imaging**

Ultrasound imaging was performed each week to measure tumor size after orthotopic tumor implantation. The mice were anesthetized by inhalation of 2–3% isoflurane with 1% oxygen. The tumors were imaged using VisualSonics Vevo 770

In Vivo High-Resolution Micro-Imaging System (VisualSonics...
Inc, Toronto, Ontario, Canada). The tumor volume \( V \) was calculated from the largest diameter \( a \) and the smallest diameter \( b \) with the equation \( V = \frac{1}{2} \times a \times b^2 \) (19).

Ex Vivo Fluorescence Imaging

To assess the level of biological activity of GFP-labeled HCC in the liver and lungs, the nude mice were sacrificed under anesthesia, 56 days after the orthotopic tumor implantation. The liver and lungs were harvested and examined with a bio-fluorescence imaging system (NightOWL II LB 983 NC100, Berthold Technologies GmbH & Co. KG, Germany) to visualize and quantify GFP-expressing cancer tissues. A normal mouse without the tumor was used as a negative control to establish the threshold.

Pathological Examination

After the livers and lungs were harvested and imaged \( \text{ex vivo} \), the specimens were treated using routine histopathological procedures, which included 10% buffered formalin processing, paraffin embedding, sectioning at 5 \( \mu \)m, and H&E staining.

Immunohistochemical Staining

Immunohistochemical staining was conducted and analyzed as described in a previous study (14). The tumor cell proliferation was determined by Ki-67 immunohistochemical staining, while tumor angiogenesis was determined by CD31 antibody staining. The paraffin sections of tumors were performed with a rabbit polyclonal anti-Ki67 antibody (Abcam, Cambridge, MA) at a dilution of 1:50 (2 \( \mu \)g/mL) in antibody diluent (DakoCytomation, Fort Collins, CO), and a rat monoclonal anti-CD31 antibody (Abcam, Cambridge, MA) at a dilution of 1:50 (2 \( \mu \)g/ml) in rabbit serum according to the manufacturer’s protocol. Areas of Ki67- or CD31-positive objects were quantified with ImagePro Plus (version 3.0, Media Cybernetics, Silver Spring, MD).

TUNEL Staining

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method is used to evaluate the apoptosis. TUNEL staining of tumor cells were conducted and analyzed according to a previous study (14). The ApopTagPlus In Situ Apoptosis Detection kit Peroxidase (Oncor, Gaithersburg, MD) was used according to the manufacturer’s protocol. Areas of TUNEL-positive objects were quantified using ImagePro Plus version 3.0 (Media Cybernetics).
Study Design and Sample Size Determination

Ten nude mice, implanted orthotopically with a highly metastatic GFP-labeled human HCC, were randomized by body weight into a sorafenib-treated group and a control group. Starting 28 days after orthotopical tumor implantation, the sorafenib-treated group was administered sorafenib orally once daily at 30 mg/kg body weight for four weeks, whereas the control group received oral administration of a solvent containing cremophor EL/ethanol/water (12.5:12.5:75). Both groups were examined with IVFC and ultrasound imaging each week. The nude mice were sacrificed on Day 56 after orthotopic tumor implantation. The primary outcome was the number of CTCs detected quantitatively by IVFC. The sample size for this step was calculated as follows: the number of CTCs was correlated with tumor progression and treatment response.

Figure 2. The number of CTCs was correlated with tumor progression and treatment response. A: Sorafenib treatment led to a significant decrease in the number of CTCs, while the number of CTCs increased in the control group with tumor progression. B: As the tumor progressed, the mice in the sorafenib-treated group exhibited no significant weight loss while the mice in the control group exhibited an observable decrease in weight. C: Sorafenib treatment inhibited tumor growth significantly. D: Ultrasound imaging showed a significant difference in tumor size between the sorafenib-treated group (8.81 × 6.47 mm²) and the control (15.35 × 9.15 mm²) in the fifth week after orthotopic tumor implantation. E: A strong correlation between the number of CTCs and the volume of liver tumors was observed (Spearman correlation coefficient r = 0.947; P < 0.001). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Circulating Tumor Cell and Treatment Response

RESULTS

Statistical Analysis

To compare the continuous data of sorafenib-treated and the control groups, Student’s t test was used for all data presented as the mean and standard deviation, while Mann-Whitney test was used for data presented as the median and range. Comparisons between groups with regards to the categorical data were analyzed using the chi-squared test. Spearman’s correlation was used to analyze the correlation between CTC count and tumor volume. All data were analyzed using the SPSS version 16.0 computer program (SPSS, Inc., Chicago, IL). The significance of these differences was defined as a P value < 0.05.

RESULTS

The Number of CTCs Was Correlated with Disease Progression and Treatment Response

Ten nude mice, implanted orthotopically with a highly metastatic GFP-labeled human HCC, were randomized into a sorafenib-treated group and a (negative) control group. The sorafenib-treated group was administered sorafenib orally once daily at 30 mg/kg body weight for four weeks, whereas the control group received oral administration of a solvent. Both groups were examined with IVFC and ultrasound imaging each week. The dynamics of CTC counts, mice body weight, and liver tumor volume in two groups were shown in Figures 2A–2C. Ultrasound imaging was used to monitor tumor growth and clearly demonstrated the change in tumor size over time after the tumor orthotopic implantation (Fig. 2D). During first 3 weeks after tumor implantation, no CTC was detected in either control group or sorafenib-treated group by IVFC. At the fourth week after tumor implantation, a low number of CTCs were detected by IVFC (Fig. 2A). However, there were no significant difference in the number of CTCs between the sorafenib-treated group and control group at this time (1.00 ± 1.00 versus 1.00 ± 0.71, P = 1.00). During the first 4 weeks after orthotopic tumor implantation, neither group underwent any form of treatment, while no significant differences in mice body weights and tumor volumes between the sorafenib-treated group and control group were observed. Thereafter, the sorafenib-treated group underwent sorafenib oral administration once daily for four weeks, whereas the control group received oral administration of a solvent. Treatment with sorafenib once daily for four weeks significantly delayed disease progression and stabilized body weight during Week 5 to Week 8 (Fig. 2B). The tumors in the sorafenib-treated group grew significantly less than those in the control group. At the fifth week after tumor implantation, the first notable difference in CTC number started to appear between the sorafenib and control groups (1.40 ± 0.55 versus 3.65 ± 0.947, P < 0.001). Collectively, a strong correlation between the number of CTCs and the volume of liver tumor was noted according to data distributions (Fig. 2F). The reduced number of CTCs was
attributed to the sorafenib treatment. In short, the number of CTCs was strongly associated with disease progression and treatment response.

The Decreased Number of CTCs, in Response to Sorafenib Treatment, Was Correlated with Low Lung Metastasis

We then investigated whether the decreased number of CTCs, in response to sorafenib treatment, was correlated with reduced metastasis. On Day 56 after orthotopical tumor implantation, the nude mice were sacrificed when there was a significant difference in the number of CTCs between the sorafenib-treated group and the control detected by the IVFC. We found out significant differences between the sorafenib-treated and the control groups regarding lung metastatic rate, the number and size of lung metastases, tumor necrosis, apoptosis, proliferation, and angiogenesis (Table 1). Ex vivo fluorescence imaging of the liver and lungs (Fig. 3) revealed that a
lower number of living cancer cells were observed in the liver and lungs in the sorafenib-treated group, compared to the control group. The mean tumor size in the sorafenib-treated group was significantly smaller than that in the control group (Table 1). The average fluorescence intensity of the liver tumor and lung metastasis in the sorafenib-treated group were lower, compared to the control group (Table 1). H&E staining confirmed that lung metastatic rate was 20% (1/5) in the sorafenib-treated group, compared to 100% (5/5) in the control (P = 0.01; Figs. 3E and 3F). The number and size of lung metastases in the sorafenib-treated group were lower and smaller than those in the control group (Table 1). Moreover, H-E staining showed a significant difference in liver tumor necrosis between the two groups (Figs. 4A and 4B). TUNEL staining showed more tumor cell apoptosis in the sorafenib-treated group (Figs. 4C and 4D). Immunohistochemical staining demonstrated that the numbers of Ki67-positive tumor cells and CD31-positive microvessels were significantly lower in the sorafenib-treated group than those in the control (Figs. 4E-4H). Taken together, sorafenib treatment induced tumor necrosis and apoptosis, and inhibited tumor proliferation and angiogenesis. The decreased number of CTCs, which was attributed to sorafenib treatment, was correlated with reduced number, size, and rate of lung metastases.

Figure 4. Sorafenib treatment induced tumor necrosis and apoptosis, inhibited tumor proliferation and angiogenesis, and reduced the number of CTCs. A: H-E imaging (magnification 40×) showed ~10% tumor necrosis in the control group (scale bar = 200 μm). B: H-E imaging (magnification 40×) showed ~75% tumor necrosis in the sorafenib-treated group (scale bar = 200 μm). C: TUNEL staining (magnification 200×) revealed a decreased rate of tumor cell apoptosis (black arrow) in the control group (scale bar = 50 μm). D: TUNEL staining (magnification 200×) revealed an increased rate of tumor cell apoptosis in the sorafenib-treated group, compared with the control (scale bar = 50 μm). E: Immunohistochemical staining (magnification 200×) demonstrated that ~80% tumor cells were Ki67-positive in the control group (scale bar = 100 μm). F: Immunohistochemical staining (magnification 200×) demonstrated that ~7% of tumor cells were Ki67-positive in the sorafenib-treated group (scale bar = 100 μm). G: Immunohistochemical staining (magnification 200×) demonstrated CD31-positive microvessels were common in the control group (scale bar = 100 μm). H: Immunohistochemical staining (magnification 200×) demonstrated that the number of CD31-positive microvessels decreased significantly in the sorafenib-treated group (scale bar = 100 μm). I: Sorafenib decreases CTCs and inhibits hematogenous metastasis by suppressing tumor proliferation and angiogenesis, and by inducing tumor necrosis and apoptosis. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
**DISCUSSION**

CTCs are present in patients with advanced-stage HCC, which may be one of the reasons why these patients have a high incidence of tumor recurrence after an apparently definitive surgical resection, even after liver transplantation (20). CTCs have therefore been a subject of investigation with the hope that their identification will aid the prognostication and that the eradication of CTCs will eliminate the recurrence and prolong the survival of patients (21). However, previous studies using alpha-fetoprotein (AFP) mRNA (22–32), albumin mRNA (33–35) and various other tumor markers (36,37) for CTCs failed to produce consistent results. The major limitation of these previous studies is most likely related to the fact that those markers are not specific to HCC and can also be detected in the blood of both healthy persons and cirrhotic patients without HCC. It is also widely questioned whether the cells releasing AFP or albumin mRNA, or cells in circulation that contain tumor markers are responsible for metastasis. In this study, we established a highly metastatic orthotopic nude mouse model of GFP-labeled HCC, and used the noninvasive IVFC to monitor GFP-labeled CTCs quantitatively and continuously. We showed sorafenib treatment reduced the number of CTCs and lung metastases significantly, induced tumor necrosis and apoptosis, and inhibited tumor proliferation and angiogenesis. The reduced number of CTCs was correlated with low lung metastasis. The possible causes underlying the observed reduce in the number of CTCs are as follows: (1) sorafenib suppresses tumor proliferation, which might reduce the CTC number as a result of the correlation of CTC counts with the size of the primary tumor; (2) sorafenib inhibits tumor angiogenesis, which decreases the entry of tumor cells from primary site into the blood significantly. Most tumor cells are limited to primary site and lack a route to get into blood vessels without increased angiogenesis; and (3) sorafenib induces tumor necrosis and apoptosis, which distinctly decreases tumor burden and results in the death of tumor cells, thereby further reducing the quantity of CTCs. Our results have demonstrated that all of these causes contributed to the decrease in the number of CTCs in the sorafenib-treated group and inhibited HCC hematogenous metastasis (Fig. 4).

The significance of this study lies in two major findings. First, our study demonstrated that the number of CTCs was strongly correlated with disease progression in HCC. The correlation coefficient between tumor size and the number of CTCs was 0.947, with a corresponding P value less than 0.001. Therefore, the number of CTCs in circulation can be used as a prognostic marker for the HCC progression. Second, our study observed that the number of CTCs was strongly correlated with treatment response in our mouse model of HCC. In clinic, a high percentage of patients with HCC have a recurrence in the liver or lung after primary resection or even after orthotopic liver transplantation. One reason for this may be the presence of a small number of tumor cells circulating in the blood prior to surgery or the release of tumor cells into the blood stream during surgical manipulation. Therefore, the detection of CTCs may have a potential application in selecting HCC patients for liver transplantation. The presence of CTCs in circulation may indicate that HCC patients are not suitable for liver transplantation because these patients have already had hematogenous metastasis. Moreover, the detection of CTCs may offer a therapeutic window prior to the development of overt metastases by guiding treatments such as neoadjuvant therapy before surgery to reduce micrometastasis and downstage tumor development, or indicate the need for adjuvant therapy after surgery to inhibit cancer recurrence and metastasis. The recent refinement of an immunomagnetic separation technology (38) that can reliably and reproducibly isolate, enumerate, and characterize CTCs in the clinic has enabled further study of the CTC as a prognostic and therapeutic marker in HCC.

Currently, there is lack of a good biomarker to monitor disease progression and therapeutic efficacy in patients with HCC. In clinic, AFP presents positive in almost sixty percent in patients with HCC in China. Therefore, AFP is not a good biomarker to monitor disease progression and therapeutic efficacy in all patients with HCC. Moreover, some HCC patients have no treatment response to expensive sorafenib, which push us to find a biomarker to judge therapeutic efficacy. Our study showed that CTCs was correlated with disease progression and treatment response, and sorafenib treatment reduced the number of CTCs significantly. This result has a potentially clinical value, which means CTCs could be a good biomarker to monitor disease progression and therapeutic efficacy in patients with HCC. In this animal study, sorafenib was intragastrical administration in mice, which is equal to oral administration in patients.

In conclusion, the number of CTCs is correlated with the disease progression and the treatment response in HCC. This study provides experimental evidence showing the considerable clinical value of CTCs as a marker in predicting the disease progression and monitoring therapeutic efficiency in patients with HCC.

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