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Research Article

Flow cytometric evaluation of cancer stem cell markers in HepG2 cells following sorafenib treatment

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Abstract

Objectives: Liver cancer is a leading cause of mortality. Sorafenib resistance and cancer stem cells (CSCs) are among the factors that contribute to a poor prognosis. Different drugs enrich different CSC populations with a variety of CSC markers. This study investigated the expression of CSC markers in HepG2 cells in response to low doses of sorafenib using flow cytometry.

Methods: The cytotoxicity of sorafenib was determined using a cell counting kit-8 assay. The expressions of the CSC markers CD44, CD90, and CD133 were measured with flow cytometry after treatment with sorafenib for 72 hours.

Results: Sofranib inhibited cell proliferation in a dose-dependent manner. Low-dose sorafenib treatment increased CD44 expression; however, there was a decrease in the expression of CD133. An increasing trend was also seen in CD90 expression, but the difference was not significant.

Conclusion: The results indicate that CSC expression varied according to the sorafenib dose administered, which supports the role of CSCs as novel pharmacological targets and highlights the importance of their characterization and the ability to identify them.

Keywords: Biomarker, cancer stem cells, flow cytometry, hepatocellular cancer, sorafenib

Malignancies are a leading cause of death in the world, with an estimated 10 million deaths from cancer in 2020. Among all localizations, liver cancer was recorded as the second most common cause of cancer death [1]. Liver metastasis is much more common than primary liver cancer, however among primary liver tumors, hepatocellular cancer (HCC) is the most frequently seen and the mortality rate is high.

There are many reasons that potentially explain the poor prognosis of liver cancer. These include multiple etiologies, concomitant hepatic disease, high rates of late diagnosis, and difficulties experienced during treatment [2]. The cells of a bulk tumor mass are not homogenous, making targeting difficult. The cancer stem cell (CSC) population has similarities to both normal stem cells and cancer cells, and the CSC subpopulation concentration may be small in comparison with other cells. It has been suggested that this stem cell population has a high capacity for self-renewal and differentiation, and that they may be the actual cancer-initiating subpopulation. It has also been reported that they are primarily responsible for resistance to chemotherapy, as well as metastasis and relapse after treatment [3]. Obviously, these stem cells have prognostic importance, in addition to tumorigenesis relevance [4]. Several cell surface biomarkers, including CD44, CD90, and

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CD133, have been used to separate, investigate, and examine the cancer stem cells of various malignancies [4-7].

Sorafenib, a multikinase inhibitor, is a systemic therapeutic agent approved for use in the treatment of renal cell carcinoma and advanced HCC. Although sorafenib appears to be effective in prolonging survival in HCC patients with limited side effects, many patients have been shown to develop resistance to this agent [8].

The present study was designed to evaluate the effect of sorafenib treatment at different doses on the expression of CSC markers. Cell proliferation was examined and flow cytometric analysis was performed to analyze the expression of CD44, CD90, and CD133 in response to low doses of sorafenib.

Materials and Methods

Cell culture and sorafenib

HepG2 cells obtained from the American Type Culture Collection were grown in Dulbecco's Modified Eagle's Medium (Biosera France SAS, Nuaille, France) containing 10% fetal bovine serum (PAN-Biotech GmbH, Wiesbaden, Germany), and 1% antibiotics (streptomycin 10 mg/mL, penicillin 10,000 U/mL, PAN-Biotech GmbH, Wiesbaden, Germany) at 37oC in a CO2 incubator (5%). Sorafenib (LC Laboratories, Woburn, MA, USA) was dissolved in dimethyl sulfoxide at a concentration of 10 mmol/L. The dilutions were prepared from stock solutions on the day of the experiment.

Cell counting kit-8 assay

Approximately 10,000 cells per well were seeded in a 96-well plate dish. The next day, the cells were exposed to a series of concentrations of sorafenib at a dilution ratio of 3:4 (30μM - 4μM) and incubated for 72 hours. Next, a cell counting kit-8 (CCK-8) reagent was added to each well and the optical density at 450 nm was measured with a Synergy microplate reader (BioTek Instruments Inc., Winooski, VT, USA) following 3 hours of incubation at 37°C. Each concentration was repeated 4 times, and each experiment was repeated 3 times.

Expression analysis of CSC markers

HepG2 cells were harvested following 72 hours of incubation with sorafenib and prepared for flow cytometric evaluation. Control and treatment groups were washed with phosphate buffer saline (PBS) and incubated with BB515 labeled-CD44 (1:100 dilution), PE-labeled CD133 (1:50 dilution), and APC labeled-CD90 (1:50 dilution) (BD Biosciences, San Diego, CA, USA) for 30 minutes at room temperature. After washing them twice with PBS, they were resuspended in PBS for measurement in a BD AccuriC6+ flow cytometer (BD Biosciences, San Diego, CA, USA). A minimum of 20,000 events were recorded for analysis using BD Accuri C6+ software.

Statistical analysis

The study data reflect 3 independent experiments and were provided as the mean±SD. The means of the groups were compared using one-way analysis of variance and posthoc Tukey tests to find the significance. GraphPad Prism 8.2.0 software (GraphPad Software Inc., San Diego, CA, USA) was used to perform the statistical analysis and to generate the graphs.

Results

Cell viability after sorafenib treatment

A CCK-8 assay was used to determine the cytotoxic effect of sorafenib on HepG2 cells. A dose-dependent decrease in cell viability was observed after sorafenib treatment for 72 hours (Fig. 1). The effects of the treatment were analyzed in comparison with 4 μM sorafenib treatment. Starting from 7.1 μM, increased concentrations exerted significantly more anti-proliferative effects on the cells. As no significant difference was observed between the 4 μM and 5.3 μM treatment groups, 5.3 μM sorafenib treatment was not included in the flow cytometric evaluations of CSC markers. Treatment with 4 μM sorafenib was associated with approximately 75% cell proliferation. High doses led to high cytotoxicity, and low viability was observed with 22.5 μM and 30 μM sorafenib treatment (22.5 μM: 6.3% viability; 30 μM: 7.9 % viability).

Evaluation of CSC marker expressions

Flow cytometric analysis was conducted to examine the expression of CSC markers CD44, CD133, and CD90 after treat-

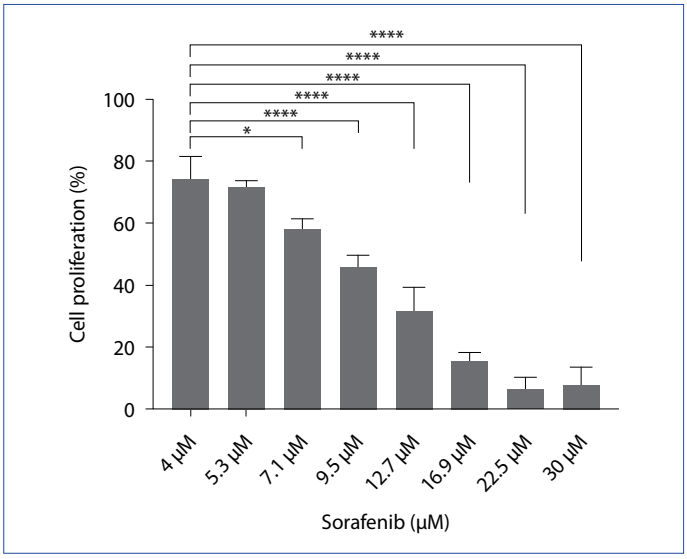

Figure 1. Dose-dependent anti-proliferative effects of sorafenib after 72 hours of incubation. The concentration ranged from 30μM to 4μM with a dilution ratio of 3:4. The differences between the treatment groups were analyzed using one-way analysis of variance and a post-hoc Tukey test. *P<0.05; ****P<0.0001 compared with cell proliferation following 4 μM sorafenib treatment.

ment with sorafenib in low doses. Groups of 4 μM, 7.1 μM, and 9.5 μM were included in the analysis; the 5.3 μM treatment group was excluded as it did not produce a significant difference in cell proliferation in comparison with the 4 μM group. Histograms illustrating the shifts in the median fluorescence in the respective channels, which were demonstrated as fold changes in comparison to the control group for evaluation of expression of each marker can be seen in Figure 2a. The administration of the lowest dose (4 μM) led to a significant increase in the expression of CD44, while no significant difference was observed with increasing doses in a comparison with the control group (Fig. 2b).

There was a decrease in the expression of CD133 in response to low doses of sorafenib (Fig. 2c). Similar to CD44 expression, CD90 expression increased after treatment with sorafenib, particularly in the 4 μM treatment group. However, this increase was not significant (Fig. 2d).

Discussion

Drug resistance can be caused by many factors, such as alterations in drug transport and intracellular metabolism, and CSCs have attracted particular attention recently [9]. Sorafenib has been the first-line systemic therapy for patients with advanced, unresectable HCC for more than 10 years. Extensive research has been carried out to identify predictive and prognostic markers [10]. Differential transcriptional patterns have been shown to be associated with sorafenib resistance in HCC [11-14]. Though numerous studies have focused on CSCs and emphasized their clinical relevance, no universal CSC markers have yet been established [15]. The CSC markers CD133, CD90, and CD44 have been proposed as markers to predict the response to treatment and survival in hepatoblastoma [16]. It has also been suggested that CD133, CD90, and CD44 may have a role as potential diagnostic, predictive, or therapeutic biomarkers in HCC [17].

The current study was an analysis of the expressions of CD44, CD133, and CD90 in sorafenib-treated HepG2 cells. After 72 hours of treatment, we observed increased CD44 levels with low-dose sorafenib treatment (4 μM), which was associated with 75% viability. We also found decreased CD133 expression after sorafenib exposure. CD90 expression did not change significantly following sorafenib administration.

High doses of sorafenib can cause acquired drug resistance and side effects. It was reported in a recent study that low-dose sorafenib treatment was associated with significant tumor growth delay in comparison to placebo and high-dose sorafenib groups in HCC, potentially reinforcing a preference for long-term low-dose treatment [18]. There is extensive ongoing research to identify novel strategies that combine low-dose sorafenib with cytotoxic drugs [19]. There is an evident need to further characterize the effects of low-dose sorafenib treatment.

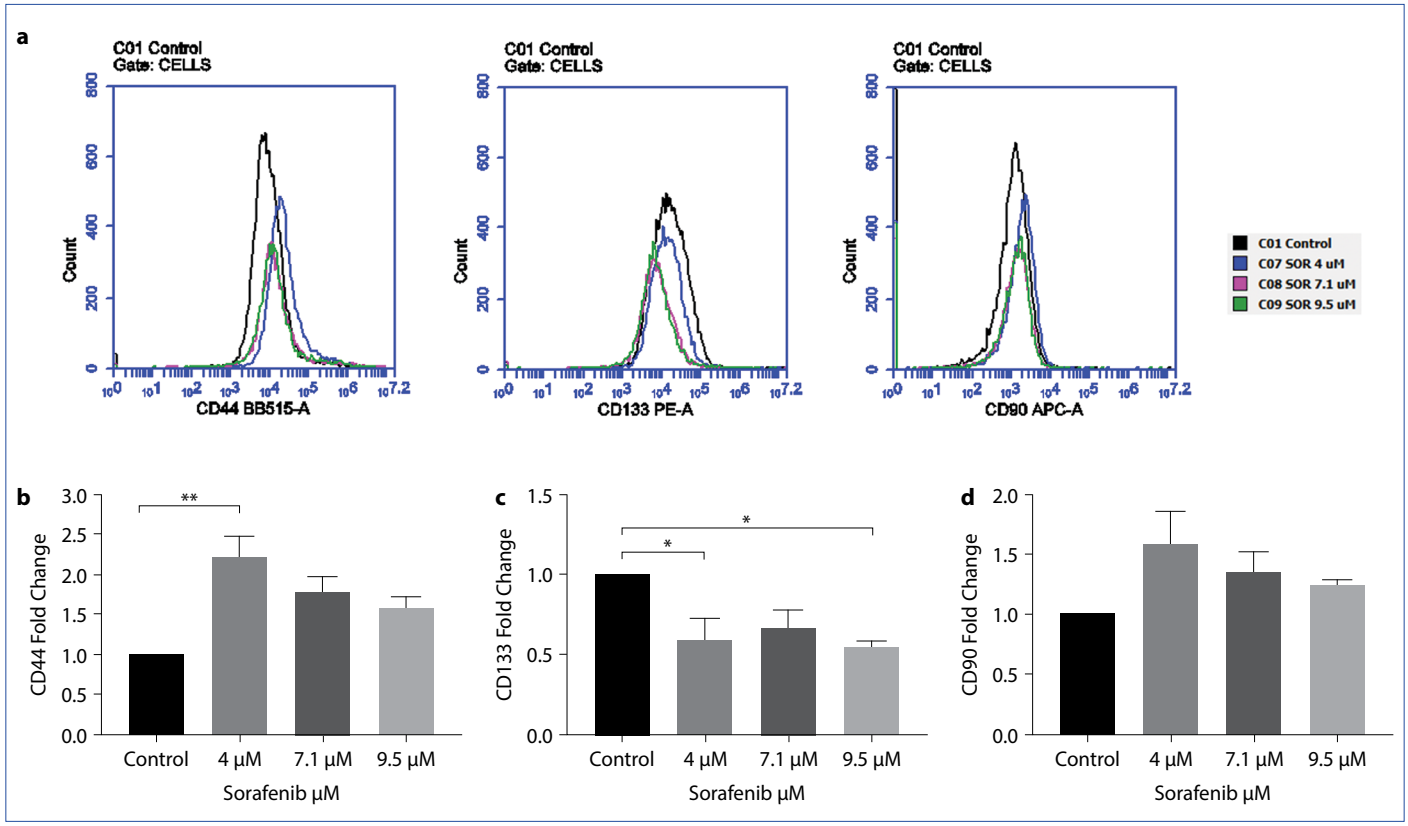

Figure 2. Flow cytometric evaluations of CSC marker expressions. (a) Histograms and (b) bar graphs with median fluorescence for CD44, (c) CD133, (d) and CD90. *P<0.05; **P<0.01 compared with the control group.

Numerous studies have reported that prolonged sorafenib treatment led to enrichment of hepatic CSCs, while the primary tumor population underwent growth suppression [9]. Hedgehog signaling has been reported to promote sorafenib resistance in CD44-positive HCC patient-derived organoids and sorafenib increased CD44 levels [20]. High CD44 levels in sorafenib-resistant HCC cells have been shown to improve cellular proliferation and migration [21]. Interestingly, mesenchymal-like HCC cells that expressed high levels of CD44 have been reported to be more resistant to sorafenib-induced apoptosis in vitro. A mesenchymal transition and expression of CD44 appear to be related to the response to sorafenib in HCC patients [14]. The authors reported that epithelial-like cells expressing CD133 were sensitive to sorafenib-induced apoptosis, which is consistent with the decrease in CD133 levels observed in our study. It has been demonstrated that CD90 was associated with cell migration, cell viability, and sphere-forming ability of HCC cells [22]. It has also been suggested that sorafenib may inhibit extrahepatic metastasis by targeting CD90-expressing CSCs [23]. Our findings revealed an increasing trend in CD90 expression after treatment with 4 μM sorafenib, however, this increase was not statistically significant.

In a previous study, our group reported increased CD44 and CD90 expression in HepG2 cells after cisplatin treatment, while no significant change was seen in CD133 levels [24]. In the current study, we observed increased CD44 and decreased CD133 expression after sorafenib administration, which indicates that the drugs have different mechanisms of action.

The mean elimination half-life following administration of sorafenib is approximately 25-48 hours in vivo [25]. In our study, we treated HepG2 cells with sorafenib for 72 hours. It has been noted previously that the in vitro half-life may be longer than the in vivo terminal half-life [26]. Sorafenib metabolites contribute to drug efficacy. Among the 8 metabolites of sorafenib, M-2, M-4, and M-5 have been reported to potently inhibit the vascular endothelial growth factor receptor signaling pathway, the platelet-derived growth factor receptor signaling pathway, and members of the mitogen-activated protein kinase pathway [27].

It should be noted that no specific marker is sufficiently comprehensive to be accepted as a universal CSC marker [28]. The most relevant known markers were chosen for this study. Other CSC markers that will adequately detect and recognize stem cell properties may be identified in future studies. Limitations of the current study include the use of 3 definitive cancer cell markers, the lack of an alternative method to flow cytometry, and not performing cell function assays. Although flow cytometry is an established method of choice to identify small cell populations and the analysis of CSCs, combined use with functional assays to assess migration, proliferation, apoptosis, or viability could provide further information.

Conclusion

Genetic heterogeneity is thought to be closely related to CSC subpopulations in tumors. Numerous studies have investigated novel therapeutic approaches targeting CSCs, and CSC markers are not just diagnostic or prognostic markers, but pharmacological targets as well. Different drugs enrich different CSC populations with a variety of CSC markers, which highlights the necessity of CSC characterization and their importance in personalized therapeutic approaches.

Conflict of Interest: The authors report that there is no potential conflict of interest relevant to this article.

Ethics Committee Approval: Not necessary.

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