CANCER BIOLOGY & THERAPY
2016, VOL. 17, NO. 6, 648–656
http://dx.doi.org/10.1080/15384047.2016.1177677

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RESEARCH PAPER

Iron depletion enhances the effect of sorafenib in hepatocarcinoma

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ABSTACT

Human hepatocellular carcinoma (HCC) is known to have a poor prognosis. Sorafenib, a molecular targeted drug, is most commonly used for HCC treatment. However, its effect on HCC is limited in clinical use and therefore new strategies regarding sorafenib treatment are required. Iron overload is known to be associated with progression of chronic hepatitis and increased risk of HCC. We previously reported that iron depletion inhibited cancer cell proliferation and conversely induced angiogenesis. Indeed iron depletion therapy including iron chelator needs to be combined with anti-angiogenic drug for its anti-cancer effect. Since sorafenib has an anti-angiogenic effect by its inhibitory targeting VEGFR, we hypothesized that sorafenib could complement the anti-cancer effect of iron depletion. We retrospectively analyzed the relationship between the efficacy of sorafenib and serum iron-related markers in clinical HCC patients. In clinical cases, overall survival was prolonged in total iron binding capacity (TIBC) high- and ferritin low-patients. This result suggested that the low iron-pooled patients, who could have a potential of more angiogenic properties in/around HCC tumors, could be adequate for sorafenib treatment. We determined the effect of sorafenib (Nexavar®) and/or deferasirox (EXJADE®) on cancer cell viability, and on cell signaling of human hepatocarcinoma HepG2 and HLE cells. Both iron depletion by deferasirox and sorafenib revealed insufficient cytotoxic effect by each monotherapy, however, on the basis of increased angiogenesis by iron depletion, the addition of deferasirox enhanced anti-proliferative effect of sorafenib. Deferasirox was confirmed to increase vascular endothelial growth factor (VEGF) secretion into cellular supernatants by ELISA analysis. In in vivo study sorafenib combined with deferasirox also enhanced sorafenib-induced apoptosis. These results suggested that sorafenib combined with deferasirox could be a novel combination chemotherapy for HCC.

ARTICLE HISTORY
Received 1 December 2015
Revised 13 March 2016
Accepted 8 April 2016

KEYWORDS
Deferasirox; deferoxamine; HCC; Iron; sorafenib

Introduction

Human hepatocellular carcinoma (HCC) is the fifth most common cancer, and is responsible for the third highest cancer mortality in the world.[1] HCC commonly appears in patients with chronic hepatitis and/or cirrhosis.[2] Sorafenib (Nexavar®), a molecular targeted drug, is most commonly used for HCC treatment.[3] Sorafenib is a multiple kinase inhibitor that directly suppresses tumor cell proliferation by blocking the activity of Raf kinases, leading to inhibition of the mitogen-activated protein kinase / extracellular signal-regulated kinase (MEK/ ERK) signaling pathway, and also inhibits angiogenesis by blocking several receptor tyrosine kinases such as vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR).[4,5] However its effect on HCC is limited in clinical use and therefore new strategies regarding sorafenib treatment are required.

Iron is an essential element for humans. Iron is taken orally and is mainly used in red blood cells to carry oxygen and is stored as ferritin in the liver.[6] Iron is also related with chronic inflammation including hepatitis. Phlebotomy has been performed to reduce iron levels, with the aim of the suppression of hepatitis virus activity. Such phlebotomy has indicated that reduction in iron suppressed to develop hepatitis. Iron plays important roles in normal cells such as in the creation of energy in mitochondria. Iron has a similar role in cancer cells. However, although both normal and cancer cells need iron to proliferate, cancer cells need more iron than normal cells because of their rapid proliferation. Iron overload is known to cause cancer in animal models [7,8] and conversely, iron depletion has been reported to suppress tumor growth.[9,10] However, monotherapy of iron reduction is not yet established as a cancer therapy for solid cancers including HCC. We have previously determined that perhaps one reason for this lack of effect of iron monotherapy for solid cancers is because iron depletion also results in induction of angiogenesis via HIF1-α and VEGF signaling.[11] Angiogenesis is thought to be a mechanism by which

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cancer cells escape from severe conditions. The fact that iron reduction inhibits tumor proliferation but enhances angiogenesis suggested that combination of iron reduction with an anti-angiogenic drug might result in increased anti-tumor effects. We thus hypothesized that iron depletion might enhance the anti-tumor effect of sorafenib. We therefore retrospectively analyzed clinical data of sorafenib and determined its potential anti-tumor capacity in combination with an iron chelator.

Materials and methods

Cell lines and cultures

The human hepatocarcinoma cell lines HepG2 (p53 wild type) and HLE (p53 mutant) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (FCS, Hyclone, Logan, UT) and 100 U/mL penicillin/streptomycin (Sigma-Aldrich) in a humidified incubator at 37°C and 5% CO2.

Reagents

Sorafenib was purchased from LKT Laboratories (St. Paul, MN). The oral iron chelator, deferasirox (EXJADE®), was purchased from Novartis Pharma (Tokyo, Japan). Compounds were dissolved in 100% DMSO (Sigma-Aldrich) and diluted with DMEM to the desired concentration for in vitro studies.

Cell viability assay

The proliferation of HepG2 and HLE cells was evaluated using the Cell Proliferation Kit II; XTT assay (Roche Diagnostics GmbH, Mannheim, Germany). Cells were plated at a density of 6000 cells per well in 96-well microplates and were incubated at 37°C in a humidified atmosphere of 5% CO2 for 24 hours. Compounds dissolved in DMEM with 0.02% FBS were added to the wells and the cells were then incubated for an additional 24 -72 hours. The absorbance of the samples was measured at 450 nm using a microplate reader after XTT solution was added to each well. The combination index was analyzed with the CalcuSyn software (BioSoft, Cambridge, UK).

Western blotting analysis

Cell lysates were extracted using cell lysis buffer (50 mmol/L Tris-HCl (pH 7.4), 30 mmol/L NaCl, and 1% Triton X-100) containing protease inhibitors (cOmplete Mini, Roche Diagnostics GmbH). Nuclear protein was extracted using NE-PER buffer (Thermo Fisher Scientific, Rockford, IL). Equal amounts of total cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride filter membranes (GE Healthcare UK Ltd, Buckinghamshire, UK). The membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies. An ECL Prime Western Blotting Detection Reagent (GE Healthcare UK Ltd) was used to detect the peroxidase activity of secondary antibodies. Antibodies against the following proteins were used for Western blotting: cyclinE (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p53 (EMD Millipore, Inc., Billerica, MA), β-actin (Sigma-Aldrich), cyclinD1, CDK4, p21, p27, caspase3, cleaved caspase3, PARP, cleaved PARP, phospho-MEK1/2 (Ser217/221), MEK1/2, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2 and HIF-1α (all from Cell Signaling Technology, Inc., Danvers, MA).

Flow cytometric analysis of the cell cycle

Cancer cells were treated with the indicated concentrations of agents for 24 hours and were then stained with 20 mg/mL propidium iodide. The effect of the agents on the cell cycle was analyzed using a fluorescence-activated cell sorter (FACScan, Becton Dickinson, Franklin Lakes, NJ) with FlowJo software (TREE STAR, Ashland, OR).

VEGF ELISA assay

HepG2 and HLE cells were seeded in 6-well plates at a density of 100,000 cells per well. After 24 hours, the cells were treated with different concentrations of deferasirox (0 - 1,000 μM) and were incubated for an additional 24 hours. VEGF secretion was evaluated by ELISA assay of VEGF in culture supernatants using the Human VEGF Quantikine ELISA Kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Absorbance was determined at 450 nm using a microplate reader.

Tumor xenograft model and experiment

All animal studies were approved by the Ethics Review Committee for Animal Experimentation of Okayama University, Okayama, Japan. BALB/c athymic mice (nu/nu) were purchased from Clea (Tokyo, Japan). All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (http://oacu.od.nih.gov/ac_cbt/guife3.htm.htm). All mice were allowed to eat a meal with water freely. Six week-old female mice were used to establish a tumor xenograft model. A total of 3×10^{7} HepG2 cells were suspended in a 50% mixture of Basement Membrane Matrix (BD Biosciences, Bedford, MA) and were injected subcutaneously into the right flank. One week after injection, the mice were randomly divided into control and 3 treatment groups ($n = 3$), and administration of sorafenib and deferasirox was started. Each agent was orally administered daily for 5 d per week. The tumor volume was measured in day 21.

Immunohistological staining

HepG2 tumors were harvested for histological analysis after sorafenib and deferasirox treatment. Resected tumors were fixed in 10% paraformaldehyde and embedded in paraffin. Paraffin sections were immunohistologically stained. Anti-cleaved PARP antibodies used for immunohistological staining were purchased from Cell Signaling Technology, Inc.

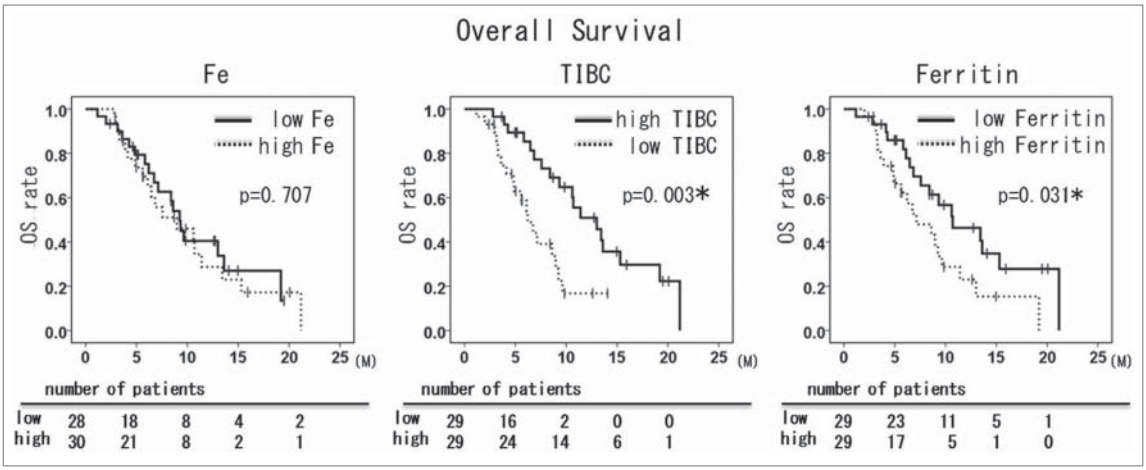

Figure 1. The relationship between overall survival and serum related markers in HCC patients treated with sorafenib HCC patients treated with sorafenib were divided into 2 groups according to serum iron related markers (cut off value; median), and overall survival was analyzed by Kaplan-Meier survival analysis. *, $p < 0.05$. Survival time of low iron patients (low Fe, high total iron-binding capacity (TIBC), low ferritin groups) is shown as continuous lines, and survival time of high iron patients (high Fe, low TIBC, high ferritin groups) is shown as dotted lines.

Patient studies

The patient study included 58 HCC patients who were treated with sorafenib in Okayama University Hospital and affiliated hospitals from February 2009 to November 2011. Clinical data including background information was reviewed retrospectively using medical records. All patients provided written informed consent and the study protocol was approved by the review committee of Okayama University, Okayama, Japan (#1452). Tumors were assessed by CT or MRI, where each tumor size was measured to evaluate the therapeutic efficacy of sorafenib was defined according to the Response Evaluation Criteria in Solid Tumors (RECIST v1.1). The patients were categorized into 2 groups according to blood serum levels of iron-related markers (cut off value; median). The survival time (Progression-Free Survival (PFS) and Overall Survival (OS)) was calculated from the date of first sorafenib treatment to disease progression or death.

Statistical analysis

Analysis of variance was performed using Student's *t*-test. Survival time was estimated by the Kaplan-Meier estimator and statistical differences were analyzed using the log-rank test. The data analysis was performed using IBM SPSS Statistics ver.22 (IBM, Armonk, NY).

Results

Low iron conditions prolonged overall survival in sorafenib treated patients

First, we retrospectively investigated HCC patients that were treated with sorafenib (n = 58) in Okayama University Hospital and affiliated hospitals. The patients were categorized into low and high groups according to the levels of serum Fe, TIBC and ferritin, and survival time was statistically analyzed (cut off value; median). The background of the patients was not significantly different between the 2 treatment groups (Supplementary Table 1). Mean survival time (MST) of the high Fe patients (mean Fe 109.7 ± 8 .3 μg/dl) was 6.8 months and that of the low Fe patients (mean Fe 47.1 ± 2 .7 μg/dl) was 8.4 months. Mean survival time (MST) of the high TIBC patients (mean TIBC 395.4 ± 10 .2 μg/dl) was 9.8 months and that of the low TIBC patients (mean TIBC 229.7 ± 11 .1 μg/dl) was 5.6 months. Mean survival time (MST) of the high ferritin patients (mean ferritin 373.5 ± 46 .2 μg/dl) was 5.7 months and that of the low ferritin patients (mean ferritin 55.7 ± 8 .3 μg/dl) was 8.7 months. OS was significantly prolonged in the high TIBC and low ferritin patients; i.e. in low iron storage patients (Fig. 1).

Sorafenib needed high concentration to induce apoptosis compared to cell cycle arrest

To determine the anti-cancer effect of sorafenib, the effect on the viability of 2 HCC cell lines were assayed using an XTT cell viability assay. This assay showed that sorafenib suppressed cell viability in a dose dependent manner (Fig. 2A). Western blot analysis showed that sorafenib decreased the expression of the cell cycle regulators, cyclinD1 of 10 μM(Fig. 2B). Sorafenib induced the expression of the apoptosis marker, Cleaved Caspase 3 of 100 μM. Sorafenib also induced the expression of another apoptosis marker, Cleaved PARP in HepG2 cells with its concentration of 100 μM. These data suggested that 10-fold high concentration of sorafenib was necessary for the induction of apoptosis compared to that of cell cycle arrest.

Iron depletion by deferasirox inhibited cancer cell proliferation

We previously reported that iron depletion by deferasirox suppressed cell proliferation in the lung cancer cell lines.[11] To determine the anti-cancer effect of iron depletion by deferasirox in the hepatocarcinoma cell lines, the effect on the viability of cell lines were assayed using an XTT cell viability assay. This assay showed that deferasirox

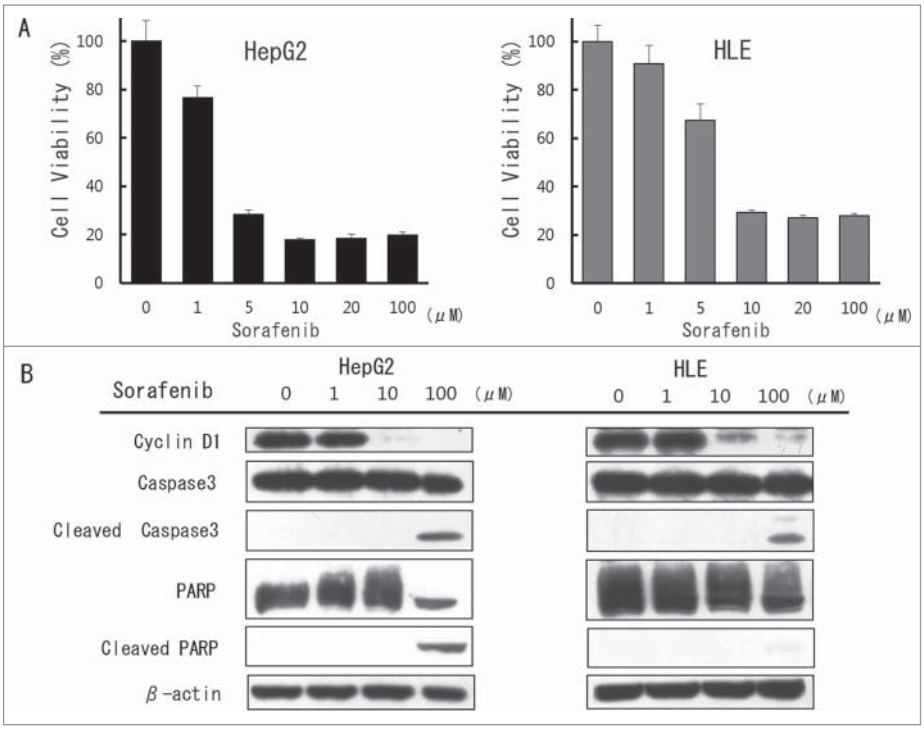

Figure 2. The inhibitory effect of sorafenib against hepatocellular carcinoma (HCC) cell lines in vitro (A) Cultured HepG2 and HLE cells were treated with different concentrations of sorafenib for 24 hours and cell viability was then evaluated using the XTT assay. Cell viability in the absence of treatment was set at 100%. Results are means + SD of 3 independent experiments. (B) Cultured HepG2 and HLE cells were treated with different concentrations of sorafenib for 72 hours and cell cycle and apoptotic effect was then evaluated using western blot analysis. Cells were then harvested and total protein in cell lysates was analyzed for expression of the indicated proteins.

suppressed cell viability in a dose dependent manner (Fig. 3A). Western blot analysis showed that Deferasirox decreased the expression of cyclinD1 in HepG2 cells with its concentration of 100 μM and in HLE cells with its concentration of 10μM (Fig. 3B). However, Deferasirox did not induce the expression of the apoptosis marker, Cleaved Caspase 3 of HepG2. Deferasirox also slightly induced Cleaved Caspase 3 and Cleved PARP in HLE cells with its concentration of 100 μM. FACS analysis showed that deferasirox increased the percentage of cells in the G0-G1 phase and decreased the percentage in the G2-M phase (Supplementary Fig. S1). These data suggested that major aspect of deferasirox on the suppression of cell proliferation would be cell cycle arrest.

Iron depletion by deferasirox induced angiogenesis via HIF-1α and VEGF signaling

As mentioned above, iron depletion only is not enough to cancer progression. One of the reasons may be that iron depletion by deferasirox induced angiogenesis via HIF-1α and VEGF signaling in compensation as we reported previously. To investigate the potential effect of deferasirox on angiogenesis in the hepatocarcinoma cell lines, we assayed the level of VEGF secreted by cells into the supernatant using an ELISA assay. Deferasirox treatment increased VEGF secretion in a dose dependent manner (Fig. 3C). Western blot analysis also indicated that deferasirox induced nuclear HIF-1α expression (Fig. 3D). These results suggested that iron depletion by deferasirox also induced angiogenesis via HIF-1α and VEGF signaling in compensation.

Deferasirox enhanced the inhibitory effect of sorafenib on cell viability

Next question is whether increased angiogenic status by iron depletion can be a preferable condition for sorafenib treatment. Cell viability assay revealed that the addition of deferasirox to sorafenib treatment strongly suppressed cell proliferation, compared to single administration of them (Fig. 4A). To better assess the synergistic effect of iron depletion combined with sorafenib, we calculated the combination index. The calculated combination index showed that deferasirox displayed significant synergy with sorafenib at a number of concentrations (Fig. 4B).

Sorafenib combined with deferasirox synergistically inhibited the cell cycle and induced apoptosis

To analyze the mechanism of synergy between sorafenib and deferasirox in greater detail, changes in the cell cycle, apoptosis and in MEK-ERK (a signaling pathway blocked by sorafenib) signaling cascades were investigated by Western blot analysis. The combination of sorafenib and deferasirox resulted in a greater decrease in the expression of cell cycle regulatory proteins (cyclinD1, cyclinE), cyclin-dependent kinase (CDK4) and cyclin-dependent kinase inhibitors (p21, p27) and a greater increase in cleaved PARP compared to the effect of either agent alone (Fig. 5). Monotherapy with deferasirox induced no significant changes in MEK-ERK signaling. FACS analysis showed that sorafenib combined with deferasirox also inhibited the cell cycle (Fig. S3). These data suggested that sorafenib combined

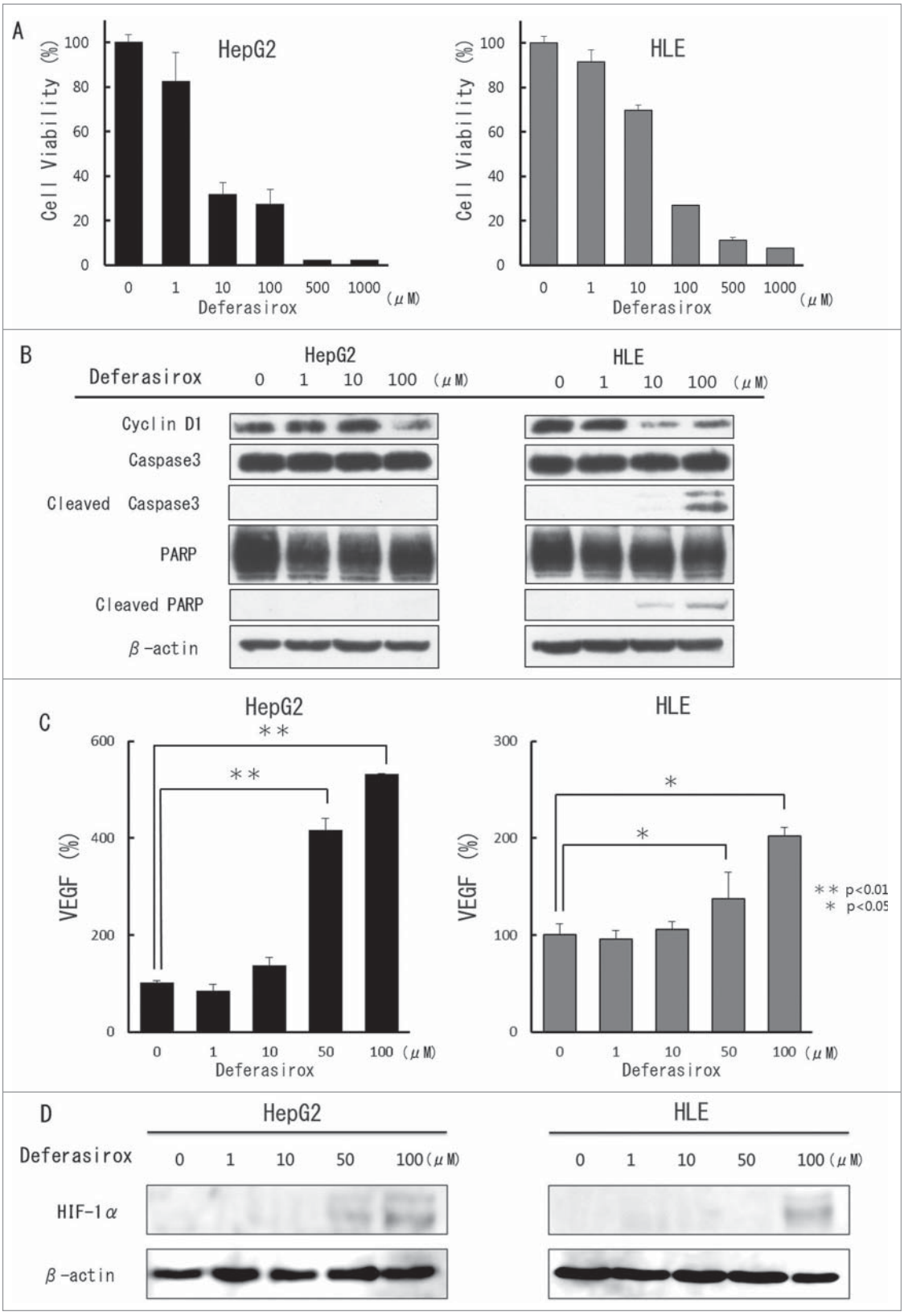

Figure 3. The inhibitory and angiogenic effects of deferasirox against hepatocellular carcinoma (HCC) cell lines in vitro (A) Cultured HepG2 and HLE cells were treated with different concentrations of deferasirox for 72 hours and cell viability was then evaluated using the XTT assay. Cell viability in the absence of treatment was set at 100%. Results are means + SD of 3 independent experiments. (B) Cultured HepG2 and HLE cells were treated with different concentrations of deferasirox for 72 hours and cell cycle and apoptotic effect was then evaluated using protein gel blot analysis. Cells were then harvested and total protein in cell lysates was analyzed for expression of the indicated proteins. (C) Cultured HepG2 and HLE cells were treated with different concentrations of deferasirox for 72 hours and the supernatant was then harvested and the amount of VEGF secreted by the cells was assessed using an ELISA assay. The level of VEGF secreted by non-treated cells was set at 100%. *, $p < 0.05$; **, $p < 0.01$. (D) Cultured HepG2 and HLE cells were treated with different concentrations of deferasirox for 72 hours. Cells were then harvested and nuclear proteins were analyzed by Western blotting to examine the expression of HIF-1α. The gels were run under the same experimental conditions.

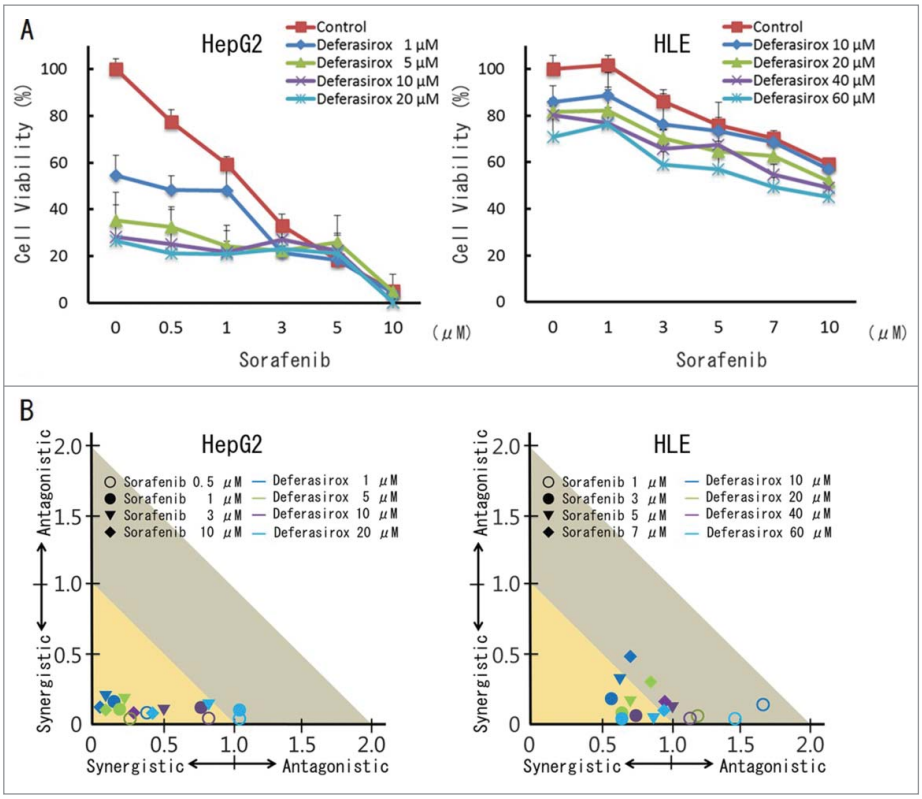

Figure 4. Synergistic inhibitory effect of sorafenib and deferasirox against HCC cells (A) HepG2 and HLE cells were treated with the indicated concentrations of sorafenib and deferasirox for 48 hours, following which cell viability was assessed using the XTT assay. (B) The combination index was defined as interaction indices, and was calculated from the data in (A) using CalcuSyn software. An index of less than 1 indicates synergistic interaction and an index greater than 1 indicates antagonistic interaction.

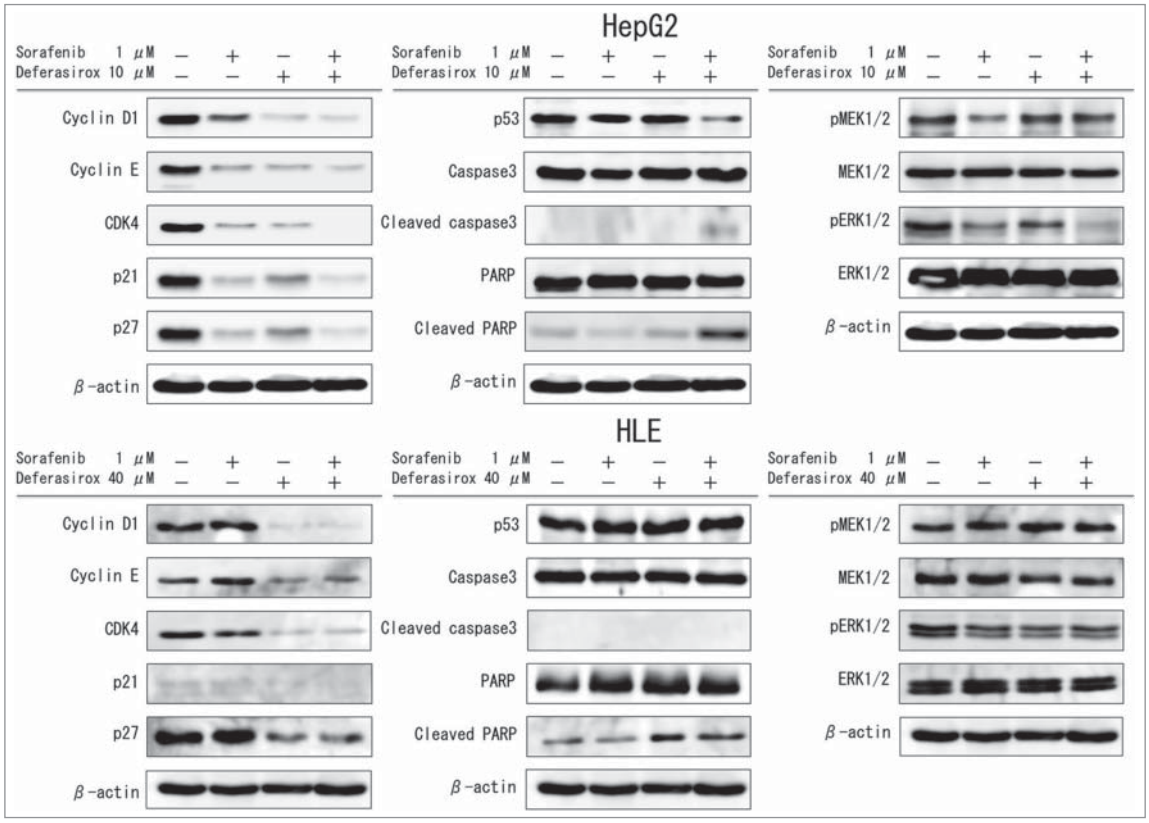

Figure 5. Changes in signaling cascades induced by sorafenib and/or deferasirox HepG2 (top) and HLE (bottom) cells were treated with the indicated concentrations of sorafenib and/or deferasirox for 48 hours. Cells were then harvested and total protein in cell lysates was analyzed for expression of the indicated proteins involved in cell cycle regulation (left), apoptosis (middle) and MAPKinase cascades (right) by Western blot analysis. The gels were run under the same experimental conditions. Protein bands were quantified by densitometry using Image J software.

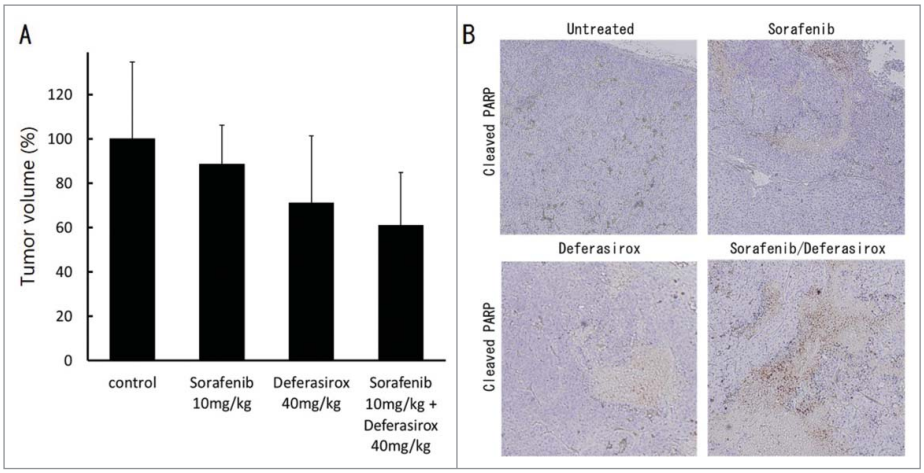

Figure 6. Synergistic inhibitory effect of sorafenib and deferasirox against HCC in vivo (A) HepG2 cells (3×10^7 per animal) were implanted subcutaneously into the right flank of mice. Sorafenib and/or deferasirox administration was initiated one week after injection. Each agent was orally administered daily for 5 d per week. Tumor measurement was started 3 weeks after injection. (B) Resected tumors were analyzed for cleaved PARP by immunohistological staining. Cleaved PARP staining showed apoptotic cells as positive spot areas.

with deferasirox synergistically inhibited the cell cycle and could facilitate sorafenib-induced apoptosis.

Sorafenib combined with deferasirox enhanced apoptosis in vivo

To confirm the enhancement of sorafenib-induced apoptosis by deferasirox in vivo, a subcutaneous tumor model with HepG2 cells was used. Oral administration of sorafenib and/or deferasirox was started one week after the injection of HepG2 cells. The tumor volume of sorafenib combined with deferasirox was smaller than the single and control treatment groups (Fig. 6A). Immunostaining of the tumors indicated that the expression of cleaved PARP, an indicator of apoptosis, was increased in the combination treatment group compared with the single and control treatment groups (Fig. 6B). These data suggested that the enhancement of anti-tumor effect of sorafenib by adding deferasirox could represent in in vivo setting.

Discussion

Iron levels are related to carcinogenesis and cancer progression. An excess of some iron compounds causes cancer.[7,8] Liver is an organ that metabolizes and stores iron, and liver diseases are often associated with iron metabolic disorders. In many hepatic diseases such as hemochromatosis, chronic hepatitis B/C and non-alcoholic steatohepatitis (NASH) iron homeostasis cannot be maintained because of the dysregulation of iron regulatory proteins.[12-15] An excess of iron in hepatocytes has recently been suggested to be closely associated with the development of HCC.[16,17] The adverse effect of iron excess is the reason why iron depletion therapy is performed to improve many hepatic diseases. Indeed, phlebotomy is performed for hepatitis C patients to lower the risk of developing HCC.[18] Therefore, iron depletion is a rational approach for the therapy of hepatic malignant disease. Iron levels in the body can be reduced by phlebotomy, an iron depleted diet, or with an iron chelator. Phlebotomy has been performed to improve inflammation of the liver in hepatitis patients. However low Hb patients could not be treated with phlebotomy in spite of their high storage of iron. Although an iron depleted diet is comparatively safe, dietary restriction over a long time is required. The use of an iron chelator is the easiest way to reduce iron and is expected to be applied to treatment of liver disease.

Deferasirox enhanced the inhibitory effect of sorafenib in in vitro study. Cancer cells require a large amount of iron because of their rapid proliferation. Indeed, iron depletion has been reported to have anti-cancer effects.[19-21] We examined cell cycle arrest and apoptosis by Western blotting and FACS analysis. Iron depletion by deferasirox inhibited cancer cell proliferation via cell cycle arrest rather than apoptosis. Single agent of sorafenib and deferasirox did not induce apoptosis strongly. However, the combination therapy of sorafenib and deferasirox synergistically inhibited cancer cell proliferation via cell cycle arrest and apoptosis. The synergistic effect of deferasirox and sorafenib on apoptosis in HepG2 cells might be associated with the notable change in cyclin-dependent kinase inhibitor p21 signals. During chemotherapy or radiation treatment cancer cells are known to repair themselves by upregulation of p21.[22,23] On the other hand, inhibition of p21 can possibly suppress cancer cell proliferation by preventing this self-repair mechanism.[24-26] Both sorafenib and deferasirox have the ability to inhibit cyclin-dependent kinase, and the combination of sorafenib and deferasirox have been shown to synergistically induce apoptosis by inhibition of p21.[27,28] These mechanisms are the reason for the synergistic effect of sorafenib in combination with deferasirox. However, we could not identify the mechanisms in the liver completely. The mechanisms including microenvironment should be examined by using orthotopic animal model or carcinogenic animal model in the next step.[29,30] Recently the imaging technology for cells and animals has been progressed.[31-34] It may be also helpful to reveal the synergistic effect of sorafenib in combination with deferasirox more detail.

Our results showed that OS was prolonged in high TIBC and low ferritin groups, which reflects low iron patients. This result is consistent with in vitro study, which iron depletion by deferasirox enhanced sorafenib via the induction of cell cycle

arrest and apoptosis. It was also shown that iron depletion by deferasirox suppressed cancer cell proliferation and induced angiogenesis via HIF-1α and VEGF signaling. These results suggest that sorafenib still exerts its anti-angiogenic effect in combination with iron depletion treatment. In clinical cases, Serum iron (Fe) levels did not significantly affect OS. In general, blood serum iron levels (Fe) can change rapidly because of diurnal variation, which might be a reason why serum iron levels are not significantly correlated with OS.[35] In addition, although low iron patients appear to have reduced inflammation, it is unclear by how much inflammation is reduced. A prospective clinical study is expected to start soon.

In conclusion, low iron conditions prolonged overall survival in sorafenib treated patients. Iron depletion by deferasirox enhanced the inhibitory effect of sorafenib via induction of cell cycle arrest and apoptosis. Sorafenib is compatible with deferasirox because of HIF1-α and VEGF induction signaling by deferasirox. These results suggest that iron depletion by deferasirox has the potential to be a novel combination chemotherapy with sorafenib for HCC.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by the Princess Takamatsu Cancer Research Fund and by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (Grant Number 26861048).

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