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Original Research

Efficacy and tolerability of Sorafenib plus metronomic chemotherapy S-1 for advanced hepatocellular carcinoma in preclinical and clinical assessments

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ABSTRACT

Objective: Although sorafenib, a molecular targeted agent, has survival benefits for advanced hepatocellular carcinoma (HCC) patients, its disease control rate remains limited. To explore the potential for augmenting its antitumor effect, we assessed the preclinical and clinical efficacy and tolerability of S-1 metronomic chemotherapy (MC) plus sorafenib.

Methods: Antitumor effects and toxicity of this combination were tested with HAK-1B xenograft and spontaneous HCC mouse models, and a prospective pilot study was performed to compare therapeutic effects and safety between sorafenib plus MC S-1 for 12 advanced HCC cases and the historical control of 363 sorafenib-treated advanced HCC patients at our hospital from July 2011 to June 2015.

Results: In mice, the combination chemotherapy enhanced anti-angiogenic effects, resulting in a stronger tumor hypoxic environment and increased tumor cell apoptosis. Clinically, the objective response rate of the combination chemotherapy was higher than that of sorafenib mono therapy (16.7%; 2/12 vs 5.2%; 19/363, $p < 0.05$); however, there were no significant differences in overall survival and time to progression. Adverse events including alopecia, thrombocytopenia, and pancreatic enzymes elevation in the combination chemotherapy were higher than those of sorafenib. No patient treated with the combination chemotherapy discontinued treatment due to severe adverse events.

Conclusions: Sorafenib plus MC S-1 seems to be effective and tolerable for patients with advanced HCC and could be considered a treatment option for these patients.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy and one of the major causes of cancer-related deaths worldwide [1]. HCC typically occurs in the setting of persistent hepatitis or cirrhosis secondary to hepatitis B/C viral infection, excessive alcohol consumption, and nonalcoholic steatohepatitis (NASH) [2,3]. Advances in the diagnosis and the treatment of hepatitis B/C viruses have contributed to a reduced risk of developing HCC; however, this disease is often diagnosed in advanced stages, such as Barcelona Clinic Liver Cancer stage B (intermediate stage) or stage C (advanced stage) [4]. Curative HCC treatments including surgical resection, ablation, and

List of abbreviations: AE, Adverse Event; Hb, Hemoglobin; HCC, Hepatocellular Carcinoma; LC, Liver Cirrhosis; MC, Metronomic Chemotherapy; MVD, Micro Vessel Density; MTA, Molecular Targeted Agent; MTD, Maximum Tolerated Dose; NASH, Non-Alcoholic Steato Hepatitis; OS, Overall Survival; PCNA, Proliferating Cell Nuclear Antigen; PDGF, Platelet-Derived Growth Factor; VEGF, Vascular Endothelial Growth Factor; VT, Vehicle Treatment; WBC, White Blood Cell.

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liver transplantation are not applicable for the patients with intermediate- and advanced- stage HCC, and their mortality rates remain high [5]. Several molecular targeted agents (MTAs) have been approved for these patients, whereas sufficient antitumor effects have not been obtained by each MTA mono therapy [4,6]. Optimal sequential or combination treatment might be necessary for these patients, and how to improve the therapeutic effect of MTAs is a pivotal issue in the future.

Sorafenib, one of the MTAs, inhibits platelet-derived growth factor (PDGF) receptors, Raf kinase, and vascular endothelial growth factor (VEGF) receptors and has been approved for the primary treatment of advanced HCC as it was found to significantly prolong overall survival (OS) compared to that with placebo in 2007 [7,8]. However, the disease control rate of Sorafenib is unsatisfactory. To improve its therapeutic effects, several trials of combination therapy with regimens such as erlotinib [9], doxorubicin [10], and hepatic arterial infusion chemotherapy [11] have been performed, but none of them have provided a survival benefit for advanced HCC patients.

S-1, a chemotherapeutic agent based on fluorouracil and an approved treatment for several types of solid tumors was also a potential candidate for combination with Sorafenib; however, the addition of S-1 did not prolong OS for advanced HCC patients with Sorafenib-refractory disease [12–14]. Treatment with Sorafenib for advanced HCC is known to result in adverse events (AEs) in approximately 80% of patients, and several patients are forced to stop therapy due to severe AEs [7]. Additionally, it was reported that the addition of S-1 to Sorafenib for advanced HCC patients doubles the incidence of severe AEs (from 21% to 41%) [14].

Metronomic chemotherapy (MC) comprising S-1, another chemotherapeutic regimen, is defined as frequent uninterrupted administration using significantly lower doses than the maximum tolerated dose (MTD) of S-1. The advantages of MC S-1 over MTD S-1 have been described as antitumor effects and anti-angiogenic effects with lower toxicity, and this has been widely approved for many cancers [15–17]. We previously reported that the antitumor effect of combination chemotherapy comprising MTA plus MC S-1 was stronger than that of MTA and MTD S-1 mono therapy with fewer AEs in an HCC xenograft mouse model [18]. Therefore, we considered the possibility that a combination of MC S-1 with Sorafenib could result in a synergistic effect with fewer AEs [19]. Hence, to more closely examine the clinical physiology of HCC, we evaluated the efficacy and tolerability of Sorafenib plus MC S-1 for advanced HCC in preclinical models and a clinical prospective study.

Materials and Methods

Cell lines and culture conditions

HAK-1B, a human hepatoma cell line, was originally established and maintained in our institute [20]. Cells were maintained in Dulbecco modified Eagle medium (Gibco Invitrogen Cell Culture Co., Auckland, New Zealand) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Biowest, Nuaille, France), 100 U/ml penicillin, and 100 mg/ml streptomycin (Nacalai Tesque, Kyoto, Japan) in a humidified atmosphere containing 5% CO2 at 37°C.

Drugs

Sorafenib tosylate was purchased from Santa Cruz Biotechnology, Inc. (Texas, USA). A Sorafenib dose of 30 mg/kg/day was used for the mice, which is equivalent to a human dose of 400 mg twice daily [21]. S-1 was provided by Taiho Pharmaceutical Co, Ltd. (Tokyo, Japan). S-1 consists of a mixture of tegafur, gimeracil, and oteracil at a molar ratio of 1:0.4:1 in 0.5% hydroxypropylmethylcellulose solution [22].

In vivo HCC Xenograft model

Male 5-week-old nude mice (BALB/c nu/nu) were purchased from Kyudo KK (Fukuoka, Japan) and housed in specific pathogen-free conditions. In total, 5×10^6 HAK1-B cells were suspended in phosphate-buffered saline and subcutaneously inoculated into the flank regions of the nude mice. When the estimated tumor volume ($0.52 \times \text{length} \times \text{width}^2$) reached 150 to 200 mm^3, the tumor-bearing mice ($n = 18$) were randomly allocated into three groups of six mice as follows: 1) Sorafenib group, 30 mg/kg of Sorafenib given orally once daily; 2) Sorafenib plus MC S-1 group, 30 mg/kg of Sorafenib and 5 mg/kg of S-1 administered orally once daily; 3) vehicle treatment (VT) group, corresponding vehicle given orally once daily. The tumor volumes and body weights were measured every 2 days. The mice were sacrificed at 3 weeks after the start of treatment and the tumors were resected and investigated.

In vivo NASH-related spontaneous HCC model

In this study, we employed a NASH-related spontaneous HCC mouse model (STAM™ mouse; SMC Laboratories, Inc., Tokyo, Japan), which was developed as described previously [23]. Briefly, 2-day-old male C57BL/6J mice were subcutaneously injected with streptozotocin (200 μg/animal) and then fed a high-fat diet (60% energy from fat, HFD-32; Clea, Tokyo, Japan) from the age of 4 weeks, which was followed by the development of NASH related HCC [23]. In this study, the development of HCC in STAM™ mice was confirmed by macroscopic observations via small laparotomy under anesthesia at the age of 14 weeks. Thereafter, STAM™ mice ($n = 30$) in which HCC developed were randomly allocated into five groups of six mice as follows: 1) MTD S-1 group, 15 mg/kg of S-1 given orally once daily for 1 week followed by a 1-week break; 2) MC S-1 group, 5 mg/kg of S-1 given orally once daily; 3) Sorafenib group, 30 mg/kg of sorafenib given orally once daily; 4) Sorafenib plus MC S-1 group, 30 mg/kg of sorafenib and 5 mg/kg of S-1 given orally once daily; 5) VT group, corresponding vehicle given orally once daily. While continuing treatment, body weights were measured every 2 days. The mice were sacrificed at 5 weeks after the start of treatment. The number of tumors was counted and the diameter of each tumor was measured using Vernier calipers upon sacrificing the animals. The number of tumors was counted as a total of five slides per mouse using hematoxylin and eosin staining slides. Peripheral white blood cells (WBCs) and hemoglobin (Hb) concentrations were also measured at this time. All mice were caged in a group of six or fewer mice per cage at the animal facility of Kurume University School of Medicine and were sacrificed via cervical dislocation under anesthesia using iso-flurane and pentobarbital. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Moreover, all animal experiments were approved by the ethical committee of the Kurume University School of Medicine (Ethical code: 2010-005-1).

Immunohistochemical staining for CD31, CA9, proliferating cell nuclear antigen (PCNA), and cleaved caspase-3 Paraffin-embedded tumor tissue sections, 5 μm-thick, were boiled for 30 min in high pH target retrieval solution (DAKO Japan, Kyoto, Japan) for antigen retrieval and subsequently incubated with primary antibodies as follows: goat anti-CD31 polyclonal antibody (1:200, #AF3628, R&D System Inc., MN, USA), rabbit anti-PCNA polyclonal antibody (1:100; #sc-7907, Santa Cruz Biotechnology, Inc., TX, USA), rabbit anti-CA9 polyclonal antibody (1:500; #ab184006, Abcam, Tokyo, Japan), and rabbit anti-cleaved caspase-3 monoclonal antibody (1:400; #9661, Cell Signaling Technology, Inc., MA, USA). The secondary antibodies were as follows: donkey anti-goat Alexa Fluor™ 555 antibody (1:200; #ab150130, Abcam, Tokyo, Japan) and Alexa Fluor™ 488 goat anti-rabbit IgG antibody (1:200; #A11034, Thermo Fischer Sciences, Tokyo, Japan). Nuclei were stained with DAPI (#H-1200, Vector Laboratories, Inc., CA, USA) for counterstaining. Immuno-reactivity was visualized using EnVision+ system HRP labelled polymer anti-rabbit (#K4003, DAKO Japan, Kyoto, Japan) and a DAB commercial kit

(Liquid DAB+ Substrate Chromogen System, #K3468, DAKO Japan, Kyoto, Japan). For quantification of the tumor microvessel density (MVD), CD31-positive vessels were counted in 30 randomly selected areas per five tumors for each treatment group at a magnification of × 200. The proliferative and apoptosis indexes were defined as the percentage of tumor nuclei showing PCNA and cleaved caspase-3 staining per 1000 total neoplastic cells, respectively, counted in five fields of 200 tumor cells. The proliferation/apoptosis (PA) index was calculated as the ratio of the proliferative index to apoptosis index. All slides were examined using a con-focal microscope (BZ-X700; Keyence Corporation, Osaka, Japan). Quantitative analyses were performed with the Adobe Photoshop CS software program (Adobe systems, Tokyo, Japan).

Clinical study design and patients

We prospectively evaluated 12 patients with advanced HCC who were treated with Sorafenib plus MC S-1 and 363 patients with advanced HCC who were treated with Sorafenib, as the historical control group, at our hospital from July 2011 to June 2015.

Eligibility criteria

Inclusion criteria were as follows: 1) histologically or radiologically diagnosed advanced (unresectable or metastatic) HCC of Child-Pugh class A; 2) age of 18 years or older; 3) Eastern Cooperative Oncology Group performance status ≤ 1; 4) total bilirubin ≤2.0′the upper limit of normal mg/dl, liver transaminases ≤5′ the upper limit of normal IU/l, albumin ≥2.8 g/dl, and creatinine < upper limit of normal mg/dl; 5) adequate bone marrow functions, WBCs > 3,000/mm^3, platelets > 75,000/mm^3, Hb concentrations >8.5 g/dl; 6) previous treatment including surgical resection and ablation completed at least 4 weeks before study entry; 7) life expectancy ≥ 12 weeks (without ≧50% liver occupation and/or obvious portal vein invasion into the trunk). Exclusion criteria were as follows: 1) previously received all kind of systemic chemotherapy; 2) brain metastases or spinal cord compression; 3) patients with a history of allergy to S-1; 4) poorly controlled or refractory hepatic encephalopathy (West Haven criteria; grade ≥ 3); 5) previous gastrointestinal hemorrhage within 1 month and/or high risk of bleeding.

MC S-1 protocol

We evaluated drug safety during the single-dose study as follow: sorafenib 400 mg × 2/day plus MC S-1 in a general schedule; to avoid the possibility of stored toxicity of S-1, we selected every-other-day S-1 (80 mg/m^2/day) administration for 4 weeks without prolonged breaks.

Disease assessments

Therapeutic response was evaluated using imaging tests according to the modified Response Evaluation Criteria in Solid Tumors guidelines [24], and time to progression and OS were calculated. AEs were assessed according to the National Cancer Institute's Common Terminology Criteria for Adverse Events version 4.0. Every patients were examined by imaging modalities once every 3 months after the treatment initiation.

Statistical analysis

All data were expressed as medians (± SEs). Differences among multiple groups were examined using one-way analyses of variance, followed by the Fisher least significant difference test. In the clinical experiment, each parameter between the Sorafenib group and Sorafenib plus MC S-1 group was compared using a chi-square test. OS and progression free survival were calculated using the Kaplan–Meier method and analyzed using the log-rank test. p-values < 0.05 and <0.001 were considered statistically significant and extremely significant, respectively. Data analysis was performed using JMP statistical analysis software (JMP Pro version 15, Tokyo, Japan).

Results

Antitumor activity and effects of Sorafenib and Sorafenib plus MC S-1 on tumor angiogenesis, hypoxia, apoptosis, and proliferation in the HCC xenograft mouse model

In terms of tumor volume assessments, both Sorafenib and Sorafenib plus MC S-1 treatments were effective compared to the VT (Fig. 1A). In addition, tumor growth in the Sorafenib plus MC S-1 group was significantly suppressed compared to that in the Sorafenib group. Tumor volumes at sacrifice were 1,428.25 mm^3 in the VT group, 924.14 mm^3 in the Sorafenib group, and 668.13 mm^3 in the sorafenib plus MC S-1 group (Fig. 1A). To assess more detailed intra tumoral changes in the HCC xenograft mouse model with each treatment, we performed immunohistochemical staining. Compared to that in the VT group, tumor MVD was substantially decreased in the Sorafenib and Sorafenib plus MC S-1 groups (Fig. 1B, C). Furthermore, compared to that in the Sorafenib group, the Sorafenib plus MC S-1 group showed a marked reduction in the MVD (Fig. 1B, C). The CA9-positive area was more abundant in the Sorafenib and the Sorafenib plus MC S-1 groups compared to that in the VT group (Fig. 1D). Further, the proliferative index in the Sorafenib plus MC S-1 group was significantly lower than that in the VT group (75.8% vs 61.9%, $p < 0.05$; Fig. 1E). However, the apoptosis index in the Sorafenib (21.9%) and the Sorafenib plus MC S-1 (32.1%) groups was significantly higher than that in the VT group (10.2%; Fig. 1F). A significant decrease in tumor cell proliferation and increase in cellular apoptosis were observed with Sorafenib plus MC S-1, leading to a decrease in the PA index (Fig. 1G).

Antitumor activity and tolerability of Sorafenib plus MC S-1 treatment in the NASH-related spontaneous HCC mice model

To evaluate the antitumor activity of Sorafenib plus MC S-1 in the liver cirrhosis (LC) mouse model, we evaluated the developing HCC in STAM™ mice (Fig. 2A). Compared to that in the VT group, Sorafenib and Sorafenib plus MC S-1 led to a marked reduction in the number of tumors in macro- and microscopically (Fig. 2B, C). There were also significant reductions in the maximum tumor diameter, maximum tumor volume, and total volume of tumors only in the Sorafenib plus MC S-1 group, as compared to those in the VT group (Fig. 2D-F). To evaluate the tolerability of Sorafenib plus MC S-1 in the LC mouse model, we assessed changes in several parameters in STAM™ mice. There were no significant differences in body weight and peripheral Hb content among the groups (Fig. 2G, H). Compared to counts in the VT group, a significant decrease in peripheral WBCs was observed only in the MTD S-1 group (Fig. 2I).

Effects of sorafenib plus MC S-1 on tumor angiogenesis, hypoxia, apoptosis, and proliferation in the NASH-related spontaneous HCC mouse model

To evaluate more detailed intra tumoral changes in the NASH-related spontaneous HCC mice model with each treatment, we performed immunohistochemical staining. Compared to that in the VT group, tumor MVD was significantly decreased in the Sorafenib and Sorafenib plus MC S-1 groups (Fig. 3A, B). Furthermore, compared to that in the Sorafenib group, the sorafenib plus MC S-1 group showed a marked reduction in the MVD (Fig. 3A, B). CA9-positive areas were more abundant in the Sorafenib and Sorafenib plus MC S-1 groups compared to those in the VT group (Fig. 3C). Further, the proliferative index in the Sorafenib plus MC S-1 group was significantly lower than that in the VT group (59.1% vs 45.6%, $p < 0.05$; Fig. 3D). However, the apoptosis index in the Sorafenib (12.4%) and Sorafenib plus MC S-1 (13.0%) groups was significantly higher than that in the VT group (6.5%; Fig. 3E). In addition, a significant decrease in tumor cell proliferation

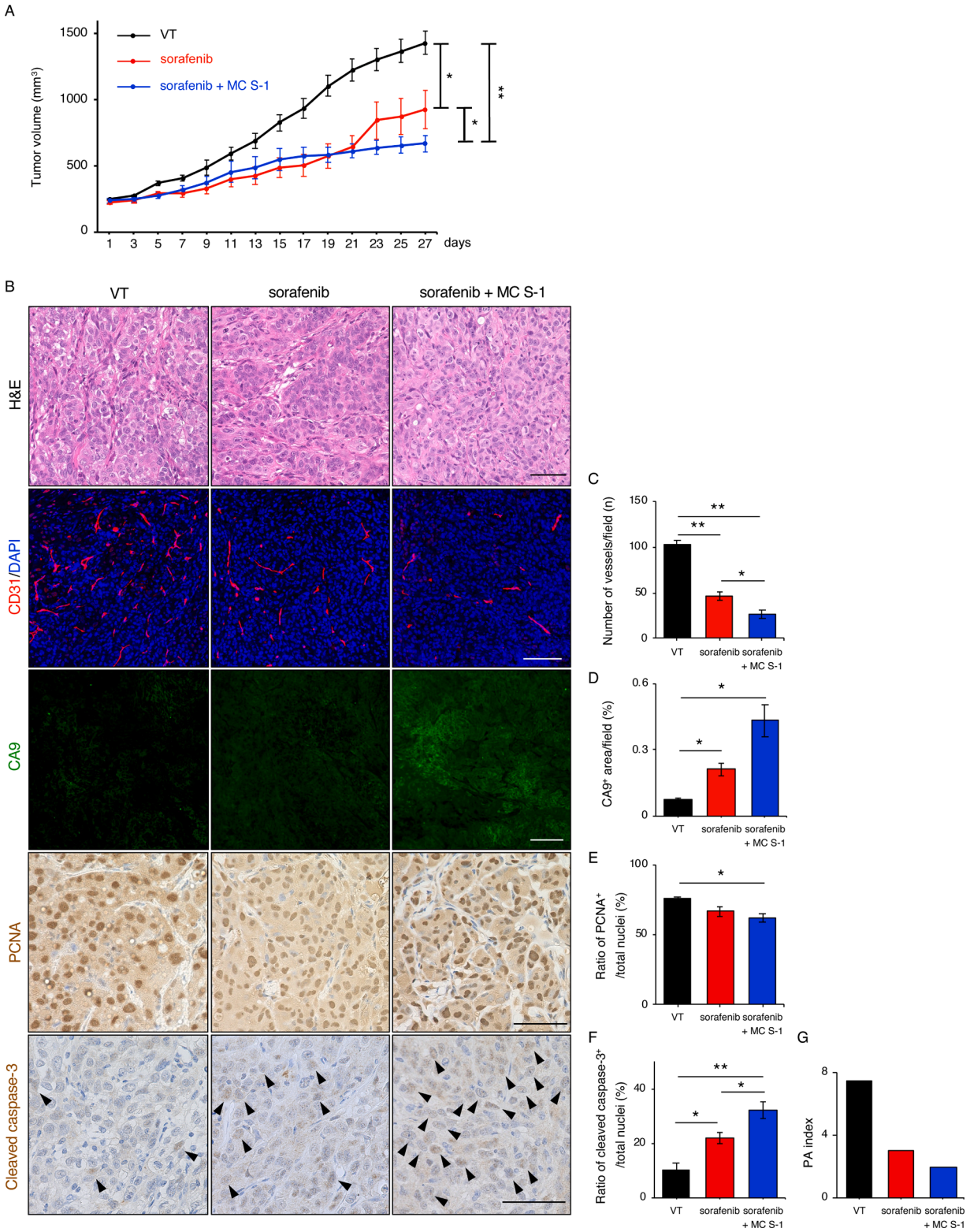

Fig. 1. Antitumor activity and changes in the MVD, hypoxia, proliferation, and apoptosis in the HCC xenograft mouse model. (A) Changes in tumor volume during drug administration. (B) Representative micrographs of CD31-positive, CA9-positive, PCNA-positive, and cleaved caspase-3-positive signals in the VT, Sorafenib, and Sorafenib plus MC S-1 groups. The arrowheads represent the cleaved caspase-3-positive cells. (C-G) Quantification of CD31-positive, PCNA-positive, cleaved caspase-3-positive areas, and PA index. Footnote: $^{*}p < 0.05$, $^{**}p < 0.001$. Data are presented as medians ± SEMs. Scale bar represents 100 μm. Abbreviations: MVD, micro vessel density; VT, vehicle treatment; MC, metronomic chemotherapy; PCNA, proliferating cell nuclear antigen; PA, proliferation/apoptosis; SEM, standard error of the mean.

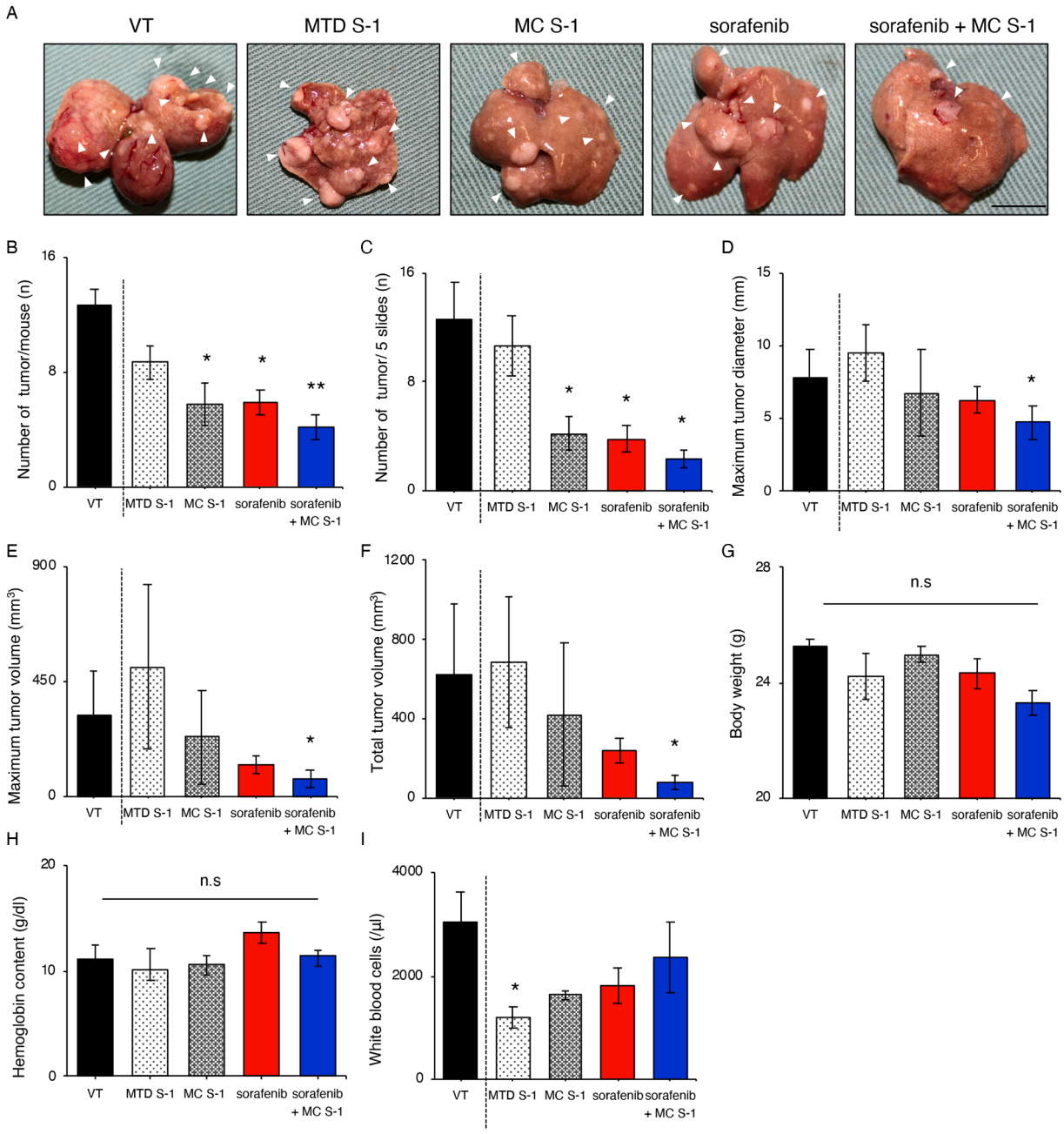

Fig. 2. Macroscopic HCC images and assessments of AEs in the NASH-related spontaneous HCC mouse model. (A) Representative macroscopic images of the VT group, MTD S-1 group, MC S-1 group, Sorafenib group, and Sorafenib plus MC S-1 group. The arrowheads represent the tumors in the liver. (B) Total tumor numbers per mouse, (C) total tumor numbers per five slides, (D) maximum tumor diameter, (E) maximum tumor volume, and (F) total tumor volume. (G) Body weight, (H) hemoglobin content, and (I) white blood cells in peripheral blood at sacrifice. Footnote: $*p < 0.05$, $**p < 0.001$. Data are presented as medians ± SEMs. Scale bar represents 1 cm. Abbreviations: HCC, hepatocellular carcinoma; AE, adverse event; VT, vehicle treatment; MC, metronomic chemotherapy; MTD, maximum tolerated dose; n.s, no significance; SEM, standard error of the mean.

and an increase in cellular apoptosis were observed with Sorafenib plus MC S-1, leading to a decrease in the PA index (Fig. 3F). We also performed Azan staining to evaluate hepatic fibrotic changes in the NASH-related spontaneous HCC mouse model with each treatment. Here, the hepatic fibrotic areas in the Sorafenib (11.7%) and Sorafenib plus MC S-1 (16.1%) groups were significantly smaller than those in the VT group (24.6%; Supplemental Fig. S1).

Clinical data for patients with advanced HCC treated with Sorafenib plus MC S-1

To evaluate the efficacy and tolerability of Sorafenib plus MC S-1 in a clinical setting, we subsequently conducted a prospective cohort study on patients with advanced HCC. The median age was 72.8 and seven patients were male. The maximum tumor diameter was 16.0 mm (Table S1). Further, there were no significant differences in time to progression and OS between the Sorafenib and Sorafenib plus MC S-1 groups (Fig. 4A, B). However, the partial response rate in the Sorafenib

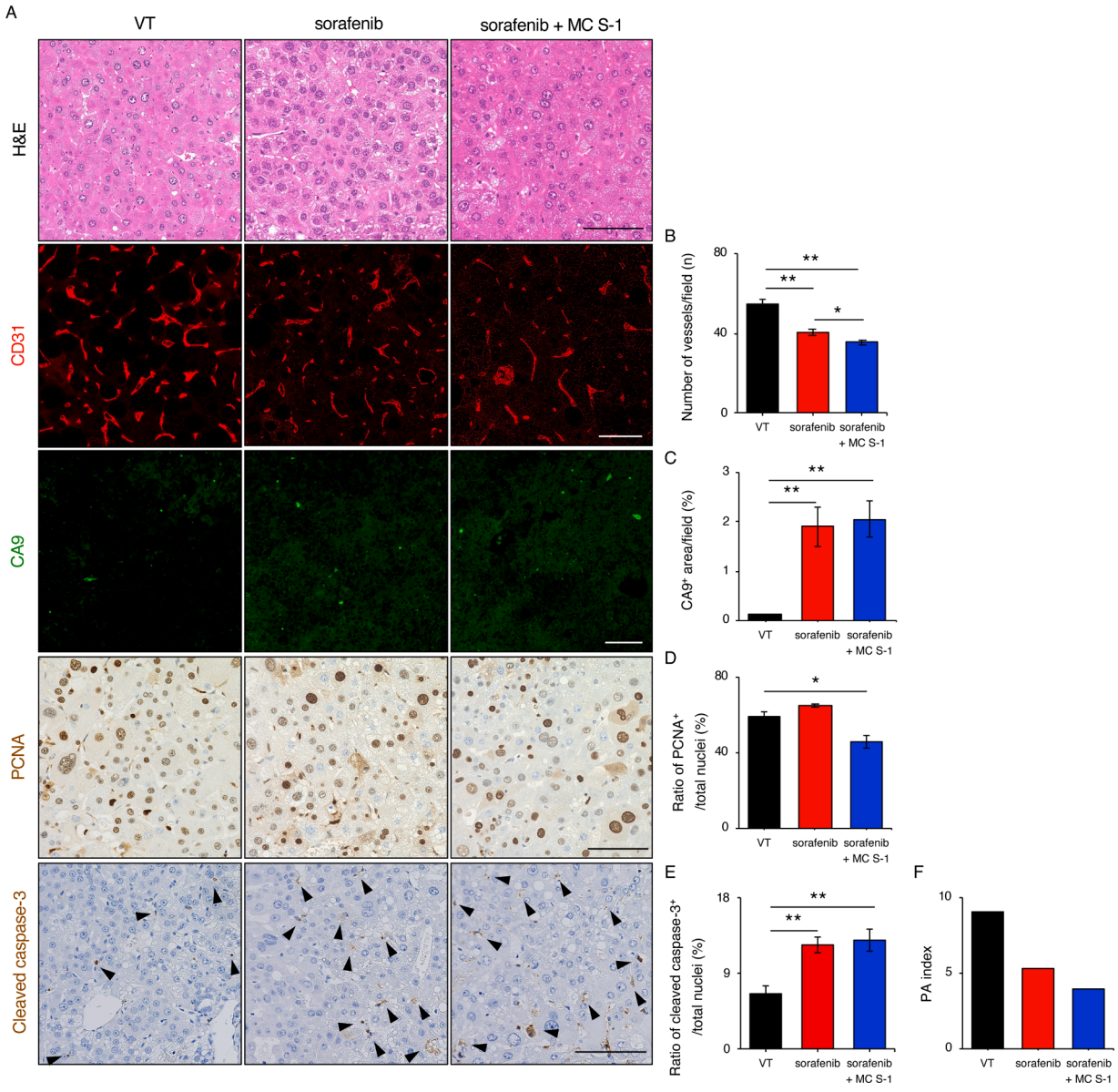

Fig. 3. Changes in the MVD, hypoxia, proliferation, and apoptosis in the NASH-related spontaneous HCC mice model. (A) Representative micrographs of CD31-positive, CA9-positive, PCNA-positive, and cleaved caspase-3-positive signals in the VT, sorafenib, and sorafenib plus MC S-1 groups. (B-F) Quantification of CD31-positive, PCNA-positive, cleaved caspase-3-positive areas, and PA index. Footnote: $*p < 0.05$, $**p < 0.001$. Data are presented as medians ± SEMs. Scale bar represents 100 μm. Abbreviations: MVD, micro vessel density; VT, vehicle treatment; MC, metronomic chemotherapy; NASH, nonalcoholic steatohepatitis; PCNA, proliferating cell nuclear antigen; PA, proliferation/apoptosis; SEM, standard error of the mean.

plus MC S-1 group (16.7%, 2/12) was significantly higher than that in the Sorafenib group (5.2%, 19/363; Table 1). Further, the disease control rate in the Sorafenib plus MC S-1 group was 41.7% (5/12), whereas that in Sorafenib group was 50.1% (182/363; Supplemental Fig. S2, Table 1). Main AEs with Sorafenib plus MC S-1 treatment were similar to those with Sorafenib treatment. The most common AE was hand-foot syndrome (58.3%, 7/12), followed by thrombocytopenia (33.3%, 4/12) (Table 2). AEs including alopecia, thrombocytopenia, and pancreatic enzymes elevation in combination chemotherapy were higher than those of Sorafenib (Table 2). There were no significant differences between the two groups in serum alanine aminotransferase and aspartate aminotransferase levels at four weeks after the treatment. Treatment was discontinued in some patients due to anorexia (6.7%, 1/12). No patient discontinued the treatment because of severe AEs in the Sorafenib plus MC S-1 group.

Discussion

In the present study, we evaluated the efficacy and tolerability of Sorafenib plus MC S-1 treatment in HCC xenograft and NASH-related spontaneous HCC mouse models. Furthermore, we also conducted a prospective study to evaluate the efficacy of this treatment for advanced HCC patients.

Principle mechanisms of MC have been described as anti-angiogenic effects and modulation of the host immune system [16] . A lower toxicity profile of MC compared to that of MTD has also been reported [16, 25-28]. We previously reported that the antitumor effect of combination chemotherapy comprising vandetanib plus MC S-1 was stronger than that of vandetanib and MTD S-1 mono therapy with fewer AEs in an HCC xenograft mouse model [18]. In addition, several rationales for the combination of Sorafenib and MC S-1 have been described as follows: 1) Sorafenib enhances the antitumor effect of S-1 through the down

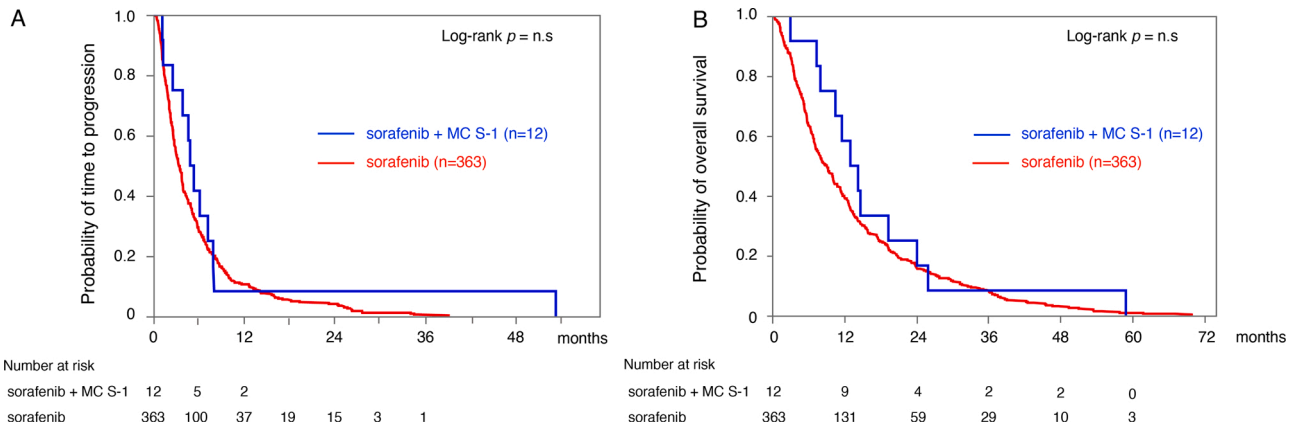

Fig. 4. Time to progression and overall survival analysis by the Kaplan–Meier method to compare sorafenib and sorafenib plus MC S-1 treatments. There were no significant differences in (A) time to progression and (B) overall survival. Footnote: *p*-values were calculated by the log-rank test. Abbreviation: n.s, no significance.

Table 1
Therapeutic effects in treatments with Sorafenib plus MC S-1 (n = 12) and sorafenib (n = 363).

	OR* [95% CI]	Sorafenib + MC S-1 n(%)	Sorafenib n (%)	*p*
Complete response	-	0(0)	0(0)	1.00
Partial response	4.07 [0.83–20.0]	2(16.7)	19(5.2)	<0.05
Stable disease	0.41 [0.11–1.54]	3(25.0)	163(45.0)	0.91
Progressive disease	1.40 [0.44–4.49]	7(58.3)	181(49.8)	0.11
Disease control rate	0.71 [0.22–2.28]	5(41.7)	182(50.2)	0.71

Abbreviation: OR, odds ratio; CI, confidence interval; MC, metronomic chemotherapy.
* OR: Odds ratio for Sorafenib + MC S-1 to Sorafenib.

Table 2
Any grade of adverse events in treatments with Sorafenib plus MC S-1 (n = 12) and Sorafenib (n = 363).

Adverse events profile	OR* [95% CI]	Sorafenib + MC S-1 n(%)	Sorafenib n (%)	*p*
Hand-foot syndrome	2.06 [0.64–6.61]	7(58.3)	147(40.5)	0.11
Thrombocytopenia	14.6 [3.86–55.4]	4(33.3)	12(3.3)	<0.01
Alopecia	11.8 [2.76–50.2]	3(25)	10(5.6)	<0.01
Pancreatic enzymes elevation	40.0 [7.07–226]	3(25)	3(0.8)	<0.01
Diarrhea	0.97 [0.21–4.54]	2(16.7)	62(17.1)	0.51
Hypertension	2.49 [0.52–11.9]	2(16.7)	27(7.4)	0.12
Stomatitis	2.91 [0.34–24.6]	1(8.3)	11(3.0)	0.16
Anorexia	1.97 [0.24–16.2]	1(8.3)	16(4.4)	0.26
ALT elevation	0.41 [0.05–3.22]	1(8.3)	66(18.2)	0.81

Abbreviations: OR, odds ratio; CI, confidence interval; MC, metronomic chemotherapy; ALT, alanine aminotransferase.
* OR: Odds ratio for Sorafenib + MC S-1 to Sorafenib.

regulation of TS [29] or E2F-1 [30]; 2) MC S-1 acts on tumor cells to up regulate the expression of an endogenous inhibitor of angiogenesis and tumor growth (thrombospondin-1), thereby enhancing anti-angiogenesis effects of Sorafenib [18]. Furthermore, it has been reported that MC UFT (uracil + tegafur), a 5-fluorouracil oral prodrug, could delay the emergence of Sorafenib resistance [31]. Therefore, in this study, we investigated whether combination therapy of Sorafenib plus MC S-1 could enhance the therapeutic effect and decrease the frequency of AEs.

First, in the evaluation of the antitumor effect of Sorafenib plus MC S-1 *in vivo*, we employed the HAK-1B xenograft model and NASH-related spontaneous HCC mouse model and treated them with Sorafenib plus MC S-1. Based on our previous study, the dose of MC S-1 in the mouse model was determined as 5 mg/kg twice daily [18]. Significantly stronger antitumor effects were shown in the Sorafenib and the MC S-1 group compared to those in the Sorafenib or VT group. Immunohistochemical staining to assess the detailed antitumor effects in each treatment group was also performed. With regard to the anti-angiogenic effects, Sorafenib was shown to exert significantly stronger anti-angiogenic effects than the VT; moreover, additional anti-angiogenic effects of the combination of Sorafenib with MC S-1 were observed compared to those with each mono therapy. Consistent with the decreased MVD, the CA9-positive staining area, known to be a marker of hypoxia, increased in the Sorafenib and Sorafenib plus MC S-1 groups. The combination of Sorafenib with MC S-1 also suppressed tumor cell growth and increased tumor cell apoptosis. We have shown that MC S-1 exerted antitumor effects via inducing thrombospondin-1 expression in tumor tissues, which inhibits the proliferation of endothelial cells [18]. Moreover, MC S-1 is known to have inhibitory effects on the viability and mobility of endothelial progenitor cells [19] and augment antitumor immune response [16]. Thus, these effects seemed to be indirectly added on the anti-angiogenic effects of Sorafenib through the PDGF and VEGF signaling blockade [7]. Taken together, we proved a synergistic anti-angiogenic effect by combining Sorafenib with MC S-1 in two different *in vivo* mouse models and provided the potential mechanisms by which Sorafenib plus MC S-1 induced apoptosis and inhibited cell proliferation.

Interestingly, regarding the maximum tumor size and volume in the MTD-S1 group were relatively larger than those in the VT and Sorafenib + S-1 group. One of the reasons were seemed that cancer cells acquired chemo resistance in during the drug-free periods in the MTD S-1 schedule [32]. In phase III trial of Sorafenib plus MTD S-1 for patients with advanced HCC, it has not been evaluated whether the drug-free periods led to tumor growth; however, this combination therapy did not prolong OS suggests that acquired chemo resistance in cancer cells during the drug-free periods might be reflected [14].

Subsequently, we evaluated the tolerability of Sorafenib plus MC S-1 in the STAM™ mouse model to more closely examine the clinical physiology of HCC, which develops from a background of NASH-related LC [23]. Only in the MTD S-1 group, peripheral WBCs were significantly

decreased, whereas the body weight, Hb concentration, and peripheral WBCs were not significantly different among the groups. These results are consistent with previous reports indicating that MC could be safely used even in an LC-HCC rat model [33]. Sorafenib is also known to exert anti-fibrotic effects [34], and we evaluated this in the STAM™ mouse model. Consistent with results of the previous report, hepatic fibrosis decreased in the Sorafenib group; however, an additional anti-fibrotic effect was not observed by combining MC S-1 and Sorafenib.

Based on these findings from preclinical experiments, we subsequently conducted a prospective study of Sorafenib plus MC S-1 for patients with advanced HCC. Although the objective response rate in the Sorafenib plus MC S-1 group (16.7%; 2/12) was significantly higher than that in the Sorafenib group (5.2%; 19/363), there were no significant differences in time to progression, OS, and disease control rates between the groups. AEs observed with Sorafenib plus MC S-1 treatment were controllable and similar to those with Sorafenib treatment.

In patients with LC, thrombocytopenia is the most general hematological abnormality [35], and therefore, the incidence of thrombocytopenia observed in the Sorafenib plus MC S-1 group was considered to be significantly higher than that in the Sorafenib group. In this study, we chose every-other-day S-1 administration for 4 weeks without prolonged breaks as the MC schedule; however, the possibility remains that once every 2 days or other schedules should be assessed to reduce thrombocytopenia. Therefore, further research to determine an appropriate schedule for patients with LC-HCC is desired.

Recently, a clinical trial revealed that a combination of the immune checkpoint inhibitor atezolizumab plus the anti-VEGF antibody bevacizumab is superior to Sorafenib, in terms of survival and progression-free survival, for patients with unresectable HCC [36]. Although this combination therapy has been approved as first-line therapy [6,36,37], it has been suggested that there is a group that is not expected to be less effective, such as patients with HCC of a non-viral etiology [38]. MC S-1 is known to have effects on augmenting antitumor immune response [16], therefore, the combination therapy of atezolizumab plus bevacizumab with MC S-1 might be also be promising for the treatment of advanced HCC.

Previously, the effectiveness of MC for HCC was published in several reports as follows: MC capecitabine for second-line HCC treatment was superior to best supportive care [39], a bio-modulatory therapy approach including MC capecitabine, COX-2 inhibitor, and PPAR-gamma agonist could efficacious and tolerable in advanced HCC [40], and MC Cisplatin and 5-fluorouracil could be safely used even in patients with poor liver function [41]. Although several MTA treatments have been approved for advanced HCC treatment, no optimal sequential treatment method has been found. In HCC treatment, it is important to extend the treatable period without deteriorating liver function [3], therefore, these advantages from MC treatment should be promising.

Limitations of the study

Although our current work uncovered the efficacy and tolerability of Sorafenib plus MC S-1 therapy for HCC in preclinical and clinical experiments, several limitations exist. First, most of our findings regarding tumor changes caused by treatments were demonstrated only in preclinical models. Second, HCC in STAM™ mice was confirmed by macroscopic observation and assessed by hematoxylin and eosin staining; however, the possibility of regenerative nodules cannot be denied. Third, although the STAM™ mouse is a diabetes-based NASH related HCC model, the effect on blood glucose level was not evaluated as AE. Finally, the present prospective study could not rule out bias due to the small sample size. However, based on our work it would be interesting in the future to design clinical trials combining Sorafenib plus MC S-1 for advanced HCC patients.

Conclusions

We verified the efficacy and tolerability of Sorafenib plus MC S-1 treatment for LC-HCC based on two *in vivo* mouse models and a clinical prospective pilot experiment. MC S-1 augmenting the antitumor effects of Sorafenib via anti-angiogenic effects with less toxicity might be promising for the treatment of advanced HCC. Accordingly, Sorafenib plus MC S-1 appears to be effective and tolerable and could thus be considered as a treatment option for patients with advanced HCC.

Ethical statement

This prospective study was conducted in accordance with the Declaration of Helsinki and reviewed and approved by the Ethical Committee of the Kurume University School of Medicine (Ethical code: 10295). Written informed consent was obtained from all subjects involved in the study.

Author Contributions

All authors discussed the results and contributed to the final manuscript. Conceptualization, H.S., H.I., and T.T. (Takuji Torimura).; methodology, H.I.; software, S.S.; vali-dation, T.S. (Tomotake Shirono)., A.M., and T.S. (Takahiko Sakaue).; formal analysis, H.S. and A.K.; investigation, Y.N., N.K., and T.T. (Toshimitsu Tanaka).; resources, S.O., R.K., and Y.N.; data curation, M.N.; writing—original draft preparation, H.S.; writing—review and editing, H.I., H.Y., H.K., and T.T. (Takuji Torimura); visualization, H.Y.; supervision, H.Y., T.N. (Toru Nakamura); project administration, D.N. and T.N. (Takashi Niizeki); funding acquisition, H.I. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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