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Silencing of secreted protein acidic and rich in cysteine inhibits the growth of human melanoma cells with G_1 arrest induction

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The overexpression of secreted protein acidic and rich in cysteine (SPARC) is associated with increased aggressiveness and poor prognosis in malignant melanoma. Its roles and underlying mechanisms on melanoma cell growth, however, are not fully clarified. To validate the potential of SPARC as a therapeutic target, we examined the effect of the knockdown of SPARC with SPARC-specific siRNA on the growth of human melanoma cell lines. SPARC siRNAs exerted a potent knockdown effect. Silencing of SPARC resulted in growth inhibition with G_1 arrest accompanied by accumulation of p21, a G_1 cyclin-dependent kinase inhibitor, in MeWo and CRL1579 cells. Moreover, the induction of p53 was observed in MeWo cells, but not in CRL1579 cells. Conditioned media containing SPARC from MeWo cells could not restore the growth of SPARC-silenced MeWo cells. This result suggests that intracellular SPARC, but not secreted SPARC, is involved in cell proliferation. In addition, silencing of SPARC induced apoptosis in MeWo and CRL1579 cells. Furthermore, when MeWo cells in which SPARC expression was transiently knocked down by SPARC siRNA were implanted in nude mice, the tumor growth was suppressed. Our findings suggest that SPARC contributes to cell growth and could be a potential target molecule for melanoma therapy. (*Cancer Sci* 2010; 101: 913–919)

The expression of secreted protein acidic and rich in cysteine (SPARC), a matricellular glycoprotein, is highly regulated during development, tissue repair, and remodeling.[1] SPARC interacts with several extracellular matrix components.[2] In addition, SPARC modulates growth factor activity, and regulates matrix metalloproteinase expression.[3–6] These reports suggest that SPARC regulates cell shape, proliferation, migration, and differentiation.

The level of SPARC expression is low in normal adult tissue, whereas this protein is overexpressed in a wide range of human cancers.[7–9] Some groups have reported that overexpression of SPARC is associated with aggressiveness and high potential of metastasis in various human cancers, including melanoma, breast, lung, esophagus, pancreas, and bladder cancers.[10–15] It has also been reported that its overexpression is related to poor prognosis in many cancers.[12,16,17] In most cancers, SPARC is produced in tumor stromal cells, such as fibroblasts and endothelial cells, rather than in cancer cells.[7,12,16] In contrast, the level of SPARC expression in melanoma and glioma cells is very high.[10,18]

Selective silencing of gene expression using siRNA has been evaluated to be not only a powerful research tool but also a potentially therapeutic approach to cancer.[19] It has been reported that silencing of SPARC directly inhibited the survival signaling pathway in glioma cells under serum reduced conditions *in vitro*.[20] Some studies using antisense RNA have showed that downregulation of SPARC abrogated a tumorigenic capacity in melanoma cells.[21–23] One of the reasons for this rejection appears to be the involvement of the antitumor activity of host polymorphonuclear cells. The underlying mechanism of SPARC on the growth of melanoma cells, however, has not been fully elucidated.

We have previously reported that the serum SPARC in melanoma patients was useful as a novel tumor marker for early diagnosis of melanoma,[24] and have shown the usefulness of SPARC as a target for cancer immunotherapy.[25] From these points of view, we hypothesized that SPARC might become a target molecule for cancer treatment, and examined whether silencing of SPARC with siRNA could influence cell growth in melanoma cells *in vitro* and *in vivo*. We found that silencing of SPARC in human melanoma cell lines induced G_1 cell cycle arrest and apoptosis. We herein report for the first time that silencing of endogenous SPARC by siRNA directly inhibits growth in melanoma cells.

Materials and Methods

Cell culture. Human melanoma cell lines MeWo, SK-MEL-28, and HMV-I were maintained in DMEM (Sigma, St Louis, MO, USA) containing 10% FBS (Hyclone, Logan, UT, USA). Human melanoma cell line CRL1579 was obtained from RIKEN Cell Bank, RIKEN BioResource Center (Tsukuba, Japan) and maintained in RPMI-1640 (Sigma) containing 10% FBS. All cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

RNAi and transfection. The siRNA duplexes were purchased from Qiagen (Valencia, CA, USA) (AllStars Neg. Control siRNA) and Invitrogen (Carlsbad, CA, USA) (SPARC). The siRNA sequences used were as follows: SPARC siRNA-1, 5′-AGUCACCUCUGCCACAGUUUCUUCC-3′; SPARC siRNA-2, 5′-AUACAGGGUGACCAGGACGUUCUUG-3′; and SPARC siRNA-3, 5′-AUUCUCAUGGAUCUUCUUCACCCGC-3′. Lipofectamine RNAiMax (Invitrogen) was used for the reverse transfection method following the manufacturer's protocol. For analysis of transfection efficiency, the cells were transfected with FITC-conjugated negative control siRNA (Qiagen) at 50 nM. After 24 h, the cells were analyzed using flow cytometry. Flow cytometry was carried out using a FACSCalibur (BD Biosciences, San Jose, CA, USA) and analyzed using CellQuest (BD Biosciences) and FlowJo (Tree Star, San Carlos, CA, USA) software.

Immunoblot analysis. The cell samples were lysed in appropriate amounts of lysing buffer (150 mM NaCl, 50 mM Tris [pH 7.4], 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM EDTA, and protease inhibitor tablet [Roche Applied Sciences, Penzberg, Germany]). Protein concentration was determined

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with DC protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysates or supernatants were heat-denatured, resolved by 10% SDS-PAGE, and electrotransferred to PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked in TBS-Tween 20 (10 mM Tris [pH 7.4], 150 mM NaCl, and 0.1% Tween 20) containing 5% non-fat milk for 2 h at room temperature and incubated overnight at 4°C with primary antibodies: anti-SPARC (Haematologic Technologies, Essex Junction, VT, USA), anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ERK (Cell Signaling Technology, Beverly, MA, USA), and anti-β-actin (Sigma), followed by reaction with HRP-conjugated secondary antibody (Jackson Immuno Reseach, West Grove, PA, USA). In addition, polyclonal HRP-conjugated anti-p21 antibody (Santa Cruz Biotechnology) was used. The bands were visualized by ECL (GE Healthcare, Little Chalfont, UK).

Cell proliferation assay. HMV-I, MeWo, CRL1579, and SK-Mel-28 cells were transfected with SPARC siRNA or negative control siRNA at indicated concentrations, then seeded in 96-well flat bottom plates at 3×10^3, 2×10^3, 4×10^3, or 2×10^3 cells/100 μL per well, respectively. The cells were cultured in the presence of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (Dojindo, Kumamoto, Japan) for 3 h, followed by measurement of absorbance at 450 nm and 650 nm. For the swapping experiment, the conditioned media from siRNA-transfected MeWo cells (1×10^5 cells in six-well plate) were harvested at 72 h post-transfection, centrifuged at 1700*g* for 5 min to remove cellular debris, and stored at −80°C until use.

Cell cycle analysis and annexin V staining. For flow cytometric cell cycle analysis, the cells treated with siRNA were collected, washed with PBS, fixed in cold 70% ethanol, and stored at −20°C until staining. After fixation, the cells were washed with PBS and incubated with 50 μg/mL RNaseA (Sigma) for 30 min at 37°C, before staining with 50 μg/mL propidium iodide (Sigma). Apoptotic cells in early and late stages were detected using an annexin V–FITC Apoptosis Detection Kit from BioVision (Mountain View, CA, USA). In brief, the cells were transfected with siRNA at 10 nM. At 96 h post-transfection, culture media and cells were collected and centrifuged. After washing, cells were resuspended in 490 μL annexin V binding buffer, followed by the addition of 5 μL annexin V–FITC and 5 μL propidium iodide. The samples were incubated in the dark for 5 min at room temperature and analyzed using flow cytometry.

***In vivo* tumor experiment.** For assessment of tumor growth *in vivo*, MeWo cells were transfected with SPARC siRNA-3 or negative control siRNA. Twenty-four hours later, the cells were trypsinized, and resuspended in serum-free DMEM. Four female athymic nude mice, ages 6- to 8-weeks-old were s.c. implanted with 1×10^6 or 3×10^5 MeWo cells per 0.1 mL into the right and left flanks resulting in two tumors per mouse. The tumor volume in mm^3 was calculated by the formula: volume = $(\text{width})^2 \times \text{length}/2$. The mice were maintained under specific pathogen-free conditions. Animal experiments in this study were approved by the Animal Research Committee of the National Cancer Center Hospital East (Kashiwa, Japan).

Statistical analysis. All data are presented as the mean ± SD. The data from the WST-8 assay were statistically analyzed by one-way ANOVA followed by Dunnett's multiple comparison test or Tukey's multiple comparison test. Tumor volume between SPARC siRNA-treated cells and negative control siRNA-treated cells was compared for statistical significance using the Mann–Whitney *U*-test or Student's *t*-test. The results were considered significant when $P < 0.05$. All tests were carried out with Dr. SPSS II for Windows (SPSS Japan, Tokyo, Japan).

Results

Silencing of SPARC expression in human melanoma cell lines. We examined the knockdown effect of SPARC siRNAs on melanoma cells in this study. To assess the knockdown efficiency of SPARC, we transfected negative control siRNA or siRNAs targeted to SPARC (SPARC siRNA-1, -2, or -3) into SPARC expressing cell lines, MeWo, CRL1579, and SK-MEL-28 (Fig. 1A,B). Transfection with three SPARC siRNAs decreased the level of SPARC protein in all tested cell lines compared with negative control siRNA. This silencing effect was enhanced in a dose-dependent manner. The level of SPARC protein was not affected among the cells transfected with negative control siRNA at each concentration. Treatment with 10 nM SPARC siRNAs resulted in the robust downregulation of SPARC expression. SPARC siRNA-3 showed the strongest

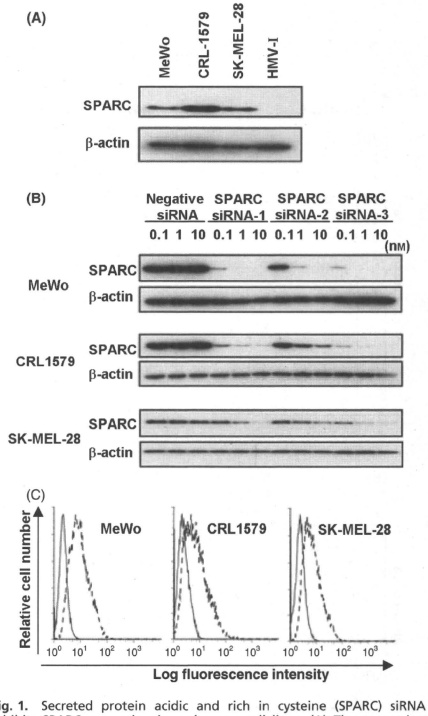

Fig. 1. Secreted protein acidic and rich in cysteine (SPARC) siRNA inhibits SPARC expression in melanoma cell lines. (A) The expression of SPARC protein in MeWo, CRL1579, SK-MEL-28, and HMV-I cells was analyzed using Western blot. β-actin was used as an internal control. (B) Knockdown efficiency of three SPARC siRNAs. At 48 h post-transfection with indicated concentrations, the expression of SPARC protein in MeWo, CRL1579, and SK-MEL-28 cells was analyzed using Western blot. β-actin was used as an internal control. (C) Transfection efficiency of siRNA on melanoma cells was assessed by flow cytometry at 24 h post-transfection with 50 nM FITC-conjugated negative control siRNA. The plot shows the relative cell number of melanoma cells (*y* axis) and the log fluorescence intensity (*x* axis).

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effect. Knockdown efficiency of SPARC protein was the highest in MeWo cells and the lowest in the SK-MEL-28 cells. These results suggest that these SPARC siRNAs successfully exert a silencing effect for SPARC expression. Transfection efficiency of siRNA was the highest in MeWo cells and the lowest in SK-MEL-28 cells (41.05%, 32.3%, and 27.97% at >10 of log fluorescence intensity in MeWo, CRL1579, and SK-MEL-28 cells, respectively) (Fig. 1C). This result was similar to knockdown efficiency among the melanoma cell lines.

Silencing of SPARC inhibits the growth of melanoma cells. We examined the effect of SPARC siRNA on the growth of melanoma cell lines. To check for non-specific side-effects of three siRNAs, we transfected SPARC siRNAs into SPARC non-producing cell line, HMV-I (Fig. 2A). As SPARC siRNA-2 showed a growth inhibition in HMV-I cells (data not shown), this siRNA was excluded from subsequent studies. SPARC siRNA-1 and -3 at 10 nM had no effect on cell growth. Therefore, we judged this concentration of SPARC siRNA to be reasonable in the experiments. SPARC siRNA-3 showed a marked growth inhibitory effect compared to SPARC siRNA-1 in MeWo and CRL1579 cells. The inhibition of the proliferation in MeWo cells was stronger than in CRL1579 cells. Silencing of SPARC hardly affected the growth of SK-MEL-28 cells. SPARC siRNA-3 inhibited the growth of MeWo cells in a dose-dependent manner, but did not significantly inhibit the growth of CRL1579 cells at 1 nM (Fig. 2B). These data indicate that silencing of SPARC can inhibit the growth of melanoma cell lines *in vitro*. Furthermore, we investigated whether the growth inhibition by silencing of SPARC in MeWo cells could be canceled by the addition of exogenous SPARC (Fig. 3A). As a source of exogenous SPARC, we prepared the conditioned media from MeWo cells transfected with negative control siRNA, or SPARC siRNA-3 (Fig. 3B). Western blot analysis revealed that the conditioned media from MeWo cells treated with negative control siRNA contained a substantial amount of SPARC protein. In contrast, SPARC protein in conditioned media from SPARC siRNA-3-treated MeWo cells was negligible. At 24 h post-transfection, conditioned media were swapped, and SPARC-silenced MeWo cells were cultured under both conditioned media. The growth of SPARC-silenced MeWo cells was not significantly different between SPARC-containing and SPARC-free conditioned media. These results indicate that intracellular SPARC, but not extracellular, is involved in the growth of melanoma cells.

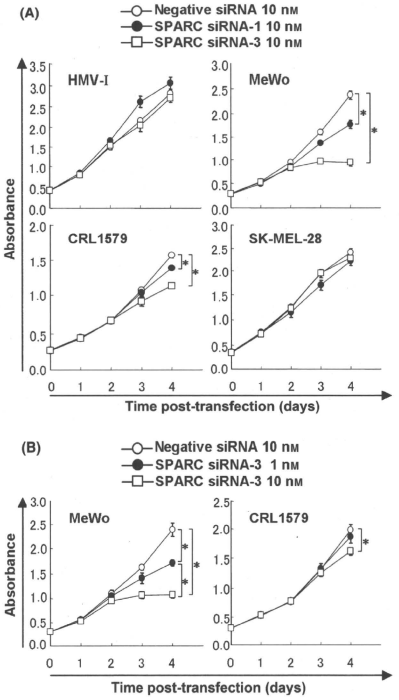

Fig. 2. Effects of secreted protein acidic and rich in cysteine (SPARC) knockdown on cell growth in melanoma cell lines. (A) HMV-I, MeWo, CRL1579, and SK-MEL-28 cells were transfected with SPARC siRNA-1, siRNA-3, or negative control siRNA at 10 nM. (B) MeWo and CRL1579 cells were transfected with SPARC siRNA-3 at the indicated concentrations. Cell growth was measured daily using WST-8 assay. The data at day 4 were statistically analyzed by one-way ANOVA followed by Dunnett's multiple comparison test (A) or Tukey's multiple comparison test (B). Error bars indicate SD. $^{*}P < 0.05$.

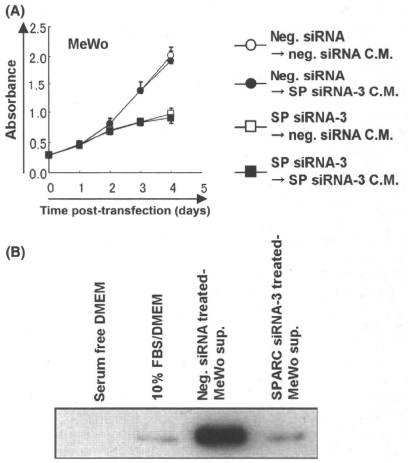

Fig. 3. Intracellular secreted protein acidic and rich in cysteine (SPARC), but not extracellular, is involved in the growth of melanoma cells. (A) Effect of exogenous SPARC on SPARC-silenced melanoma cells. MeWo cells were transfected with SPARC (SP) siRNA-3 (squares) or negative control (neg.) siRNA (circles) at 10 nM. After 24 h, cell culture media were swapped for MeWo cells treated with SP siRNA-3 (closed squares and circles) or neg. siRNA (open squares and circles). Cell growth was measured by using WST-8 assay. The data were statistically analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Error bars indicate SD. C.M., conditioned media. (B) The preparation of siRNA-treated MeWo cell conditioned media. The amount of SPARC protein in conditioned media was analyzed using Western blot. sup., supernatants.

Inhibition of SPARC expression induces cell cycle arrest in melanoma cells. We examined the effects of SPARC siRNA on cell cycle progression. Silencing of SPARC in MeWo cells increased G_1 and decreased S phase populations at 72 h post-transfection with SPARC siRNA-1 or -3 (Fig. 4A). These results indicate that silencing of SPARC induces G_1 arrest. Similarly, the induction of G_1 arrest was observed in SPARC-silenced CRL1579 cells. However, no change was observed in SPARC-silenced SK-MEL-28 cells. To confirm G_1 arrest induced by SPARC siRNA, p21 and p53 protein expressions were investigated (Fig. 4B). When SPARC siRNA induced G_1 arrest in MeWo and CRL1579 cells, notable p21 induction was observed. The base level of p21 protein was very low in SK-MEL-28 cells. The accumulation of p21 protein was not observed in SPARC-silenced SK-MEL-28 cells. In SPARC-silenced MeWo cells, the level of p53 protein was increased compared with negative control siRNA-treated cells. However, it was not observed in SPARC-silenced CRL1579 or SK-MEL-28 cells. These results indicate that there is no correlation between p21 and p53 induction in CRL1579 and SK-MEL-28 cells. Taken together, these results indicate that the downregulation of SPARC induces growth inhibition with G_1 arrest and p53-dependent or -independent p21 accumulation in some melanoma cells.

Inhibition of SPARC expression enhances apoptosis in melanoma cells. We next tested whether silencing of SPARC induced cell death in melanoma cell lines. The treatment of

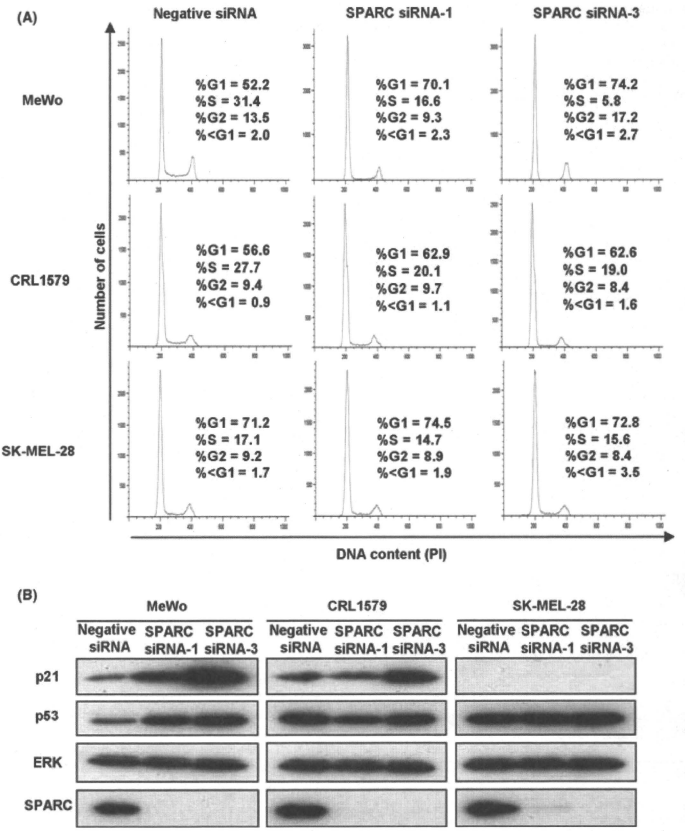

Fig. 4. Silencing of secreted protein acidic and rich in cysteine (SPARC) induces cell cycle arrest at G_1 phase in MeWo and CRL1579 melanoma cells. (A) Cell cycle distribution of melanoma cell lines transfected with SPARC siRNA. MeWo, CRL1579, and SK-MEL-28 cells were transfected with 10 nM SPARC siRNA-1, siRNA-3, or negative control siRNA. At 72 h post-transfection, DNA content was measured using propidium iodide (PI) staining on flow cytometry. The percentage of cells in each phase of the cell cycle is shown in each panel. (B) The expression of p21 and p53 proteins in MeWo, CRL1579, and SK-MEL-28 cells transfected with 10 nM SPARC siRNA-1, siRNA-3, or negative control siRNA. At 72 h post-transfection, total protein was analyzed using Western blot. ERK was used as an internal control.

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MeWo and CRL1579 cells with SPARC siRNA-3 increased (more than two-fold) early apoptotic cells as well as late apoptotic cells, compared with negative control siRNA treatment (Fig. 5). In SK-MEL-28 cells, the increase of apoptotic cells was not observed. Similarly, no difference was observed in the ratio of apoptosis between negative control siRNA and SPARC siRNA-3-treated HMV-I cells. These findings suggest that SPARC is involved in apoptosis to maintain cellular survival in some melanoma cells.

Silencing of SPARC inhibits growth of melanoma cells *in vivo*. We attempted to examine the effect of silencing of SPARC on tumor growth *in vivo* with a xenograft model. To assess the persistence of SPARC siRNA-mediated silencing, the kinetics of the downregulation of SPARC protein in MeWo cells *in vitro* was shown using Western blot analysis (Fig. 6A). The duration of the downregulation by SPARC siRNA was 6 days *in vitro*. At 8 days post-transfection, SPARC expression increased slightly. On the basis of these findings, we examined whether silencing of SPARC inhibited tumor growth *in vivo* using a xenograft model. A similar number of MeWo cells, transfected with SPARC siRNA-3 or negative control siRNA, were injected into both flanks of four nude mice. When tumors were palpable, their size was determined until 8 days post-implantation. As seen in Figure 6(B,C), the growth of tumors transfected with SPARC siRNA-3 was significantly suppressed compared with tumors transfected with negative control siRNA. At 8 days post-implantation, the growth inhibition of mice implanted with 1×10^6 or 3×10^5 cells transfected with SPARC siRNA-3 was 49% and 48%, respectively, as compared with negative control siRNA transfected cells ($P < 0.05$). These results indicate that downregulation of SPARC suppresses tumor growth *in vivo*.

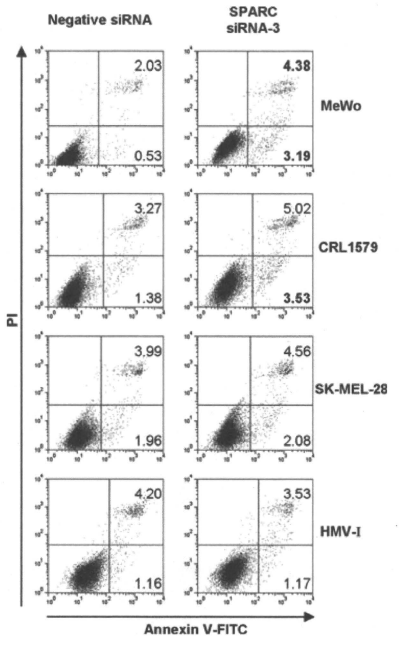

Fig. 5. Silencing of secreted protein acidic and rich in cysteine (SPARC) results in induction of early apoptosis in melanoma cell lines. For flow cytometric analysis, cells were harvested at 96 h after transfection with 10 nM SPARC siRNA-3 or negative control siRNA, then stained with annexin V–FITC and propidium iodide (PI). The percentages of annexin V^+/PI^- (early apoptotic) and annexin V^+/PI^+ (late apoptotic) cells is shown in each panel. Values in bold indicate more than a two-fold increase in apoptotic cells using SPARC siRNA-3, compared with negative control siRNA.

Discussion

The underlying mechanisms of growth regulation by SPARC in tumor cells are complicated. We focused on cell growth, and showed that SPARC produced from melanoma cells functionally linked to their own growth in this study. The most significant finding was that the downregulation of SPARC expression induced growth inhibition with G_1 arrest. This growth inhibitory effect by silencing of SPARC was maintained in an *in vivo* xenograft model.

We showed that there was a correlation with the level of p21 accumulation and the growth inhibition by silencing of SPARC. To examine whether induced p21 was involved in growth arrest by the silencing of SPARC, p21 was knocked down in MeWo cells using three siRNAs targeted to p21. The transfection with p21 siRNAs resulted in the reduction of the level of p21 protein accumulated using SPARC siRNA-3 (data not shown). However, the induction of p21 protein did not contribute to cell cycle arrest in our model, because growth inhibition by silencing of SPARC was maintained in cells even after knockdown of the p21 protein level (data not shown). These results suggest that p21 is not a major player in mediating the growth inhibition by silencing of SPARC. It is well known that G_1 arrest is regulated by Ink4 and Cip/Kip family proteins.[26] We have not yet examined the correlation between other Ink4 or Cip/Kip family proteins and G_1 arrest caused by silencing of SPARC. To better understand the mechanism of G_1 arrest induction, further investigations are needed to examine the expression of these proteins in SPARC-silenced cells.

It has been described that p21 is a transcriptional target of p53.[27] The expression of p21 was positively correlated with the expression of p53 in SPARC siRNA transfected MeWo cells, whereas p53 expression was not changed in SPARC siRNA transfected CRL1579 or SK-MEL-28 cells. Therefore, we speculate that p53-dependent or -independent p21 induction occurred in the melanoma cells treated with SPARC siRNA. The mechanisms for p53 induction in SPARC-silenced MeWo cells are unclear. Many forms of stress have now been shown to activate p53.[28,29] Although not examined in this study, it is possible that the loss of the protective effect of SPARC against some stress might affect p53 induction. Weaver *et al.* reported that SPARC protects lens epithelial cells from cell death induced by exposure to intracellular stressor, tunicamycin.[30] In addition, it has been reported that SPARC promotes glioma cell survival through Akt activation through integrin signaling under serum-free conditions.[20] These reports strongly suggest that SPARC plays a role as an antistress factor.

How does SPARC act? We showed that exogenous SPARC in culture conditioned media could not cancel the growth inhibition of MeWo cells with SPARC siRNA treatment. Thus, extracellular SPARC released from melanoma cells had no effect on cell proliferation in this system. We suggest that intracellular SPARC, but not secreted extracellular SPARC, contribute to cell growth or survival advantages. Martinek *et al.* have proposed intracellular SPARC functions as collagen-specific molecular

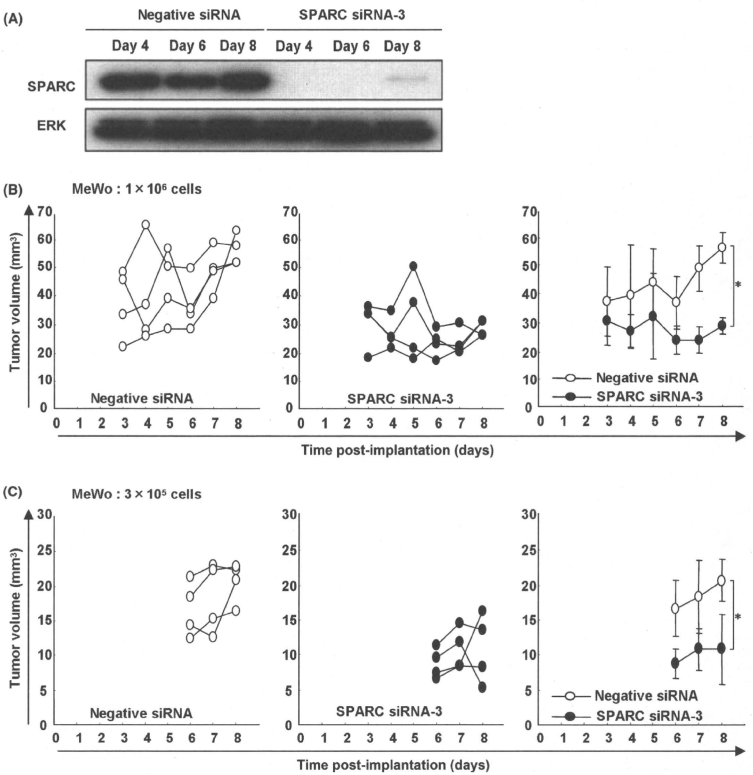

Fig. 6. Effect of secreted protein acidic and rich in cysteine (SPARC) knockdown on melanoma cell growth *in vivo*. (A) The duration of the knockdown effect of SPARC siRNA *in vitro*. At 4, 6, and 8 days post-transfection, SPARC protein was analyzed using Western blot. ERK was used as an internal control. MeWo cells were transfected with SPARC siRNA-3 or negative control siRNA in culture dishes. Twenty-four hours later, 1×10^6 (B) or 3×10^5 (C) tumor cells were s.c. implanted into both flanks of four nude mice. Individual tumor growth was measured with a caliper every day until 8 days post-implantation. Statistical differences were determined by Mann–Whitney *U*-test (B) or Student's *t*-test (C). The mean tumor volumes ± SD. $^*P < 0.05$ compared to negative control siRNA treatment at day 8.

chaperone, prior to their export from the endoplasmic reticulum.[31] Further study is needed to elucidate the roles of intracellular SPARC.

In our *in vivo* experiments, silencing of SPARC inhibited tumor growth, but did not lead to tumor rejection. This result might be due to the modest induction of apoptosis caused by silencing of SPARC. Other investigators showed that SPARC-silenced melanoma cells were abolished in *in vivo* xenograft models.[21] Their strategies for SPARC knockdown used SPARC downregulated stable cell lines. Their report suggests that it is important for the persistence of SPARC knockdown to abolish tumor cells. We need to further confirm the efficacy of the sequential administration of SPARC siRNA in the *in vivo* xenograft model.

Our results showed that there were differences in the degree of growth inhibition among SPARC siRNA-treated cell lines. Unlike MeWo and CRL1579 cells, the silencing of SPARC in SK-MEL-28 cells did not show growth inhibition. From our results, a reason for this might have been that the degree of knockdown of SPARC by siRNA in SK-MEL-28 cells was weaker than other cell lines. Second, the SPARC dependency on cell growth in SK-MEL-28 cells might have been less. We found that p53 was not induced by silencing of SPARC in CRL1579 and SK-MEL-28 cells. It is well known that p53 is important in the regulation of cell cycle checkpoints. Therefore, these results suggest that the regulation of cell cycle arrest by the checkpoint system in these cell lines might have been partial, compared with SPARC siRNA-treated MeWo cells. In addition, the antiproliferative effect of SPARC siRNA in CRL1579 cells was not clearly manifested, because the cell growth rate was lower in CRL1579 cells than in MeWo cells under ordinary culture conditions (data not shown). The differences of these

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inherent features of cell lines may have influenced the outcome of SPARC siRNA treatment.

Although the functional role of SPARC in cancer have been controversial, there are reports that it might play antitumorigenic roles in ovarian cancer.[32–34] It has been indicated that SPARC induces apoptosis in ovarian cancer cells.[32] In addition, SPARC normalizes the ovarian cancer microenvironment through vascular endothelial growth factor (VEGF) signaling modulation.[33] Furthermore, it has been reported that SPARC attenuates integrin-mediated signaling and Akt survival signaling in ovarian cancer cells.[34] The possible causes of these contradictory roles of SPARC in cancers might be the difference in tumor origin, properties of malignant cells, and tumor microenvironment. There is a need for further studies to clarify the roles of SPARC not only on cancer cells, but also on the interplay of tumor cells and tumor stroma.

In conclusion, we provided a new finding regarding the functional roles of SPARC on the regulation of cell proliferation in melanoma cell lines. The SPARC siRNAs we used exerted a potent knockdown effect. These siRNAs will be useful tools for therapeutic intervention by targeting SPARC. Further studies will elucidate the roles of SPARC on the growth of melanoma cells. All of this information contributes to a better understanding of SPARC as a potential therapeutic target for the treatment of melanoma.

Acknowledgments

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Phase I/II study of the pharmacokinetics, safety and efficacy of S-1 in patients with advanced hepatocellular carcinoma

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S-1, an oral fluoropyrimidine derivative, has been shown to be clinically effective against various solid tumors, and preclinical studies have demonstrated activity against hepatocellular carcinoma. We conducted a phase I/II study in patients with advanced hepatocellular carcinoma to examine the pharmacokinetics, recommended dose, safety and efficacy of S-1. In phase I, the administered dose of S-1 was approximately 64 mg/m^2 per day in three patients (level 1) and approximately 80 mg/m^2 per day in six patients (level 2). There was no dose-limiting toxicity at level 1, but two patients had dose-limiting toxicity at level 2 (grade 3 anorexia and grade 2 rash requiring eight or more consecutive days of rest). The recommended dose was finally estimated to be 80 mg/m^2 per day. There were no significant differences in the pharmacokinetics of S-1 between patients with Child-Pugh A and those with B. In phase II, five of 23 patients (21.7%) had partial responses. The median progression-free survival and overall survival were 3.7 and 16.6 months, respectively. The most common toxicities of grade 3 or 4 were elevated serum aspartate aminotransferase levels, hypochromia and thrombocytopenia. In conclusion, S-1 showed an acceptable toxicity profile and promising antitumor activity for hepatocellular carcinoma, warranting further evaluation in randomized clinical trials. (*Cancer Sci* 2010; 101: 2606–2611)

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Outcomes remain poor because the disease is usually advanced and associated with hepatic impairment at diagnosis, and because of the high rate of recurrence resulting from either intrahepatic metastases from the primary tumor or multicentric lesions. As for therapy, surgical resection and percutaneous ethanol injection (PEI) or radiofrequency ablation (RFA) are considered the mainstays of treatment in patients with potentially curable disease. Transcatheter arterial chemoembolization (TACE) is the treatment of choice for noncurative HCC. Despite numerous clinical trials of a wide variety of cytotoxic agents, survival remains dismal in HCC.[1] Recently, sorafenib, an oral multi-kinase inhibitor that targets mainly Raf kinases and receptor tyrosine kinases associated with angiogenesis (vascular endothelial growth factor receptor [VEGFR]-2/-3 and platelet-derived growth factor receptor [PDGFR]-β), provided a significant survival benefit in patients with advanced HCC enrolled in placebo-controlled, randomized, phase III trials, including Asian as well as European subjects.[2,3] An initial phase I study in Japanese patients with HCC associated mainly with hepatitis C virus (HCV) infection showed promising antitumor activity and a favorable tolerability profile.[4] However, further improvement in the treatment of advanced HCC is essential.

S-1 is a novel, orally administered drug that combines tegafur (FT), 5-chloro-2,4-dihydroxypyridine (CDHP) and oteracil potassium (Oxo) in a molar concentration ratio of 1:0.4:1.[5] CDHP is a competitive inhibitor of dihydropyrimidine dehydrogenase (DPD), a metabolizing enzyme of 5-fluorouracil (5-FU) that is expressed in the liver. Inhibition of DPD by CDHP results in prolonged effective concentrations of 5-FU in plasma and tumor tissue.[6] Oxo, a competitive inhibitor of orotate phosphoribosyltransferase, inhibits the phosphorylation of 5-FU in the gastrointestinal tract, thereby reducing serious 5-FU-related gastrointestinal toxicity.[7] Clinically, S-1 has been shown to be effective against a variety of solid tumors, with response rates ranging 21–49% in late phase II studies conducted in Japan.[8] S-1 has yet to be evaluated in patients with HCC. However, in nude rats with human HCC xenografts, S-1 has been confirmed to have antitumor activity.[9]

Patients with HCC usually have various degrees of liver dysfunction because of associated liver disease and replacement of liver tissue by tumor, leading to pathophysiological changes that influence drug disposition. Decreased hepatic blood flow, extrahepatic and intrahepatic blood shunting and hepatocyte loss also alter drug metabolism, and decreased protein synthesis reduces drug binding to plasma proteins. In fact, the maximal tolerated dose (MTD) of 5-FU given as a 5-day continuous infusion in patients with HCC is approximately 50% of that in patients with normal organ function, and patients with cirrhosis have significantly lower clearance of 5-FU than those without cirrhosis.[10] We therefore conducted a multicenter phase I/II study to evaluate the pharmacokinetics, safety and efficacy of S-1 monotherapy in patients with advanced HCC.

Materials and Methods

Eligibility. Eligible patients had histologically or cytologically proved HCC that was not amenable to treatment by resection, liver transplantation, RFA, PEI or percutaneous microwave coagulation therapy (PMCT) and was not expected to respond to TACE. A hypervascular mass on computed tomography (CT) or magnetic resonance imaging (MRI) associated with a serum alpha-fetoprotein level or a serum protein induced by vitamin K absence or antagonist (PIVKA-II) level of more than the upper limit of normal (ULN) was considered a sufficient non-invasive diagnostic criterion for HCC. At least one measurable lesion on CT or MRI (not including necrotic lesions caused by prior treatment) was required. Other eligibility criteria included: age of at least 20 years; Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2; estimated life expectancy of at least 60 days; adequate

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hematological function (white blood cells [WBC] ≥3000/mm^3, hemoglobin ≥ 9.0 g/dL, platelets ≥ 7.0 × 10^4/mm^3); adequate hepatic function (aspartate aminotransferase [AST] and alanine aminotransferase [ALT] ≤5 times the ULN, total bilirubin ≤2.0 mg/dL, serum albumin ≥2.8 g/dL, prothrombin activity ≥40%); adequate renal function (serum creatinine ≤ULN); and a Child-Pugh class of A or B. Prior treatment for HCC, such as resection, liver transplantation, RFA, PEI, PMCT and TACE was permitted if the treatment had been performed 30 or more days before registration in the study. Patients were excluded if they had: tumor involving more than 50% of the liver; brain or bone metastasis or vascular invasion of the main trunk and first-order branch(es) of the portal vein, hepatic veins, hepatic arteries or bile duct; severe complications; other malignancies; or inability to comply with the protocol requirements. Written informed consent was obtained from each patient. The study was approved by the local institutional review boards at all participating centers.

Study design. S-1 was supplied by Taiho Pharmaceutical Co., Ltd (Tokyo, Japan) in capsules containing 20 or 25 mg of FT. Individual doses were calculated according to body surface area. The calculated dose was rounded to derive the daily dose and the number of capsules to be dispensed per patient. At each dose level, S-1 was administered orally twice daily (after breakfast and dinner) for 28 consecutive days, followed by a 14-day recovery period. Each treatment cycle was 42 days. If grade 3 or higher hematological toxicity, grade 2 or higher non-hematological toxicity, grade 3 or higher elevations of AST or ALT, or grade 2 or higher increases in the serum creatinine concentration occurred, treatment with S-1 was temporarily suspended, the dose of S-1 was reduced, or both (minimum dose, 50 mg/day). Treatment continued until there was evidence of disease progression, or if the recovery period exceeded 28 days, the patient requested treatment to be discontinued or unacceptable toxicity developed and treatment was terminated at the discretion of the investigator. Drug compliance and accountability were carefully monitored; patients were requested to record their intake of S-1 and other medications in a diary.

During phase I, the starting dose of S-1 (level 1) was approximately 64 mg/m^2 per day twice daily (80% of the standard dose), level 2 was approximately 80 mg/m^2 per day and level 0 was approximately 50 mg/m^2 per day (80% of level 1). Patients were enrolled in cohorts of three for each dose level. The dose was escalated according to the cohort and was not increased in the same patient. If none of the first three patients had dose-limiting toxicity (DLT) during the first cycle, the dose was increased to level 2. If one or two of the first three patients had DLT, three additional patients were entered at the same dose level; if only one or two of the first six patients at level 1 had DLT, the dose was increased to level 2; if all of the first three patients or three or more of the first six patients had DLT, the dose was decreased to level 0; if none of the first three patients had DLT at level 0 or level 2, three additional patients were assigned to receive the same dose level. The DLT was defined as any of the following: (i) hematological toxicity ≥grade 4; (ii) non-hematological toxicity ≥grade 3; (iii) AST, ALT ≥15 times the ULN; or (iv) a rest period of 8 or more consecutive days was required. The recommended dose (RD) determined in the phase I part of this study was used in phase II.

Pharmacokinetics. Blood samples (5 mL) were obtained from each patient assigned to receive level 2 in the phase I part of the study. The samples were taken before and 1, 2, 4, 6, 8, 10 and 12 h after administration of S-1 on days 1 and 8 of the first treatment cycle. Plasma was separated from the whole-blood samples by centrifugation and stored at −20°C until analysis. Plasma FT concentrations were measured by high-performance liquid chromatography with ultraviolet detection. Plasma concentrations of 5-FU, CDHP and Oxo were measured by gas chromatography-negative ion chemical ionization mass spectrometry, as described previously.[11]

Pharmacokinetic data, including the maximum plasma concentration (C_{max}, ng/mL), time to reach C_{max} (T_{max}, h), area under the plasma-concentration–time curve for 0–12 h (AUC_{0-12}, ng h/mL) and the elimination half-life ($T_{1/2}$, h) were calculated by noncompartment model analysis using WinNonlin software, version 4.1 (Pharsight, Cary, NC, USA).

Assessment of efficacy and toxicity. All patients who received at least one dose of the study drug were included in the evaluations of response and toxicity. During each course of treatment, tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) by computed tomography (CT) or magnetic resonance imaging (MRI), with a slice thickness of no more than 5 mM.[12] The primary efficacy end-point in the phase II part of this study was the overall response rate, assessed on the basis of changes in tumor dimensions. The other end-points were overall survival (OS) and progression-free survival (PFS). The PFS was defined as the interval between the date of initiating treatment and the date on which disease progression was first confirmed or the date of death from any cause. Overall survival was defined at the interval from the date of initiating treatment to the date of death from any cause. Median OS and median PFS were

Table 1. Patient characteristics

	Level 1 (n = 3)	Level 2 (n = 23)
	n (%)	n (%)
Median age (range) (years)	67.0 (63–68)	68.0 (45–78)
Gender		
Male	2 (66.7)	21 (91.3)
Female	1 (33.3)	2 (8.7)
Virus marker		
HBs (+)	1 (33.3)	3 (13.0)
HCV (+)	1 (33.3)	14 (60.9)
HBs(−), HCV(−)	1 (33.3)	6 (26.1)
Child-Pugh classification		
A	3 (100)	16 (69.6)
B	0 (0)	7 (30.4)
Stage		
Stage II	1 (33.3)	3 (13.0)
Stage III	1 (33.3)	10 (43.5)
Stage IVB	1 (33.3)	10 (43.5)
Vascular invasion	0 (0)	2 (8.7)
ECOG PS		
0	3 (100)	21 (91.3)
1	0 (0)	2 (8.7)
Pretreatment		
TA(C)E	2 (66.7)	17 (73.9)
Surgery	1 (33.3)	8 (34.8)
RFA	0 (0)	7 (30.4)
HAI	2 (66.7)	6 (26.1)
PEI	0 (0)	4 (17.4)
Radiation	0 (0)	4 (17.4)
PMCT	0 (0)	3 (13.0)
Systemic chemotherapy	0 (0)	3 (13.0)
BCLC staging		
Early	0 (0)	1 (4.3)
Intermediate	2 (66.7)	11 (47.8)
Advanced	1 (33.3)	11 (47.8)

BCLC, Barcelona Clinic Liver Cancer Group; ECOG, Eastern Cooperative Oncology Group; HAI, hepatic arterial infusion; HBs, hepatitis B surface antigen; HCV, hepatitis C virus antibody; PEI, percutaneous ethanol injection; PMCT, percutaneous microwave coagulation therapy; PS, performance status; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization.

Table 2. Toxic effects

Toxicity	Level 1 (n = 3)		Level 2 (n = 23)		Child Pugh A (n = 16)		Child Pugh B (n = 7)	
	All grades	≥G3	All grades	≥G3	All grades	≥G3	All grades	≥G3
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
All adverse events	3 (100.0)	0 (0.0)	23 (100.0)	10 (43.5)	16 (100.0)	8 (50.0)	7 (100.0)	2 (28.6)
Hematological								
Erythropenia	1 (33.3)	0 (0.0)	21 (91.3)	1 (4.3)	14 (87.5)	1 (6.3)	7 (100.0)	0 (0.0)
Hypochromia	1 (33.3)	0 (0.0)	19 (82.6)	4 (17.4)	12 (75.0)	4 (25.0)	7 (100.0)	0 (0.0)
Leukopenia	2 (66.7)	0 (0.0)	18 (78.3)	1 (4.3)	12 (75.0)	1 (6.3)	6 (85.7)	0 (0.0)
Lymphopenia	2 (66.7)	0 (0.0)	12 (52.2)	3 (13.0)	7 (43.8)	3 (18.8)	5 (71.4)	0 (0.0)
Neutropenia	1 (33.3)	0 (0.0)	17 (73.9)	1 (4.3)	12 (75.0)	1 (6.3)	5 (71.4)	0 (0.0)
Reduced hematocrit	1 (33.3)	0 (0.0)	19 (82.6)	1 (4.3)	12 (75.0)	1 (6.3)	7 (100.0)	0 (0.0)
Reduced prothrombin content	1 (33.3)	0 (0.0)	19 (82.6)	0 (0.0)	14 (87.5)	0 (0.0)	5 (71.4)	0 (0.0)
Thrombocytopenia	1 (33.3)	0 (0.0)	18 (78.3)	4 (17.4)	12 (75.0)	4 (25.0)	6 (85.7)	0 (0.0)
Non-hematological								
Elevated alkaline phosphatase	0 (0.0)	0 (0.0)	8 (34.8)	1 (4.3)	7 (43.8)	1 (6.3)	1 (14.3)	0 (0.0)
Elevated lactate dehydrogenase	0 (0.0)	0 (0.0)	15 (65.2)	0 (0.0)	9 (56.3)	0 (0.0)	6 (85.7)	0 (0.0)
Elevated serum AST	1 (33.3)	0 (0.0)	8 (34.8)	4 (17.4)	6 (37.5)	3 (18.8)	2 (28.6)	1 (14.3)
Elevated serum bilirubin	0 (0.0)	0 (0.0)	18 (78.3)	3 (13.0)	13 (81.3)	2 (12.5)	5 (71.4)	1 (14.3)
Hyponatremic	0 (0.0)	0 (0.0)	8 (34.8)	0 (0.0)	5 (31.3)	0 (0.0)	3 (42.9)	0 (0.0)
Reduced cholinesterase	2 (66.7)	0 (0.0)	18 (78.3)	0 (0.0)	13 (81.3)	0 (0.0)	5 (71.4)	0 (0.0)
Reduced serum albumin	0 (0.0)	0 (0.0)	18 (78.3)	2 (8.7)	12 (75.0)	1 (6.3)	6 (85.7)	1 (14.3)
Reduced total protein	0 (0.0)	0 (0.0)	11 (47.8)	0 (0.0)	8 (50.0)	0 (0.0)	3 (42.9)	0 (0.0)
Anorexia	1 (33.3)	0 (0.0)	18 (78.3)	2 (8.7)	13 (81.3)	1 (6.3)	5 (71.4)	1 (14.3)
Ascites	0 (0.0)	0 (0.0)	7 (30.4)	0 (0.0)	3 (18.8)	0 (0.0)	4 (57.1)	0 (0.0)
Diarrhea	0 (0.0)	0 (0.0)	10 (43.5)	0 (0.0)	8 (50.0)	0 (0.0)	2 (28.6)	0 (0.0)
Fatigue	0 (0.0)	0 (0.0)	19 (82.6)	2 (8.7)	13 (81.3)	2 (12.5)	6 (85.7)	0 (0.0)
Pigmentation	0 (0.0)	0 (0.0)	20 (87.0)	0 (0.0)	14 (87.5)	0 (0.0)	6 (85.7)	0 (0.0)
Rash	0 (0.0)	0 (0.0)	8 (34.8)	0 (0.0)	5 (31.3)	0 (0.0)	3 (42.9)	0 (0.0)
Stomatitis	0 (0.0)	0 (0.0)	7 (30.4)	0 (0.0)	5 (31.3)	0 (0.0)	2 (28.6)	0 (0.0)

Dosage level, level 1, 2 (n = 3, 23); AST, aspartate aminotransferase.

Table 3. Efficacy in patients who received dose level 2

	Child-Pugh A (n = 16)	Child-Pugh B (n = 7)	Total (n = 23)
Partial response†	4	1	5
Stable disease‡	5	2	7
Progressive disease	7	3	10
Not evaluable	0	1	1
Response rate (90%CI)§ (%)	–	–	23.1 (9.0–40.4)
Response rate (95%CI) (%)	25.0 (7.3–52.4)	14.3 (0.4–57.9)	23.1 (7.5–43.7)
Median PFS (95% CI) (months)	3.3 (2.3–5.1)	3.7 (2.5–7.4)	3.7 (2.5–5.1)
Median OS (95% CI) (months)	17.8 (14.0–NA)	14.5 (9.6–18.7)	16.6 (14.0–24.5)
1-year survival (95% CI) (%)	–	–	69.6 (50.8–88.4)
1.5-years survival (95% CI) (%)	–	–	43.0 (22.6–63.5)
Disease control rate¶			
6W (95% CI) (%)	–	–	47.8 (26.8–69.4)
12W (95% CI) (%)	–	–	26.1 (10.2–48.4)
24W (95% CI) (%)	–	–	21.7 (7.5–43.7)

†Partial response was re-evaluated after at least 4 weeks in patients with a partial response. ‡Stable disease was reassessed after at least 6 weeks. §Response rate (90% CI) is a primary end-point. ¶Disease control rates were respectively estimated by dividing the number of patients with no disease progression by the total number of patients. Disease control was defined as a response of complete response, partial response or stable disease. CI, confidence interval; NA, not available; OS, overall survival; PFS, progression-free survival.

estimated using the Kaplan–Meier method. Physical findings and the results of serum chemical and urine analyses were assessed at 2-week intervals; vital signs were assessed as necessary. Patients were observed until death or at least 1 year after registration to determine survival status. The severity of all adverse events was evaluated according to the Common Terminology Criteria for Adverse Events, version 3.0 (CTCAE, Ver. 3.0). The duration of all adverse events and their relation to S-1 were initially assessed by the attending physicians. Subsequently, an independent review committee reviewed data on objective response and adverse events.

Statistical considerations. With the response rate as the primary end-point, a total sample size of at least 23 patients was estimated to be required in the phase II portion to allow the

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study to have a one-sided 5% significance level of 0.05 and a power of 70%, assuming a threshold response rate of 5% and an expected response rate of 20%.

Results

Patient characteristics and treatment. Between May 2006 and April 2007, a total of 26 patients (nine in phase I and 17 in phase II) were enrolled at four centers in Japan. All patients were eligible for the evaluation of toxicity and efficacy. The first six patients who received dose level 2 (80 mg/m^2 per day) during the phase I part of this study were included in the phase II assessment, along with the 17 other patients (a total of 23 patients in the phase II assessment). The characteristics of patients are summarized in Table 1. At the study entry, 11 of 26 (42.3%) had metastatic disease. Six patients (23.1%) had single extrahepatic metastases (lung metastases, three patients; lymph node metastasis, three patients). Four patients had two sites of metastases, including the lung, lymph nodes and adrenal glands. Of the 26 patients, 23 had received some prior treatment, including three who had received systemic chemotherapy.

Dose-limiting toxicity and RD. None of the three patients who received dose level 1 (64 mg/m^2 per day) in the phase I part of the study had DLT. At dose level 2 (80 mg/m^2 per day), one patient with Child-Pugh class B had grade 3 anorexia during the first course of treatment, but the other two patients in the same cohort had no DLT. Three additional patients were enrolled to confirm safety, and one patient with Child-Pugh class B had a grade 2 rash; recovery required eight or more consecutive days of rest. Because two of the six patients who received level 2 had DLT, level 2 was defined as the RD for the phase II part of the study.

Treatment delivered. Twenty-three patients received a total of 85 cycles of treatment at dose level 2 (median, three cycles per patient; range, 1–15). The dose of S-1 was reduced in seven patients (30.4%) or a total of nine cycles (10.6%). The most common reasons for dose reductions were rash in four patients, and elevated serum bilirubin concentrations and anorexia in two patients each (some overlap among patients). Treatment was delayed because of toxicity in 12 patients (20 cycles), most often in cycles 1 or 2. The most common reasons for toxicity-related treatment delays were fatigue (five patients), rash (four patients) and elevated serum bilirubin concentrations (three patients). The reasons for terminating treatment were progressive disease in 19 patients (82.6%), adverse reactions in two patients (8.7%) and other reasons in two patients (8.7%; one required 28 or more consecutive days of rest, and one withdrew consent).

Toxicity. Drug-related adverse events occurring in all 26 patients in the phase I/II portion of the study are shown in Table 2. Treatment with S-1 was generally well tolerated throughout the study. Grade 3 or 4 toxicity occurred in 10 of the 23 patients (43.5%) who received level 2. Most toxic effects were laboratory abnormalities. There was no grade 3 or 4 toxicity at level 1. The most common grade 3 or 4 hematological toxic effects were hypochromia (17.4%), thrombocytopenia (17.4%) and lymphopenia (13.0%); the most common grade 3 or 4 nonhematological toxic effects were elevated serum AST levels (17.4%) and elevated serum bilirubin concentrations (13.0%).

Efficacy. A response could be evaluated in 26 patients in the phase I/II portion of the study. In the phase I part of the study (dose level 1), one patient had a partial response, one had progressive disease and the other was not evaluable. Of the 23 patients in the phase II part of the study, five (21.7%; 90% confidence interval [CI], 9.0–40.4%) responded to treatment Among the 23 patients in whom a response could be evaluated, five had a partial response, seven had stable disease, and 10 had progressive disease (Table 3). The remaining patient underwent imaging studies, but treatment was completed after one course, and continuation of stable disease for at least 6 weeks could not be

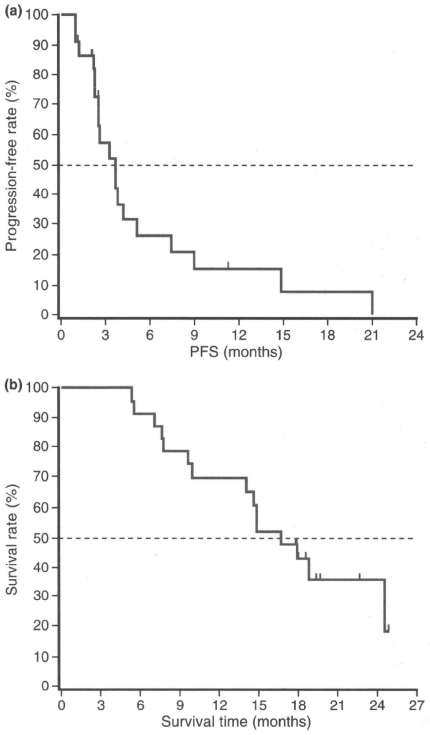

Fig. 1. Progression-free survival (PFS) (a) and overall survival (b) in patients who received dose level 2 of S-1 (n = 23). The median progression-free survival and overall survival were 3.7 and 16.6 months, respectively.

Table 4. Pharmacokinetics of FT, 5-FU, CDHP and Oxo on day 1 and day 8 in patients with HCC who received dose level 2

		C_{max} (ng/mL)	T_{max} (h)	$AUC_{0\text{-}12}$ (ng h/mL)	$T_{1/2}$ (h)
FT	Day 1	2032 ± 437	3.3 ± 1.0	17070 ± 5139	10.1 ± 2.8
	Day 8	4365 ± 1712	3.7 ± 0.8	42399 ± 18137	12.7 ± 5.0
5-FU	Day 1	114.5 ± 35.5	4.3 ± 0.8	695.3 ± 223.6	2.3 ± 1.0
	Day 8	145.8 ± 31.4	4.3 ± 0.8	936.6 ± 292.3	2.4 ± 1.0
CDHP	Day 1	267.2 ± 76.8	3.3 ± 1.0	1424.8 ± 414.2	3.3 ± 0.9
	Day 8	281.0 ± 113.8	3.3 ± 1.0	1694.4 ± 603.5	3.4 ± 0.9
Oxo	Day 1	38.5 ± 1.8	3.7 ± 0.8	231.6 ± 69.8	4.0 ± 2.1
	Day 8	33.4 ± 9.5	4.0 ± 0.0	241.5 ± 115.6	4.0 ± 2.0

Parameters are represented as mean ± SD. CDHP, 5-chloro-2,4-dihydroxypyridine; 5-FU, 5-fluorouracil; FT, tegafur; Oxo, oteracil potassium.

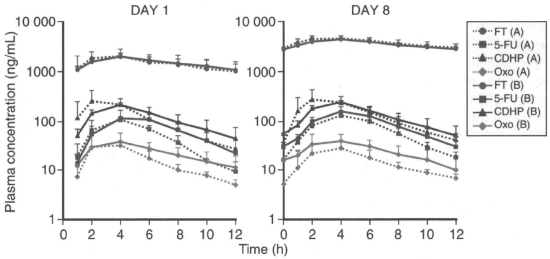

Fig. 2. Plasma-concentration–time profiles of tegafur (FT), 5-fluorouracil (5-FU), 5-chloro-2,4-dihydroxypyridine (CDHP) and oteracil potassium (Oxo) on day 1 and day 8 were similar in patients with Child-Pugh class A ($n = 3$) and those with Child-Pugh class B ($n = 3$).

confirmed. The duration of the five responses was 42, 147, 188, 238 and 371 days, respectively.

The median PFS was 3.7 months (95% CI, 2.5–5.1 months). The disease control rates at 6, 12 and 24 weeks were 47.8% (95% CI, 26.8–69.4%), 26.1% (95% CI, 10.2–48.4%) and 21.7% (95% CI, 7.5–43.7%), respectively. The PFS and OS are shown in Figure 1. The median OS was 16.6 months (95% CI, 14.0–24.5 months). Survival rates were 69.6% (95% CI, 50.8–88.4%) at 1 year and 43.0% (95% CI, 22.6–63.5%) at 1.5 years.

Pharmacokinetic analysis. Table 4 shows the pharmacokinetic data for the components of S-1 and 5-FU at level 2 on days 1 and 8. Compared with day 1, the C_{max} and $AUC_{0\text{-}12}$ of FT increased markedly on day 8; however, these increases were within the expected range given the slow elimination of FT, and repeated administration of S-1 had no effect on the T_{max} or $T_{1/2}$ of FT. There was no evidence of accumulation of 5-FU, CDHP or Oxo on day 8.

Figure 2 compares the plasma-concentration–time profiles of S-1 components and 5-FU between patients with Child-Pugh class A and those with Child-Pugh class B on days 1 and 8. The plasma-concentration–time profiles of FT, 5-FU, CDHP and Oxo were similar in patients with Child-Pugh class A and those with Child-Pugh class B on both days.

Discussion

There has been no established standard therapy for patients with advanced HCC refractory to surgery, transplantation, local ablation and TACE.[13,14] Some cytotoxic regimens have produced encouraging response rates, but survival benefits have been minimal compared with control groups, at the cost of clinically unacceptable adverse effects.[1,15]

S-1 is an anticancer drug consisting of FT, CDHP and Oxo. The conversion of FT to 5-FU is mediated mainly by hepatic cytochrome CYP2A6.[16] 5-FU is rapidly metabolized by DPD in the liver after the intravenous administration of 5-FU alone, but S-1, which includes a DPD inhibitor (i.e. CDHP), produces prolonged, effective concentrations of 5-FU in the blood. Thus, the liver plays an important role in the metabolism of FT.

The RD of S-1 in patients with HCC was estimated to be 80 mg/m^2 per day in phase I, which is similar to the dose recommended for the treatment of other solid tumors. However, in patients with HCC, Ueno *et al.*[10] reported that the DLT of 5-FU administered as a 5-day continuous infusion was stomatitis. Moreover, the MTD was equivalent to approximately 50% of that of 5-FU in patients with normal organ function,[10] suggesting that 5-FU-related gastrointestinal toxicity was reduced by Oxo in the formulation of S-1. We did not determine the MTD in this study because S-1 was approved for the treatment of other cancers. The pharmacokinetic properties of S-1 components and 5-FU in patients with HCC were similar to those in patients with pancreatic cancer or biliary tract cancer.[17,18]

Hematological toxic effects and symptomatic events such as pigmentation (87.0%), fatigue (82.6%), anorexia (78.3%) and ascites (30.4%) were more common than previously reported for S-1 in patients with other cancers. Nonetheless, severe toxic effects were comparable among patients with HCC and those with other cancers. Nonhematological toxic effects related to hepatic function were also more frequent than reported previously for S-1 in patients with other types of cancer, but such effects may have been caused by differences in underlying liver disease.

The pharmacokinetics of S-1 did not obviously differ between patients with Child-Pugh class A and those with Child-Pugh class B, suggesting that hepatic dysfunction associated with Child-Pugh class B did not affect the pharmacokinetics of S-1 components or 5-FU. The sample size of the pharmacokinetic evaluations was small because the primary end-point was to determine the RD as the evaluation of DLT in phase I. At dose level 2, DLT occurred in two patients with Child-Pugh class B (Grade 3 anorexia in one, and a Grade 2 rash requiring 8 or more consecutive days of rest in the other). There was no DLT at level 1 (given only to patients with Child-Pugh class A). However, the patient who had DLT of grade 3 anorexia had renal dysfunction at baseline, and the plasma 5-FU concentrations in this patient on day 8 were higher than those in other patients, perhaps contributing to the development of DLT (data not shown). In addition, there were no obvious differences in the incidence or grade of drug-related adverse events between patients with Child-Pugh class A and those with Child-Pugh class B, consistent with the results of pharmacokinetic analysis. These results suggested that there were no clinically meaningful differences in pharmacokinetics or safety according to Child-Pugh class or between patients with HCC and those with other cancers, and that S-1 was well tolerated in patients with HCC, similar to patients with other cancers. However, our study had several limitations: only a very small number of patients with Child-Pugh class B were included; among the patients with Child-Pugh class B, the score was heterogeneous, ranging from 7 to 9; and only patients with better scores were studied. Therefore, extra care should be taken when S-1 is given to patients with Child-Pugh class B.

As for efficacy, five of 23 patients had partial responses at dose level 2. Compared with previously reported response rates obtained with single-agent chemotherapy in patients with HCC, our results are good. In particular, the median OS appeared to be longer than that obtained with other agents in non-Japanese studies. The reason for the better OS in Japanese patients might be similar to that previously reported for sorafenib.[4] The median OS in our study was similar to that in a Japanese phase I study of sorafenib.[4] In studies of sorafenib in non-Japanese and

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Japanese patients with HCC, the median TTP and response rates were comparable, but the median OS was 15.6 months in Japanese patients compared with only 9.2 months in non-Japanese patients.[4] Differences in various treatments, including hepatic arterial infusion chemotherapy, and the palliative care of patients with progressive disease who had conditions such as hepatic decompression and variceal bleeding might be related to the longer survival time in Japanese rather than non-Japanese patients with HCC.

In conclusion, our results suggested that S-1 is effective and has an acceptable toxicity profile in patients with advanced HCC. Nonetheless, S-1 should be used with caution in the presence of liver dysfunction. Sorafenib has been established to be a standard treatment for advanced HCC. Perhaps, systemic chemotherapy with S-1 plus molecular-targeted therapies such as sorafenib will further improve survival in patients with advanced HCC or monotherapy with S-1 will be useful as a second-line regimen for chemotherapy.

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Disclosure Statement

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ORIGINAL ARTICLE

A phase I/II trial of the oral antiangiogenic agent TSU-68 in patients with advanced hepatocellular carcinoma

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Abstract
Purpose We studied the safety and effectiveness of TSU-68, an oral tyrosine kinase inhibitor of vascular endothelial growth factor receptor-2, platelet-derived growth factor receptor and fibroblast growth factor receptor, in patients with advanced hepatocellular carcinoma (HCC).
Methods Patients with unresectable or metastatic HCC were eligible for enrollment. In phase I, the safety, tolerability and pharmacokinetics were assessed in patients stratified based on liver function, from no cirrhosis to Child–Pugh class B. The safety and effectiveness were assessed in phase II at the dose determined in phase I.
Results Twelve patients were enrolled in phase I. Dose-limiting toxicities were found with TSU-68 at the dose of 400 mg bid in Child–Pugh B patients, and 200 mg bid was established as the phase II dose. Phase II included 23 additional patients, and the safety and efficacy were evaluated in a total of 35 patients. One patient (2.9%) had a complete response. Two patients (5.7%) had a partial response, and 15 patients (42.8%) showed a stable disease. The median time to progression was 2.1 months, and the median overall survival was 13.1 months. Common adverse events were hypoalbuminemia, diarrhea, anorexia, abdominal pain, malaise, edema and AST/ALT elevation. The analysis of angiogenesis-related parameters suggests that serum-soluble vascular cell adhesion molecule-1 is a possible marker to show the response.
Conclusions TSU-68 at a dose of 200 mg bid determined by stratification into liver function, showed promising preliminary efficacy with a high safety profile in patients with HCC who had been heavily pre-treated.

Keywords Advanced HCC · Liver function · TSU-68 · Pharmacokinetics · Tolerability · Angiogenesis

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide, with ~626,000 new cases reported annually [1]. Potentially curative treatments such as surgical therapy (resection and liver transplantation) and locoregional procedures (radiofrequency ablation) are indicated in early stage HCC. However, disease that is

diagnosed at an advanced stage or with progression after locoregional therapy has a dismal prognosis owing to the underlying liver disease [2]. Although no systemic therapy was effective for advanced HCC, two randomized, placebo-controlled studies have proven the survival benefits of sorafenib in such patients [3, 4].

TSU-68 is an orally administered, small-molecule, multiple receptor tyrosine kinase inhibitor that targets vascular endothelial growth factor receptor-2 (VEGFR-2), platelet-derived growth factor receptor (PDGFR) and fibroblast growth factor receptor (FGFR) [5–9]. As HCC is a highly vascular tumor, several antiangiogenic agents have been tested for the treatment of HCC [3, 4]. Since it is a potent antiangiogenic agent, TSU-68 is also expected to be effective against HCC. However, most patients with HCC have accompanying liver cirrhosis or hepatitis. Therefore, its safety must be reevaluated in the presence of liver function impairment [10, 11]. In particular, concerns have been expressed about impairment of the pharmacokinetics of TSU-68, which is eliminated predominantly through hepatic metabolism, oxidation and glucuronidation [12, 13].

From three phase I studies that have been conducted in Japan on patients with solid tumors, the administration of TSU-68 twice daily after meals was selected as the recommended dose regimen [14, 15]. In this regimen, although no dose-limiting toxicity (DLT) exists at dose levels of 200–500 mg/m^2/dose, the higher dose showed some unacceptable adverse events for an antitumor drug that is administered for long-term consecutive treatment. No obvious dose-dependent increases were detected in the maximum concentration (C_{max}) or the area under the curve ($AUC_{0\text{-}t}$) over the dose range, which was probably due to a saturation of absorption. Consequently, a dose of 400 mg/dose bid was determined to be the recommended dosage of TSU-68 [14, 15].

In the phase I step of our trial, the safety, tolerance and pharmacokinetics (PK) of TSU-68 at the recommended dose were assessed in successive cohorts of patients with various degrees of liver function: no cirrhosis, Child–Pugh class A and Child–Pugh class B cirrhosis, allowing for dose reduction when necessary. In phase II, we evaluated the effectiveness of TSU-68 against advanced HCC.

Patients and methods

Eligibility criteria

The eligibility criteria were histologically confirmed HCC; no indication for or no response to resection, ablation or transcatheter arterial chemoembolization (TACE); age 20–74 years old; World Health Organization performance status of ≤2; life expectancy of ≥90 days; and white blood cells ≥3,000/μl or neutrophils ≥1,500/μl; hemoglobin ≥8.0 g/dl; platelets ≥75,000/μl; liver function Child–Pugh A or B; total bilirubin ≤2.5 mg/dl; AST and ALT ≤ 200 U/l; albumin ≥3 g/dl; prothrombin time [%] ≥40 and serum creatinine ≤1.5 mg/dl. The criteria for patients in Level 1 of phase I were platelets ≥130,000/μl, AST and ALT ≤ 100 U/l; total bilirubin below or equal to the upper limit of normal and albumin equal to or over the lower limit of normal.

Patients were not eligible if they had received ablation, TACE, chemotherapy or radiotherapy within 4 weeks or surgery within 6 weeks. Patients were excluded if they had clinical evidence of central nervous system metastasis, severe cardiovascular disorders, hepatic encephalopathy, uncontrollable pleural effusion or ascites or a serious infection. Patients who needed prophylactic variceal ligation or sclerotherapy were excluded.

All patients were informed of the purpose and methods of the study and provided written informed consent in accordance with national and institutional guidelines. The study was approved by the institutional review board at each of the three participating hospitals and was performed in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines.

Study design and treatment

This was an open-label phase I/II study. In phase I, eligible patients were stratified into three groups based on hepatic function: Level 1, no cirrhosis; Level 2, Child–Pugh class A; and Level 3, Child–Pugh class B. The safety, tolerability and PK were evaluated in each successive cohort. DLT was defined as grade 3 or 4 non-hematological toxicity or grade 4 hematological toxicity according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 2. As shown in Fig. 1a, the dosage of 400 mg bid was first assessed in three patients at Level 1, each treated for one cycle (28 days). If no DLT was observed, three patients at Level 2 were treated with the same dosage. However, if one patient developed DLT, another three patients at Level 1 were added, based on a 3 + 3 study design [16]. If DLT was observed in no more than two of the six patients, three patients at Level 2 were enrolled. By contrast, if more than one of the first three patients or more than two of the six patients developed DLT, the other three patients at Level 1 were treated with half the dosage. The level transition and dose reduction were planned similarly. Drug administration was continued until no evidence of disease progression was observed, unacceptable drug-related toxicity occurred or the patient withdrew consent.

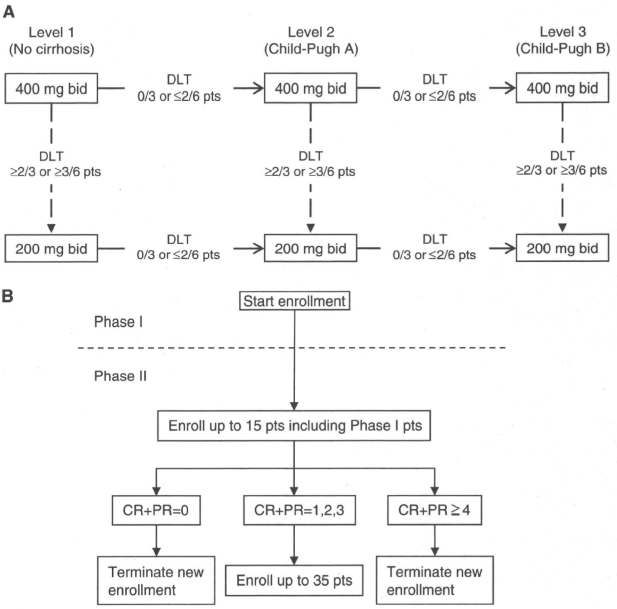

Fig. 1 TSU-68 phase I/II study schema. **a** In phase I, patients were stratified into three groups based on hepatic function, and the toxicity and pharmacokinetics were assessed from Level 1 (no cirrhosis) to Level 3 (Child–Pugh B) by enrolling three patients at each level. *Bid* twice daily, *DLT* dose-limiting toxicity, *pts* patients. **b** Patient enrollment procedure based on the two-step method of Fleming [17]

Patients were accrued using Fleming's optimal two-stage method [17], allowing for an interim evaluation that would be performed when 15 patients (including phase I) were enrolled (Fig. 1b). TSU-68 would be judged "effective" if efficacy (complete or partial response) was observed in four or more patients and "ineffective" if efficacy was observed in none. If efficacy were confirmed in one to three patients, phase II would be performed at the dosage determined in phase I using 20 additional patients (35 patients in total).

Drug administration

TSU-68 (Z)-3-[2,4-dimethyl-5-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1H-pyrrol-3-yl]-propionic acid was obtained from Taiho Pharmaceutical Inc. Co. (Tokyo, Japan). Twice-daily administration was given within 1 h after meals with about 12-h intervals between doses. TSU-68 was taken for 28 consecutive days and was continued in case of stable disease or disease remission after this period for as long as no disease progression and/or no unacceptable drug-related toxicity were seen. TSU-68 administration was immediately interrupted upon the occurrence of DLT.

Response assessment

The objective response was assessed using the Response Evaluation Criteria in Solid Tumors (RECIST). Naïve untreated lesions were selected as targets for evaluation. At the end of each cycle, a three-phase computed tomography protocol consisting of early arterial, late arterial and portal venous phases was performed, obtaining contiguous transverse sections with a thickness of 5–7 mm. Responses were assessed independently.

Pharmacokinetics

In phase I, blood samples were collected from a total of 12 patients at 0 (pre-dose), 1, 2, 3, 4, 6 and 9 h post-dose on days 1 and 2 of cycle 1 and at pre-dose on day 1 of cycle 2. The plasma TSU-68 concentration was determined using high-performance liquid chromatography (HPLC). Briefly, an aliquot of plasma was mixed with acetate buffer and methanol including an internal standard. After centrifugation, the supernatant was mixed with ammonium acetate and applied to a Zorbax Eclipse XDB C18 column (3.5 μm, 3 cm × 4.6 mm; Agilent Technologies, Mississauga, ON, Canada) of a Waters Alliance 2690 HPLC

system (Waters, Milford, MA, USA), and the effluent was monitored at 440 nm. The lower limit of quantification was 0.1 μg/ml. Non-compartmental PK parameters, including AUC, C_{max}, time to maximum concentration (T_{max}) and elimination half-life ($T_{1/2}$), were calculated using PhAST (version 2.3; MDS Pharma Services, Montreal, Quebec, Canada).

Angiogenesis-related markers

Blood samples were collected at baseline and at day 28 of cycle 1. The following were measured; platelet-derived growth factor (PDGF)-BB, basic fibroblast growth factor (bFGF), soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble endothelial-leukocyte adhesion molecule-1 (sELAM-1) in serum and vascular endothelial growth factor-A (VEGF-A) in plasma were analyzed using enzyme-linked immunosorbent assays (ELISAs; R&D Systems, Minneapolis, MN, USA); plasma interleukin-8 (IL-8), with ELISA (BioSource Europe, Nivelles, Belgium); plasma tissue plasminogen activator (t-PA), with a soluble t-PA ELISA kit (Oncogene Science, Cambridge, MA, USA); plasma plasminogen activator inhibitor-1(PAI-1), with a latex photometric immunoassay (LPIA; LPIA t-PAI test, Mitsubishi Kagaku Iatron, Tokyo, Japan); and plasma factor VIII, with Pathromtin SL (Dade Behring, Marburg, Germany).

Statistical analysis

The primary endpoint of phase I was to evaluate the safety and PK, whereas the primary endpoint of phase II was to determine the best overall response rate based on RECIST. Secondary endpoints of both phases were to evaluate the tumor necrotic effect and the relationship between blood angiogenesis-related molecules and clinical effects. We adopted the 3 + 3 study design generally used in phase I dose-escalation studies [16]. Patients were accrued using Fleming's method [17]. The target number of patients was 35, with an interim evaluation planned for the first 15 patients. The statistical power was 86% with an expected response rate of 20%, and the lower margin of efficacy and one-sided α-level were both 5%. Time to progression (TTP) was defined as the interval between the first day of treatment and tumor progression or death due to any cause. Overall survival (OS) was calculated from the first day of treatment to death. TTP and OS were calculated using the Kaplan–Meier method.

The basal level of angiogenesis-related parameters to predict the response was evaluated by receiver operating characteristic (ROC) analysis. The optimal cut-off value for differentiation of responders and non-responders was defined by the point of the ROC curve (Youden index method). After ROC analysis, logistic regression analysis was performed. The *t* test was used to compare baseline levels of angiogenesis-related parameters in term of responders.

This study is registered at ClinicalTrials.gov, number NCT 00784290.

The data were analyzed using SAS version 8.1 (SAS Institute, Cary, NC, USA).

Results

Patient characteristics

From September 2003 through February 2007, 35 patients were enrolled at the University of Tokyo Hospital, Mitsui Memorial Hospital and the National Cancer Centre, all located in Tokyo, Japan. Baseline demographics and disease characteristics are summarized in Table 1. Phase I consisted of 12 patients: three patients each at Level 1 (no cirrhosis) and Level 2 (Child–Pugh A), and six patients at Level 3 (Child–Pugh B). The other 23 patients were enrolled in phase II.

In the overall study population, 29 (82.9%) of 35 patients were HCV-positive, and four (11.4%) were HBV-positive. For liver function, three (8.6%) of 35 patients were non-cirrhotic; 24 (68.6%) had Child–Pugh A cirrhosis; and eight (22.9%) had Child–Pugh B cirrhosis. Extrahepatic metastasis was found in 19 (54.3%) patients. Table 1 shows the disease stages according to the TNM classification [18, 19]: 20 (57.1%) patients were stage C (advanced), and 15 (42.9%) patients were stage B (intermediate) according to the Barcelona Clinic Liver Cancer (BCLC) Staging System [2, 20]. The patients had been treated previously a mean of 8.2 (range, 1–20) times using various modalities, including surgery, RFA and TACE. No patients ever received Sorafenib.

Safety and pharmacokinetics

The toxicity of TSU-68 was assessed using NCI-CTC (version 2.0) in 12 patients enrolled in phase I (Table 2). Since no DLT was found with 400 mg bid at Level 1 (no cirrhosis) or Level 2 (Child–Pugh A), the same dosage was used in Level 3 (Child–Pugh B) patients (Fig. 1a). However, patients at Level 3 on 400 mg bid experienced DLT (grade 3 abdominal pain and ascites); the dose was reduced by half, to 200 mg bid, in an additional three patients at Level 3, among whom DLT was not observed. The most common drug-related adverse events observed in phase I were hypoalbuminemia, diarrhea, abdominal pain, fever and AST/ALT elevation.

Table 1 Patient characteristics

	Phase I		Phase II	All
	400 mg bid	200 mg bid	200 mg bid	
No. of patients	9	3	23	35
Gender				
Male	8	2	19	29
Female	1	1	4	6
Age, years				
Median	66	73	69	68
Mean	66.0	68.7	65.2	65.7
Range	53–74	60–73	49–74	49–74
ECOG performance status				
0	6	3	21	30
1	3	0	2	5
Viral markers				
HBs Ag^+, HCV Ab^-	2	0	2	4
HBs Ag^-, HCV Ab^+	6	3	20	29
HBs Ag^-, HCV Ab^-	1	0	1	2
Child–Pugh status				
Chronic hepatitis	3	0	0	3
A (5/6)[a]	3 (3/0)	0	21 (15/6)	24 (18/6)
B (7/8/9)[a]	3 (2/1/0)	3 (3/0/0)	2 (2/0/0)	8 (7/1/0)
Prior treatments[b]				
Median	8	4	9	8
Mean	8.9	6.0	8.2	8.2
Range	5–16	3–11	1–20	1–20
Disease stage[c]				
II	2	1	3	6
III	3	1	5	9
IVa	0	0	1	1
IVb	4	1	14	19
Extrahepatic metastasis				
Yes	4	1	14	19
No	5	2	9	16
Portal vein thrombosis				
Yes	0	0	1	1
No	9	3	22	34

[a] Child-Pugh score (points)

[b] Number of pre-treatments with surgery, radio-frequency ablation, transcatheter arterial chemoembolization, chemotherapy or radiotherapy

[c] Stage is based on the TNM classification [18, 19]

The PK levels were examined in nine patients (3 each at Levels 1–3) receiving 400 mg bid and in three patients (Level 3) receiving 200 mg bid, after the first dose (day 1) and the third dose (day 2; Table 3). The C_{max} and AUC_{0-9h} did not increase with poorer liver function. In all patients, the C_{max} and AUC_{0-9h} on day 2 were lower than those on day 1. In Level 3, in which both 200 and 400 mg TSU-68 were evaluated, no appreciable difference in the exposure was observed on day 2 between the two dose levels. TSU-68 had not accumulated at any level when measured immediately before administration on day 29 (data not shown).

Table 2 shows all of the drug-related adverse events reported in ≥10% of the patients. The most common adverse events, regardless of grade, were hypoalbuminemia (57%), diarrhea (37%), anorexia (34%), abdominal pain (31%), malaise (29%), edema (29%), AST/ALT elevation (29%) and fever (23%); most were grade 1 or 2. Four patients (11.4%) experienced grade 3 or higher toxicity, and the most common grade 3–4 adverse event was AST/ALT elevation (14%). Reducing the dose of TSU-68 from 400 to 200 mg bid decreased the incidence of diarrhea, abdominal pain, fever and hypoalbuminemia. TSU-68 administration was discontinued in one patient because of anemia. However, this patient was later diagnosed with bleeding from the peritoneal dissemination of HCC invading into the colon. Most adverse events were mild, and TSU-68 was well tolerated at the dose of 200 mg bid.

Efficacy and survival

The antitumor effect of TSU-68 was assessed independently in the 35 patients using RECIST (Table 4). One patient at 200 mg bid achieved a complete response (CR; Fig. 2, patient 1), two patients at 200 mg bid had a partial response (PR), 15 patients had stable disease (SD), and 16 patients had progressive disease (PD). The response rate (CR + PR) was 8.6%, and the disease control rate (CR + PR + SD) was 51.4%. Disease control was maintained for >6 months in six patients. One patient did not complete the first cycle and was not evaluated (NE).

Tumor necrosis (TN) was confirmed by independent radiologists in nine patients (25.7%). Figure 2 (patient 2) is an example in which the lack of contrast enhancement and marked central hypoattenuation within the metastatic masses were consistent with TN. The magnitude of necrosis in nine patients was quantified with bi-dimensional measurements of target lesions (RECIST). The baseline mean TN was 0%, and the follow-up mean TN was 35% (5–71%). In the overall study population of 35 patients, the median TTP was 2.1 months (95% confidence interval, 1.2–2.9 months; Fig. 3a), and the median OS was 13.1 months (95% confidence interval, 6.9–26.6 months; Fig. 3b).

Angiogenesis-related markers

Multiple logistic regression analysis was performed. Independent variables were the data for VEGF, t-PA, sVCAM-

Table 2 Drug-related adverse events and laboratory abnormalities by grade occurring in at least 10% of patients ($n = 35$)

	Phase I ($n = 12$)								Phase II ($n = 23$)			All ($n = 35$)					
	Level 1 ($n = 3$) 400 mg bid		Level 2 ($n = 3$) 400 mg bid		Level 3 ($n = 3$) 400 mg bid		Level 3 ($n = 3$) 200 mg bid		200 mg bid								
Common toxicity criteria grade	All	3	All	3	All	3	All	3	All	3	4	All		3		4	
Adverse event	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	%	No.	%	No.	%
Treatment-related adverse events																	
Diarrhea	2		2		2		2		5			13	37				
Anorexia					2				10			12	34				
Abdominal pain	2				3	1	1		5			11	31	1	3		
Malaise	2								8			10	29				
Edema					1		1		8			10	29				
Fever	1		1		2				4			8	23				
Ascites					2	1	1		3			6	17	1	3		
Nausea					1				4			5	14				
Abdominal distension									4			4	11				
Laboratory abnormalities																	
Albumin decrease	2		3		3		1		11			20	57				
AST increase	1						2	1	7	4		10	29	5	14		
ALT increase	1						2	1	7	4		10	29	5	14		
Total bilirubin increase					1		1		6			8	23				
Alkaline phosphatase increase									7	1		7	20	1	3		
Erythropenia									7			7	20				
Hematocrit decrease	1				1				4	1		6	17	1	3		
Hemoglobin decrease	1				1				4	1	1	6	17	1	3	1	3
LDH decrease	1								5			6	17				
Thrombocytopenia	1								4	2		5	14	2	6		

Results are expressed as the worst adverse event possibly related to TSU-68 per patient based on the NCI-CTC version 2.0

Table 3 Pharmacokinetic parameters of TSU-68 corresponding to liver function levels (mean ± SD)

Hepatic function level ($n = 3$)	Dosing	T_{max} (h)	C_{max} (μg/mL)	AUC_{0-9h} (μg·h/mL)	$T_{1/2}$ (h)
Level 1 (400 mg bid)	Day 1 (1st)	3.7 ± 2.1	16.8 ± 7.1	70.1 ± 28.6	2.0[a]
	Day 2 (3rd)	3.0 ± 1.0	9.5 ± 1.8	44.4 ± 11.9	2.5 ± 0.8
Level 2 (400 mg bid)	Day 1 (1st)	4.7 ± 1.2	11.7 ± 2.5	60.6 ± 19.0	2.6[a]
	Day 2 (3rd)	4.0 ± 0.0	7.8 ± 1.4	36.7 ± 7.7	2.2 ± 0.9
Level 3 (400 mg bid)	Day 1 (1st)	4.0 ± 2.0	8.6 ± 4.1	46.4 ± 20.6	2.8[a]
	Day 2 (3rd)	3.7 ± 0.6	5.1 ± 1.6	26.0 ± 6.9	3.0 ± 1.4
Level 3 (200 mg bid)	Day 1 (1st)	4.0 ± 0.0	5.1 ± 1.6	28.9 ± 5.2	8.2[a]
	Day 2 (3rd)	3.7 ± 2.5	4.3 ± 1.4	20.7 ± 4.0	6.9[a]

AUC_{0-9h}, area under the concentration versus time curve for 0–9 h

[a] $n = 2$

1, PAI-1, sELAM-1, IL-8, PDGF, bFGF and plasma factor VIII levels, and dependent variables were the two groups based on each cut-off level (0, below the cut-off value or 1, above the cut-off value). By logistic regression analysis, we found that the sVCAM-1 level was an independent factor ($P = 0.014$; Table 5), and sVCAM-1 (odds ratio 16.0) had the strongest influence on responders (patients with CR + PR + SD). None of the rest of the

Table 4 Tumor response

Best response	Phase I ($n = 12$)		Phase II ($n = 23$)	Total ($n = 35$)	
	400 mg bid ($n = 9$) No.	200 mg bid ($n = 3$) No.	200 mg bid No.	No.	%
Complete response	0	0	1	1	2.9
Partial response	0	0	2	2	5.7
Stable disease	2	2	11	15	42.8
Progressive disease	6	1	9	16	45.7
Not evaluated[a]	1	0	0	1	2.9

[a] This patient did not complete cycle 1

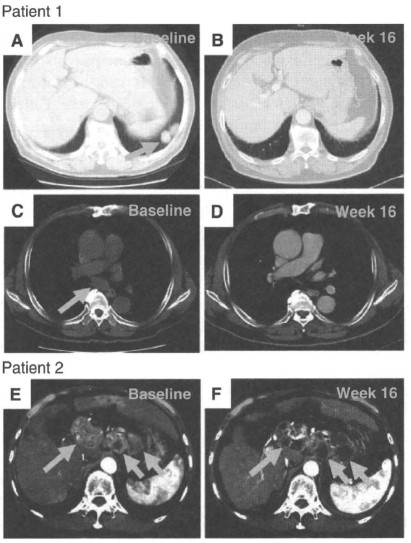

Fig. 2 Computed tomography images of responding lesions from patient 1, who achieved a complete response. Metastatic lesions in the lung (**a**) and lymph node (**c**) disappeared after four cycles (16 weeks) of TSU-68 treatment (**b**, **d**). Representative computed tomography images of a tumor showing necrosis in patient 2. Before treatment, several abdominal lymph node metastases were apparent (**e**). After four cycles of treatment (16 weeks), the lesions demonstrated a lack of enhancement and markedly lower attenuation, consistent with tumor necrosis (**f**)

angiogenesis-related parameters showed any variation with treatment (as the variation of the data for PAI-1 was so large, they were not analyzed; Table 5). The mean values of sVCAM-1 for responders (patients with CR + PR + SD; 1,944 pg/ml) were higher than that for non-responders (patients with PD + NE; 1,422 pg/ml), which was statistically significant ($P = 0.026$, t test).

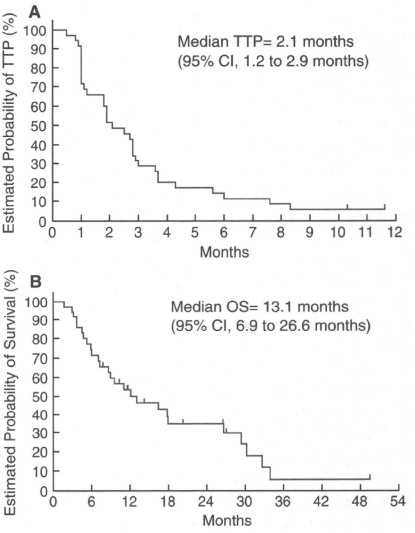

Fig. 3 **a** The independently assessed median time to progression in all 35 patients treated with TSU-68 was 2.1 months. **b** The investigator-assessed median overall survival in all 35 patients treated with TSU-68 was 13.1 months

Discussion

In this trial, special attention was paid to patients with HCC, who often have impaired liver function and might have the potential for reduced clearance of TSU-68, which is eliminated mainly by the liver [12, 13]. This study suggests that the adverse-event profile of TSU-68 in this trial was comparable to observations in other phase I trials examining patients with solid tumors [14, 15]. Although half of the patients experienced exacerbation of pre-existing hypoalbuminemia during the treatment, this was