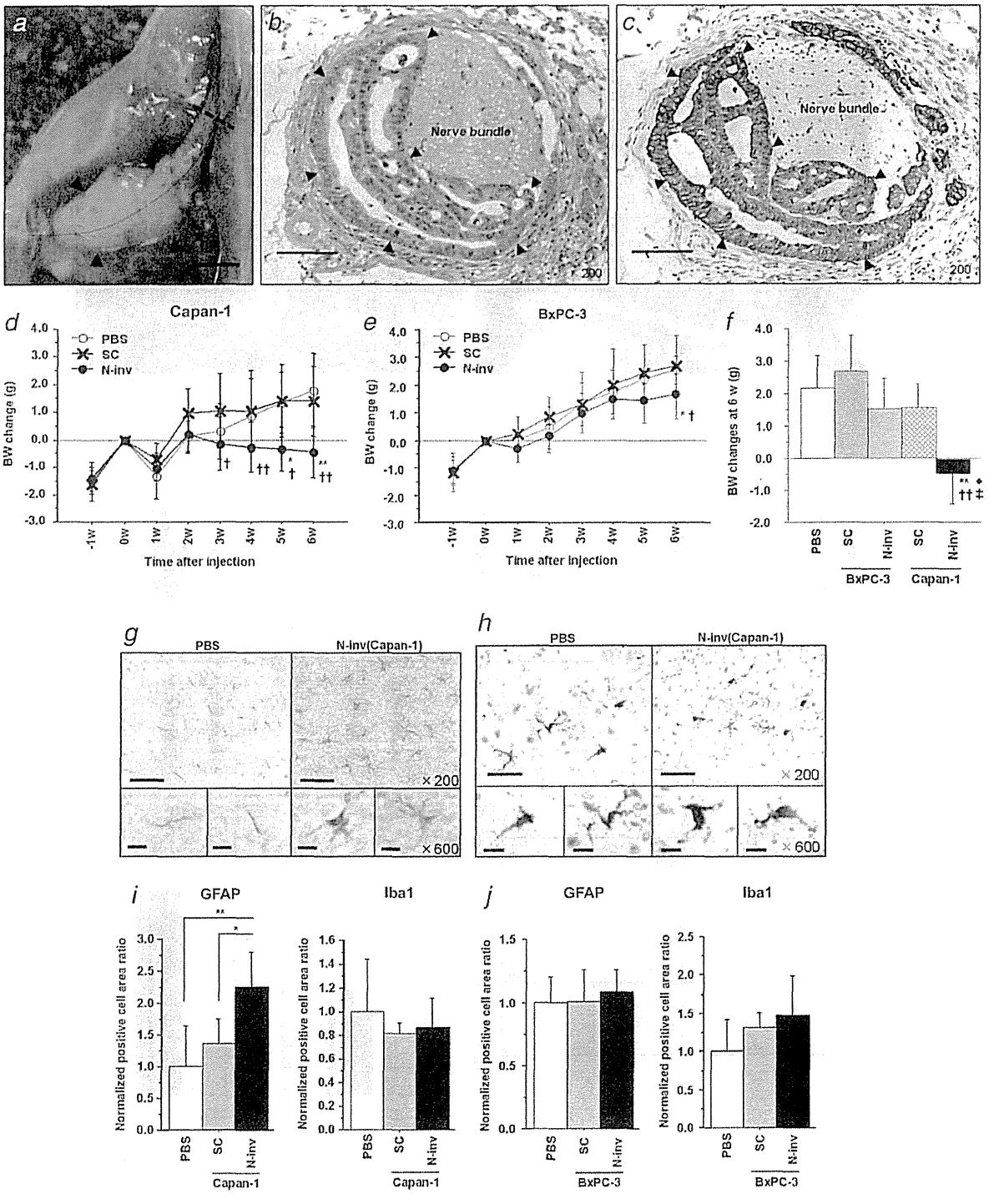

Figure 3. Cachectic changes in the N-inv model. (*a*) Macroscopic images of the left sciatic nerve in N-inv mice (Capan-1) at 6 week. Pancreatic cancer cells formed a spindle tumor (arrow head). Black curved line shows original left sciatic nerve. Scale bar: 5 mm. (*b*) Histological images of invasive pancreatic cancer cells were obtained at the level of the broken line in (*a*). Pancreatic cancer cells formed a ductal shape (arrow head) along the nerve bundle. Scale bar: 100 μm. (*c*) The same section as (*b*) was stained with cytokeratin AE1/3, an epithelial marker. Scale bar: 100 μm. (*d* and *e*) chronological BW changes from the original weight in PBS ($n = 4$ each), SC ($n = 8$, $n = 7$, respectively), and N-inv mice with Capan-1 cells ($n = 8$) (*d*) and BxPC-3 cells ($n = 7$) (*e*). $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* PBS mice; $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ *vs.* SC mice. (*f*) Total BW changes from the original weight at 6 week. $^{**}p < 0.01$ *vs.* PBS mice, $\dagger\dagger p < 0.01$ *vs.* SC (BxPC-3) mice, $\blacklozenge p < 0.05$ *vs.* N-inv (BxPC-3) mice, $\ddagger p < 0.05$, *vs.* SC (Capan-1) mice. (*g* and *h*) IHC images for GFAP (*g*) and Iba1 (*h*) in Th13 were taken from PBS and N-inv (Capan-1) mice at 6 week. Top, scale bar: 50 μm. Bottom, scale bar: 10 μm. (*i* and *j*) Quantification of the GFAP-positive and Iba1-positive cell area ratio in the ipsilateral dorsal horn relative to the N-inv in Th13 in mice given Capan-1 (*i*) and BxPC3 (*j*) cells. $^{*}p < 0.05$, $^{**}p < 0.01$. The ratios are presented as the fold changes relative to the PBS group. Data are expressed as the means ± standard deviation.

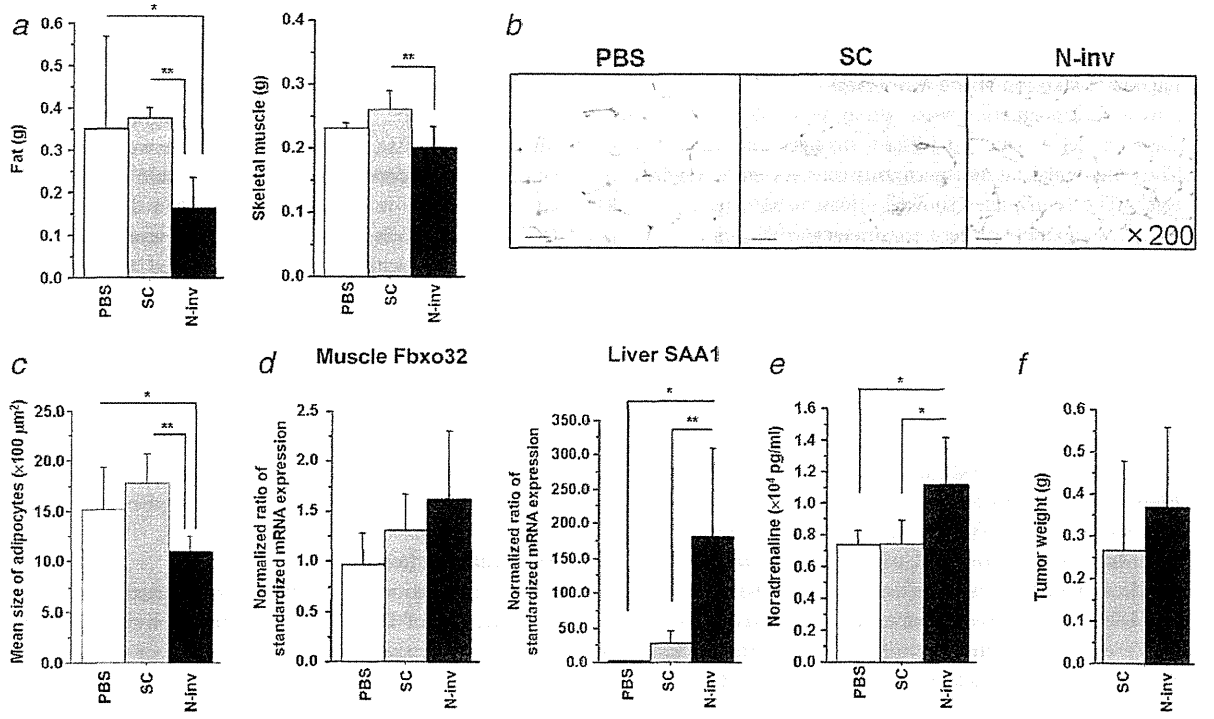

Figure 4. Cachectic features of the N-inv model (Capan-1). (*a* and *f*) Epididymal fat tissue, greater pectoral muscle and tumors were harvested and weighed at 6 week. (*b*) HE stained sections of epididymal adipose tissue from PBS ($n = 4$), SC ($n = 8$) and N-inv ($n = 8$) mice at 6 week were assessed with light microscopy. Scale bar: 50 μm. Adipocyte size, as determined by the sectional area, of N-inv mice was smaller than that in PBS and SC mice. (*c*) Adipocyte size in epididymal adipose tissue was quantified. (*d*) mRNA expression of Fbxo32 in muscle and SAA1 in liver at 6 week is shown. (*e*) The serum noradrenaline level at 6 week was measured with HPLC. $*p < 0.05$, $**p < 0.01$. mRNA expression of target genes normalized to GAPDH is presented as fold change to PBS mice. The results are expressed as the means ± standard deviations.

0.8 g, $p < 0.05$; 1.6 ± 0.9 g, $p < 0.01$, respectively). The BW of N-inv mice given BxPC-3 cells ($n = 7$) did not increase after 4 week, and the gain in BW (1.4 ± 0.9 g) was very small at 6 week, compared to that of mice given PBS ($n = 4$, 2.5 ± 0.5 g, $p < 0.05$) and SC ($n = 7$, 2.7 ± 1.1 g, $p < 0.05$) (Fig. 3*e*). N-inv mice given Capan-1 cells exhibited a marked BW loss at 6 week, compared to N-inv mice given BxPC-3 cells ($p < 0.05$) (Fig. 3*f*). No differences in food intake were observed among the three experimental groups for either Capan-1 or BxPC-3 cells (Supporting Information 1 and Table 4).

Microarray analysis using spinal cords of N-inv mice

At the beginning of the evaluation of spinal cords from N-inv mice, microarray analysis was performed using the first lumbar cord (L1) from PBS, SC and N-inv mice with Capan-1 cells at 6 week ($n = 2$ each) (Supporting Information Table 2). Overexpressed genes in the N-inv mice at L1 included galanin, prodynorphin (PDYN) and GFAP. These genes are known to be upregulated in the spinal cord after injury to the spinal nerve.[24–26] The mRNA levels of these three genes evaluated with real-time RT-PCR at 6 week were higher in the spines of N-inv mice ($n = 2$) than those in the PBS ($n = 2$) and SC ($n = 1$) mice (Supporting Information Fig. 4). The damage caused by N-inv may be responsible for the elevation of galanin, PDYN and GFAP.

Activation of astrocytes in the spinal cords of N-inv mice

Next, spinal cord glial activation was investigated with IHC and RT-PCR analyses. GFAP-positive cells in the spinal cords of N-inv mice with Capan-1 cells exhibited thickened branches and enlarged cell bodies, compared to mice given PBS (Fig. 3*g*). The GFAP-positive area in the N-inv mice was 2.2-fold larger than that in the spinal cords of PBS mice ($p < 0.01$) and 1.6-fold larger than that in the spinal cords of SC mice ($p < 0.05$) (Fig. 3*i*). Morphological features of Iba1-positive cells in the spinal cords did not show evident difference among three groups (Fig. 3*h*). Iba1-positive area was not significantly different among the PBS, SC and N-inv mice. These results were consistent with the mRNA expression of GFAP and Iba1 in the spinal cord (Supporting Information Fig. 2). In contrast, no obvious differences were observed in

the GFAP-positive areas among the three experimental groups given BxPC-3 cells (Fig. 3*j*).

Cachectic features of the N-inv model

Further investigations were done with N-inv mice given Capan-1 cells, which produced stronger cachectic changes. The epididymal fat and pectoralis muscles were weighed at 6 week. The N-inv mice showed a loss of both fat and muscle weight compared to these parameters in SC mice ($p < 0.01$, <0.01, respectively) (Fig. 4*a* and Supporting Information Table 5). Between the N-inv and PBS mice, the weight loss in the N-inv mice was evident in the fat ($p < 0.05$) and tended to be lower in the skeletal muscle, but the difference was not statistically significant ($p = 0.095$). The adipocytes were smaller in the N-inv mice compared to the PBS ($p < 0.05$) and SC mice ($p < 0.01$) (Figs. 4*b* and 4*c*).

Upregulation of mRNA for a marker of muscle atrophy,[27] F-box protein (Fbxo) 32, was seen in muscle of N-inv mice, compared to the PBS ($p = 0.111$) and SC mice ($p = 0.283$) (Fig. 4*d*). The mRNA level of uncoupling protein (UCP) 2, which is elevated in muscle tissue during cachexia,[28] was elevated in the muscles of N-inv mice, compared to the PBS ($p = 0.178$) and SC mice ($p = 0.148$) (Supporting Information Fig. 3). The inflammatory response is represented by the mRNA level of serum amyloid A (SAA) in the liver. The SAA1 mRNA expression level in the N-inv mice was higher than that in the PBS ($p < 0.05$) and SC mice ($p < 0.01$) (Fig. 4*d*). The serum level of noradrenaline in the N-inv mice was significantly higher than that in the PBS ($p < 0.05$) and SC mice ($p < 0.05$) (Fig. 4*e*). The tumor weight was similar between the N-inv and SC mice at 6 week. The mean tumor weight in the N-inv mice was 0.37 ± 0.19 g, corresponding to 1.78% of the BW (Fig. 4*f*). BxPC-3 cells formed intraneural tumors that were smaller than those formed by Capan-1 cells (0.13 ± 0.09 g, data not shown).

Ligation of the nerve route in N-inv mice

We ligated the proximal nerve to disrupt the connection between the N-inv and the CNS in N-inv mice given Capan-1 cells (Ligated N-inv mice or N-inv + ligation, $n = 5$). The BW changes in the ligated N-inv mice were similar to those in ligated PBS mice ($n = 4$), which were ligated at a site proximal to the PBS injection (Fig. 5*a* and Supporting Information Table 3). The BW loss in the N-inv mice ($n = 5$) and the increased BW of the PBS mice ($n = 3$) were reproducible. The BWs of the N-inv mice were markedly low at 5 week (−1.6 ± 1.1 g) and 6 week (−2.0 ± 1.3 g), compared to the PBS (2.0 ± 0.9 g, $p < 0.01$; 2.9 ± 0.8 g, $p < 0.01$), ligated PBS (1.0 ± 0.5 g, $p < 0.01$; 0.7 ± 0.9 g, $p < 0.01$) and ligated N-inv mice (1.0 ± 1.1 g, $p < 0.01$; 0.8 ± 1.1 g, $p < 0.01$, respectively). No significant differences were seen in food intake among the four groups during the observation period (Supporting Information Fig. 1 and Table 4). The activation of astrocytes in N-inv mice was reproducible in the experiments with ligation (Figs. 5*b*, 5*c* and Supporting Information Fig. 2). Hypertrophy, increased size of the GFAP cell area, and increased GFAP mRNA expression were observed in the N-inv mice and these GFAP changes were attenuated by the ligation of the sciatic nerve proximal to the N-inv. Compared to the N-inv mice, the ligated N-inv mice had higher fat weights ($p < 0.05$) and muscle weights ($p = 0.136$) (Fig. 5*d* and Supporting Information Table 5). The adipocyte size in ligated N-inv mice was larger than that in N-inv mice ($p < 0.05$) (Fig. 5*e*). The mean mRNA levels of Fbxo32 and UCP2 in the ligated N-inv mice tended to be lower than those in the N-inv mice ($p = 0.177$, $p = 0.288$, respectively) in muscle tissue (Fig. 5*f* and Supporting Information Fig. 3). The upregulation of SAA1 mRNA as a result of N-inv was significantly suppressed by ligation ($p < 0.01$). The mean tumor weight of the N-inv mice was 0.49 ± 0.09 g, which was similar to that in the other N-inv mice (Figs. 4*f*, 5*g* and Supporting Information Table 5). Ligation provided a 76.3% reduction in tumor weight, compared to the N-inv mice.

PPF treatment in vivo

Spinal cord astrocytic activation is reportedly suppressed by i.p. injection of PPF.[14,29] The hypertrophy of GFAP-labeled astrocytes observed in the spinal cords of saline-treated N-inv mice with Capan-1 cells ($n = 3$) was attenuated in the spinal cords of PPF-treated N-inv mice ($n = 3$) (Fig. 6*b*). The GFAP-positive area in the spinal cord of PPF-treated N-inv mice was 44.1% smaller than that of saline-treated N-inv mice (Fig. 6*c*). PPF administration caused a 23.4% reduction in the mRNA expression of GFAP ($p = 0.089$) (Supporting Information Fig. 2). The BW of the PPF-treated N-inv mice had not decreased after 2 week, but the BW of the saline-treated mice began to decrease after 5 week. A significant difference in BW was observed between the saline-treated and PPF-treated N-inv mice at 6 week (Fig. 6*a* and Supporting Information Table 3). Food intake was similar in the two experimental groups (Supporting Information Fig. 1 and Table 4). The PPF-treated N-inv mice had a higher muscle tissue weight ($p = 0.0152$), compared to saline-treated N-inv mice. The differences in fat mass and tumor weight were not significantly different between the saline and PPF-treated N-inv mice (Fig. 6*d*, 6*g* and Supporting Information Table 5). The mean size of adipocytes in PPF-treated N-inv mice was similar to that in saline-treated N-inv mice (Fig. 6*e*). Fbxo32 and UCP2 mRNA levels in the muscles of PPF-treated N-inv mice were markedly reduced, compared to levels in saline mice ($p = 0.0792$, $p < 0.05$) (Fig. 6*f* and Supporting Information Fig. 3).

Discussion

N-inv mice and patients with severe N-inv show a BW loss. Subcutaneous tumors and intraneural injection of PBS did not impact weight loss in mice. Intraneural tumors, but not extra-neural tumors or the manipulation of an intraneural injection, led to the reduction in body mass. A wasting condition with elevated inflammatory status is recognized as

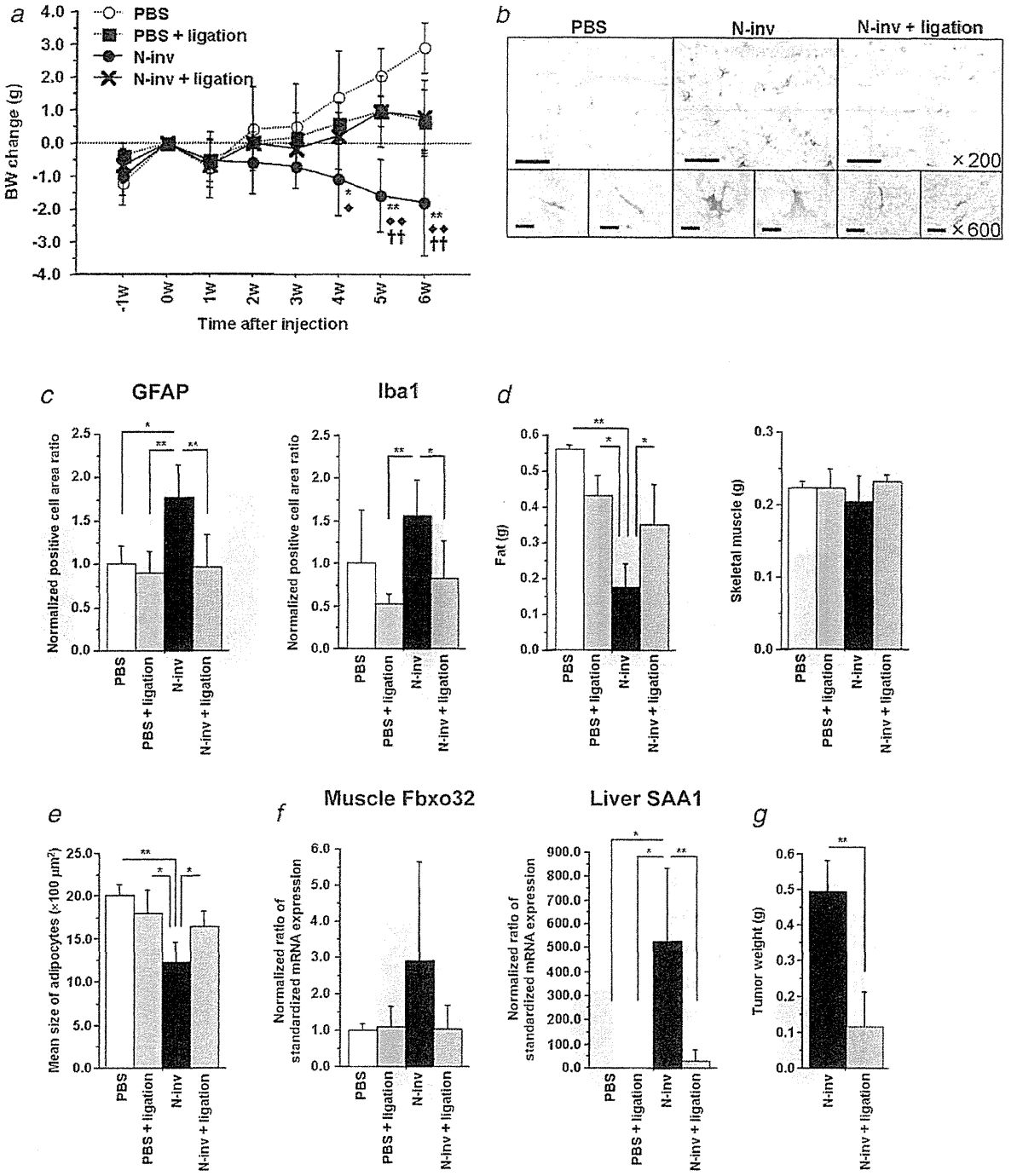

Figure 5. Ligation of the nerve route in N-inv mice (Capan-1). (*a*) Ligation of the sciatic nerve at a site proximal from the site of inoculation was performed in PBS ($n = 4$) and N-inv ($n = 5$) mice. Nonligated PBS ($n = 3$) and N-inv ($n = 5$) mice were used as controls. BW changes from the original weight were plotted weekly. $*p < 0.05$, $**p < 0.01$ *vs.* PBS group; ◆$p < 0.05$, ◆◆$p < 0.01$ *vs.* ligated PBS group; ††$p <$ 0.01 *vs.* ligated N-inv group. (*b*) The hypertrophic features of the GFAP-positive cells in the N-inv mice in Figure 3*g* were reproducibly observed in the N-inv mice at and were attenuated in the ligated N-inv mice. Top, scale bar: 50 μm. Bottom, scale bar: 10 μm. (*c*) Quantification of the GFAP-positive and Iba1-positive cell area ratio in the ipsilateral dorsal horn relative to the N-inv in Th13 was performed. (*d* and *g*) Fat, muscle and tumors were harvested and weighed at 6 week. (*e*) Adipocyte size in epididymal adipose tissue was calculated. (*f*) mRNA expression of Fbxo32 in muscle and SAA1 in liver at 6 week is shown. $*p < 0.05$, $**p < 0.01$. mRNA expression of target genes normalized to GAPDH is presented as fold changes to PBS group. The results are expressed as the means ± standard deviations.

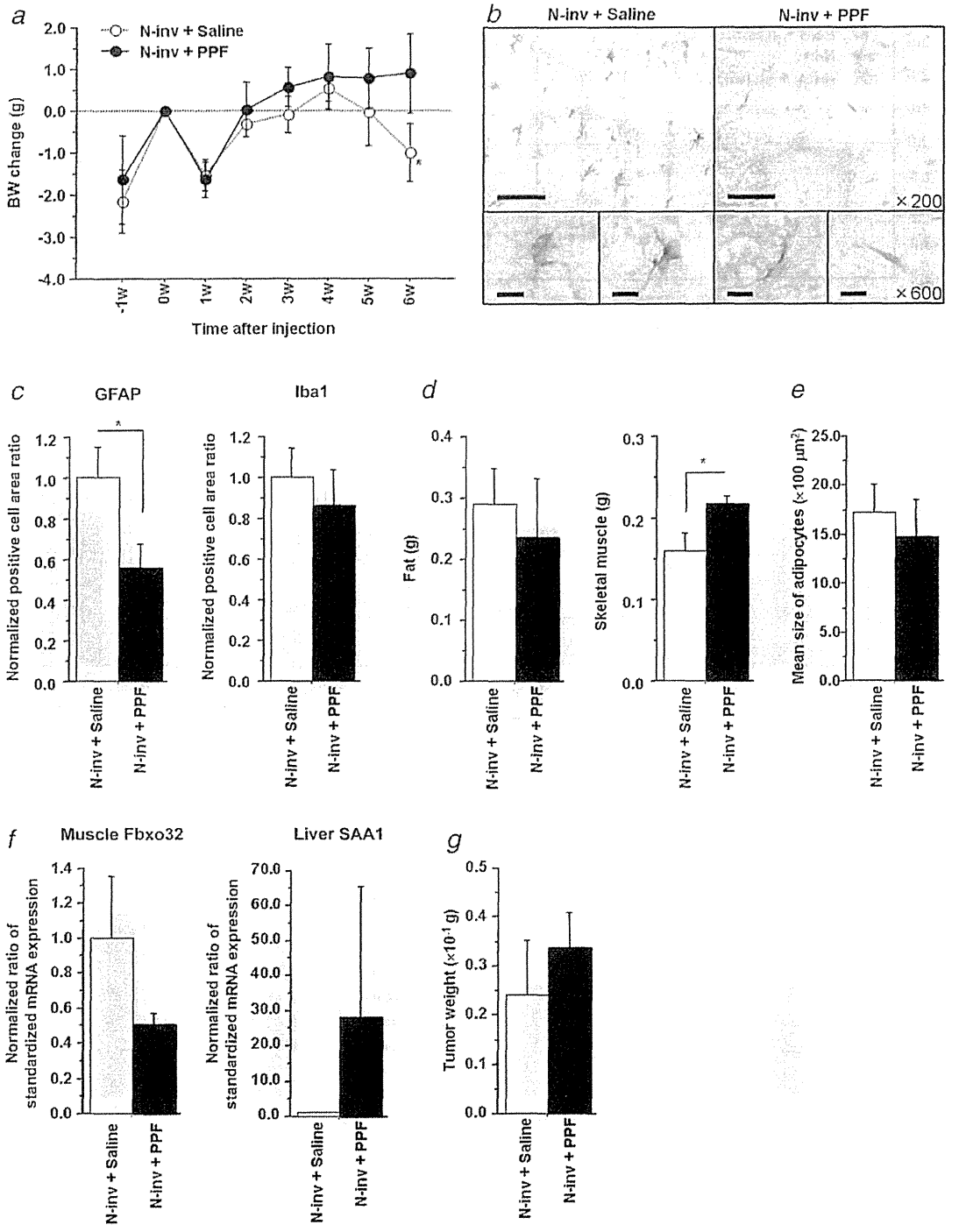

Figure 6. Effects of PPF in N-inv mice. (*a*) PPF (10 mg/kg) or the saline vehicle ($n = 3$/treatment) was administered to N-inv mice (Capan-1) by i.p. injection. The BW changes were then measured weekly. (*b*) IHC for GFAP in Th13 spinal cord dorsal horns showed that relatively thick GFAP-positive cells were seen in the saline group, but these cells were slimmer in the PPF group. Top, scale bar: 50 μm. Bottom, scale bar: 10 μm. (*c*) The GFAP-positive and Iba1-positive cell area ratios in the ipsilateral dorsal horns of N-inv were quantified. (*d* and *g*) The weights of the fat, muscle and tumors in each group at 6 week are shown. (*e*) Mean adipocyte size in epididymal fat was quantified. (*f*) mRNA expression of Fbxo32 in muscle and SAA1 in liver at 6 week was evaluated using real-time RT-PCR. $^*p < 0.05$. Positive cell area ratios and mRNA expression of target genes normalized to GAPDH are presented as the fold changes relative to the saline-treated N-inv group. The results are expressed as the means ± standard deviations.

cachexia.[20] Elevated SAA1 mRNA expression in liver and increased levels of circulating CRP were observed in N-inv mice and in patients with pancreatic cancer with high N-inv, respectively. Our study revealed that N-inv was a novel factor that induces cachexia in pancreatic cancer.

Cachexia involves catabolic changes in fat and muscle.[1] The body tissues that were decreased in N-inv mice were adipose and muscle tissue. Catabolic changes in muscle were reported after denervation or suppression of the activin receptor IIB in muscle due to upregulation of Fbxo32, a ubiquitin-protein ligase specific to muscle.[27,30,31] The increased mRNA level of Fbxo32 in the pectoral muscle in N-inv mice indicates the activation of muscle catabolism by N-inv. The loss of BMI, a good index of body fat,[32] in the high N-inv patients reinforced the idea that atrophy of adipose tissue was due to N-inv. Small lipid droplets and loss of fat mass were observed in N-inv mice. Noradrenaline induces lipolysis *via* adrenaline receptors and was elevated in the plasma of N-inv mice and the high N-inv patients.[33,34] These phenomena may indicate that lipolysis was due to N-inv. Anorexia is prevalent in patients with cachexia,[1] and this disorder affects lipid and amino acid metabolism.[35] Starvation results in fat and muscle catabolism.[36] The food intake in N-inv mice was similar to that in PBS and subcutaneous tumor model mice. The wasting condition of N-inv mice was independent of starvation. Experimental cachexia is preferably induced by a small amount of tumor (<5% of BW)[22] because the burden rarely exceeds 5% of the BW in patients with cancer.[37] In our N-inv mice injected with Capan-1 cells, the mean tumor weight was 1.78% of the BW. N-inv mice are a good model for cachexia regarding catalytic changes that are independent of calorie intake and that involve a small tumor burden.

Many studies have established that sciatic nerve ligation causes astrocytic activation in the spinal dorsal horn,[38,39] whereas our ligation experiment showed no obvious differences in the GFAP-positive areas between the PBS mice and ligated PBS mice. The difference in the observation period may be one possible explanation for this complication. Spinal cord astrocytes expression by sciatic nerve ligation was higher on 7 days than on 3 or 17 days in Andreea's study, suggesting that the peak of astrocytic activation by ligation was around day 7. Our observation period was much longer (42 days after ligation) than others and might be the phase after peak of astrocytic activation by sciatic nerve ligation. The persistent astrocytic activation in N-inv mice was likely due to repeated stimulation by tumor, compared to single stimulation by ligation.

N-inv mice injected with Capan-1 cells exhibited a marked BW loss and activation of spinal cord astrocytes compared to those injected with BxPC-3 cells. The differences between Capan-1- and BcPC-3-treated mice were a longer N-inv distance of Capan-1 cells than that of BxPC-3 cells,[21] mutations in KRAS and SMAD4, and many other factors.[40] We focused on the reaction of the host to N-inv and examined the status of astrocytic activation. The degree of astrocytic activation is mediated by damage to the peripheral nerve.[16] The spinal cords of human autopsy cases with N-inv also showed activation of spinal cord astrocytes compared to those without N-inv. The coexistence of N-inv and spinal cord glial activation was observed in both experimental and clinical N-inv. The activation of spinal cord astrocytes in N-inv mice was suppressed by ligation at a site proximal from the N-inv. The ligation of the sciatic nerve disrupted the transmission of the injury signal from the N-inv to the spinal cord. The neural damage signal caused by N-inv may mediate activation of astrocytes.

Systemic administration of PPF, a pan-phosphodiesterase (PDE) inhibitor, to N-inv mice suppressed the activation of spinal cord astrocytes and the loss of BW and skeletal muscle weight. PDE inhibitors downregulate the activation of astrocytes and microglia[14,29] and induce hypertrophy of skeletal muscle.[41] Another PDE inhibitor, pentoxifylline, failed to improve the appetite and BW loss in patients with cancer cachexia,[42] and the hypertrophic effect in muscle due to the PDE inhibitor was limited in cancer cachexia. Therefore, prevention of the wasting condition in N-inv mice by the PDE inhibitor implied a direct effect of PDE inhibition in muscle and an indirect effect due to suppression of astrocytic activation.

Mean relative level of SAA1 mRNA in PPF-treated group (5.2 ± 7.0) tends to be higher than that in saline-treated group (0.2 ± 0.0). Each values of SAA1 mRNA level in PPF group were extremely varied. We think this issue is difficult to discuss at present and interpret there is no significant difference between hepatic SAA1 mRNA level of PPF and vehicle group in our study. If hepatic SAA1 level truly tends to be elevated in PPF mice, a direct effect of PPF on hepatocyte is possibly associated with. The expressions of SAA1 mRNA and protein synthesis in hepatocytes are known to be mainly induced by inflammatory cytokines, such as IL-1, TNFα and IL-6.[43,44] Upregulation of SAA1 mRNA expression in PPF-treated N-inv mice may be due to a direct effect of PPF on hepatocyte, because increased intracellular cAMP leads to elevation of IL-6 production in hepatocyte.[45] The levels of cytokine and cAMP in hepatocyte were not examined in our study. Additional examinations are required to solve this issue.

The mechanism of astrocyte activation that subsequently causes cachexia was not examined in our study. Sympathetic activity may be a candidate. The mean level of blood noradrenaline, which is known to correlate with systemic sympathetic activity,[46] was high in severe N-inv patients and N-inv mice. Sympathetic activation induces lipolysis in adipose tissue[34] and muscle atrophy,[33,41] and thus causes BW loss. Because efferent fibers from the spinal cord to peripheral tissues include sympathetic nerves, spinal cord glial activation may affect sympathetic activity *via* neural routes. Astrocyte-derived cytokines are the other candidates. Astrocytic activation leads to the production and release of inflammatory cytokines, such as IL-1β, IL-6 and TNF-α.[47] IL-1 plays an

important role in lipid metabolism.[48] IL-6 and TNF-α are known to stimulate lipolysis in adipose tissue.[49] Astrocyte-derived cytokines are possible to induce lipolysis in adipose tissue in N-inv mice.

Our study has some limitations. First, a wide variety of effects of PPF made interpretation of our results complex. The combined action of PPF as a PDE inhibitor and a transporter of extracellular adenosine strengthens cyclic adenosine-5′,3′-monophosphate (cAMP) signaling.[29] Systemic administration of PPF to N-inv mice did not suppress the weight loss in adipose tissue. Stimulation of intracellular cAMP signaling leads to lipolysis in adipose tissue.[50] The failure to gain fat mass may have been caused by the lipolytic effect in adipose tissues, exceeding the downregulation of activated astrocytes in the spinal cord. Furthermore, the inhibitory effect of PPF on spinal cord microglial activation was not examined in this work, but should be investigated in the future. Finally, the difference in BW changes between N-inv mice and ligated N-inv mice was not evaluated under conditions of similar tumor weights. The reason for the decrease in tumor weights in N-inv mice by ligation was not examined in our study, but the reduction in intraneural tumors likely improved the cachectic changes. Further studies are required to examine the influences of ligation on the environment around the tumor, including neurotrophic factors.

In conclusion, our study showed (1) characteristics of cachexia by N-inv in patients with pancreatic cancer and in an experimental mouse model, (2) a relationship between N-inv and spinal cord astrocytic activation *via* a neural route in patients with pancreatic cancer and the experimental mouse model and (3) the anti-cachectic effect of suppression of spinal cord astrocytic activation in the experimental model. This is the first evidence that spinal cord astrocytes mediate cancer cachexia *via* neural routes. In addition, the cachectic animal model induced by N-inv has been established for the first time. Future studies on N-inv and spinal cord glial activation may provide insight into strategies for anti-cachectic therapy.

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ORIGINAL ARTICLE

Phase I/II study of gemcitabine as a fixed dose rate infusion and S-1 combination therapy (FGS) in gemcitabine-refractory pancreatic cancer patients

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Abstract
Purpose There is no standard regimen for gemcitabine (Gem)-refractory pancreatic cancer (PC) patients. In a previous phase II trial, S-1 was found to exhibit marginal efficacy. Gem administration by fixed dose rate infusion of 10 mg/m^2/min (FDR-Gem) should maximize the rate of intracellular accumulation of gemcitabine triphosphate and might improve clinical efficacy. We conducted the phase I/II of FDR-Gem and S-1 (FGS) in patients with Gem-refractory PC.
Methods The patients received FDR-Gem on day 1 and S-1 orally twice daily on days 1–7. Cycles were repeated every 14 days. Patients were scheduled to receive Gem (mg/m^2/week) and S-1 (mg/m^2/day) at four dose levels in the phase I: 800/80 (level 1), 1,000/80 (level 2), 1,200/80 (level 3) and 1,200/100 (level 4). Forty patients were enrolled in the phase II study at recommended dose.
Results The recommended dose was the level 3. In the phase II, a partial response has been confirmed in seven patients (18%). The median overall survival time and median progression-free survival time are 7.0 and 2.8 months, respectively. The common adverse reactions were anorexia, leukocytopenia and neutropenia.
Conclusion This combination regimen of FGS is active and well tolerated in patients with Gem-refractory PC.

Keywords Chemotherapy · Pancreatic carcinoma · Second-line · Gemcitabine · S-1 · Salvage · Fixed dose rate infusion

Introduction

Gemcitabine monotherapy or gemcitabine-containing combination chemotherapy is the standard first-line therapy for advanced pancreatic cancer. In the recent phase III study, the first-line FOLFIRINOX regimen (5-fluorouracil, leucovorin, irinotecan and oxaliplatin) led to a median survival of 11.1 months compared with 6.8 months in the gemcitabine group [4]. However, the FOLFIRINOX regimen was quite toxic (e.g., 5.4% of patients had grade 3 or 4 febrile neutropenia), and a survival benefit was shown only among a highly select population with a good performance status, an age of 75 years or younger, and normal or nearly normal bilirubin levels [13]. Therefore, this combination therapy was considered to be one of the treatment options for patients in good general condition, and gemcitabine remains the mainstay of care for patients with advanced pancreatic cancer. However, after disease progression during first-line gemcitabine-containing chemotherapy, the

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options for further anticancer treatment are limited. S-1 is an orally administered anticancer drug that consists of a combination of tegafur, 5-chloro-2,4-dihydroxypyridine and oteracil potassium in a 1 : 0.4 : 1 molar ratio [27]. The antitumor effect of S-1 has already been demonstrated in a variety of solid tumors including pancreatic cancer [7, 11, 12, 14, 20, 21, 25, 26, 32, 33]. In patients with chemo-naïve pancreatic cancer, an overall response rate of 21.1% was achieved, and the median time-to-progression and median overall survival period were 3.7 and 8.3 months, respectively [32]. In gemcitabine-refractory metastatic pancreatic cancer, our recent phase II study of S-1 yielded results that demonstrated marginal activity including a response rate of 15%, a median progression-free survival time of 2.0 months and a median overall survival time of 4.5 months, with a favorable toxicity profile [17]. In addition, other reports also demonstrated marginal antitumor activity [1, 28]. Gemcitabine administration via infusion at a fixed dose rate of 10 mg/m^2/min (FDR-Gem) has been found to increase the intracellular drug concentrations, compared with gemcitabine at a standard dose rate infusion over a period of 30 min. A recent phase II study of combination therapy consisting of FDR-Gem and oxaliplatin (GEMOX) yielded results that demonstrated activity in gemcitabine-refractory advanced pancreatic cancer [5], although oxaliplatin is inactive against pancreatic cancer when used as a single agent [6]. The increased intracellular concentrations of gemcitabine as a result of FDR infusion and/or the synergistic effect of gemcitabine and oxaliplatin may play an important role in the antitumor effect of GEMOX. This finding is of interest when considering the effect of combination therapy consisting of FDR-Gem and some other agent that exhibits a synergistic effect with gemcitabine in patients with metastatic pancreatic cancer who failed standard dose rate gemcitabine.

The inhibition of ribonucleotide reductase by gemcitabine is considered to enhance the effect of the 5-FU metabolite 5-FdUMP by reducing the concentration of its physiological competitor [10]. Preclinical studies have demonstrated a synergy between gemcitabine and 5-FU in tumor cell lines, including pancreatic cancer cells [3, 23]. S-1 is a fluoropyrimidine, and several phase II studies of S-1 and gemcitabine combination therapy have yielded results that demonstrated a promising activity in chemo-naïve advanced pancreatic cancer patients, including a response rate of 32–48% and a median survival times of 7.89–12.5 months [16, 18, 19, 31].

Therefore, we conducted the present phase I/II study to determine the recommended doses of FDR-Gem and S-1 (FGS) to use for combination therapy and to evaluate the toxicity and efficacy at the recommended doses in patients with gemcitabine-refractory pancreatic cancer.

Materials and methods

Eligibility criteria

The eligibility criteria were histologically proven pancreatic adenocarcinoma with measurable metastatic lesions, disease progression during gemcitabine-based first-line chemotherapy, age 20 years or over, ECOG performance status of 0–2 points, more than 2-week interval between the final dose of the prior chemotherapy regimen and study entry, adequate bone marrow function (leukocyte count $\geq$ 3,500/mm^3, neutrophil count $\geq$ 1,500/mm^3, platelet count $\geq$ 100,000/mm^3, hemoglobin concentration $\geq$ 9.0 g/dL), adequate renal function (serum creatinine level $\leq$ 1.1 mg/dL) and adequate liver function (serum total bilirubin level $\leq$ 2.0 mg/dL, transaminase levels $\leq$ 100 U/L). Patients with obstructive jaundice or liver metastasis were considered eligible if their total bilirubin level $\leq$ 3.0 mg/dL and transaminase levels could be reduced to 150 U/L by biliary drainage. The exclusion criteria were regular use of phenytoin, warfarin or flucytosine, history of fluorinated pyrimidine use, severe mental disorder, active infection, ileus, watery diarrhea, interstitial pneumonitis or pulmonary fibrosis, refractory diabetes mellitus, heart failure, renal failure, active gastric or duodenal ulcer, massive pleural or abdominal effusion, brain metastasis, and active concomitant malignancy. Pregnant or lactating women were also excluded. Written informed consent was obtained from all patients. This study was approved by the institutional review board of the National Cancer Center of Japan.

Treatment

Considering the patients' quality of life, we adopted biweekly schedule. Gemcitabine (Eli Lilly Japan K.K., Kobe, Japan) was administered by FDR intravenous infusion of 10 mg/m^2/min on day 1. S-1 (Taiho Pharmaceutical Co., Ltd., Tokyo, Japan) was administered orally twice daily on day 1 to day 7, followed by a 1-week rest. Treatment cycles were repeated every 2 weeks until disease progression or unacceptable toxicity occurred. If blood examination revealed leukocytopenia $<$ 2,000/mm^3, thrombocytopenia $<$ 75,000/mm^3, total bilirubin $>$ 3.0 mg/dL, aspartate aminotransferase or alanine aminotransferase level $>$ 150 U/L, or creatinine $>$ 1.5 mg/dL, both gemcitabine and S-1 were withheld until recovery. If a patient experienced dose-limiting toxicity (DLT), the dose of gemcitabine and S-1 was reduced by one level in the subsequent cycle. If a rest period of more than 15 days was required because of toxicity, the patient was withdrawn from the study. Patients were scheduled to receive gemcitabine and S-1 at four dosage levels (Table 1). Two dosage levels of S-1 were established according to the body

Table 1 Dosage levels of gemcitabine and S-1

Dosage level	Gemcitabine	S-1
Level 0	600 mg/m^2/60 min	Dosage A
Level 1[a]	800 mg/m^2/80 min	Dosage A
Level 2	1,000 mg/m^2/100 min	Dosage A
Level 3	1,200 mg/m^2/120 min	Dosage A
Level 4	1,200 mg/m^2/120 min	Dosage B

[a] Starting dosage

surface area as dosage A, about 80 mg/m^2/day, and dosage B, about 100 mg/m^2/day (Table 2). At the first dose level (level 1), gemcitabine was administered at a dosage of 800 mg/m^2 administered as a 80-min infusion, and S-1 was administered at dosage A. At the next dose level (level 2), the gemcitabine dosage was increased to 1,000 mg/m^2 administered as a 100-min infusion, and S-1 was administered at the same dosage. At the next dose level (level 3), the gemcitabine dosage was increased to 1,200 mg/m^2 administered as a 120-min infusion, and S-1 was administered at the same dosage. At the final dosage level (level 4), gemcitabine administered at the same dosage, and S-1 was administered at dosage B.

Study design

This study was an open-label, four-center, single-arm phase I/II study performed in two steps. The objective of step 1 (phase I) was to evaluate the frequency of DLT during first 2 cycles (4 weeks) and then use the frequency of DLT to determine which of the four dosages tested to recommend (Table 1). At least 3 patients were enrolled at each dosage level. If DLT was observed in the initial three patients, up to three additional patients were entered at the same dosage level. The highest dosage level that did not cause DLT in 3 of the 3 or $\geq$3 of the 6 patients treated at that level during the first two cycles of treatment was considered the maximum-tolerated dosage (MTD). DLT was defined as (1) grade 4 leucopenia or grade 4 neutropenia or febrile neutropenia, (2) grade 4 thrombocytopenia or thrombocytopenia requiring transfusion, (3) grade 3 or 4 non-hematological toxicity excluding hyperglycemia and electrolyte disturbances, (4) serum transaminases levels, γ-glutamyl transpeptidase level and alkaline phosphatase level $\geq$10 times UNL, (5) serum creatinine level $\geq$ 2.0 mg/dL and (6) any toxicity that necessitated a treatment delay of more than 15 days. Toxicity was graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. In step 2, the recommended dosages (RD) of FGS were then administered, and the effect of this combination therapy on objective tumor response was evaluated in patients who were given the RD (phase II). The number of patients to be enrolled in phase II was determined by using a SWOG's standard design (attained design) [8, 9]. The phase II included the patients who received the RD in the step 1. The null hypothesis was that the overall response rate would be $\leq$5%, and the alternative hypothesis was that the overall response rate would be $\geq$20%. The α error was 5% (one-tailed), and the β error was 10% (one-tailed). The alternative hypothesis was established based on the preferable data in previous reports [5, 15, 24, 30, 34]. Interim analysis was planned when 20 patients were enrolled. If none of the first 20 patients had a partial response or complete response, the study was to be ended. If a response was detected in any of the first 20 patients, an additional 20 patients were to be included in a second stage of accrual to more precisely estimate the actual response rate. If the number of objective responses after completing the trial was 5 or more among the 40 patients, then we would reject the null hypothesis and conclude that FGS was effective, and we would proceed to the next large-scale study. The severity of adverse events and progression-free survival and overall survival were investigated as secondary objectives in phase II.

Table 2 Dosage of S-1 (tegafur equivalent)

Body surface area (m^2)	Dosage A ($\fallingdotseq$80 mg/m^2/day)	Dosage B ($\fallingdotseq$100 mg/m^2/day)
<1.25	40 mg × 2/day	50 mg × 2/day
1.25–<1.5	50 mg × 2/day	60 mg × 2/day
$\geq$1.5	60 mg × 2/day	75 mg × 2/day

Results

Patient characteristics

Between June 2006 and March 2009, 49 patients were enrolled in this study. Fifteen patients (level 1: 3 patients, level 2: 3 patients, level 3: 6 patients, level 4: 3 patients) were enrolled into the phase I (STEP 1), and an additional 34 patients were enrolled into the phase II (STEP2) at dose level 3. Table 3 shows the baseline characteristics of the patients in step 1 and step 2. A total of the 40 patients who were given the recommended dose, 6 patients and 34 patients who entered into the study at phase I and phase II, respectively, were evaluated for efficacy and detailed safety profile.

Phase I (STEP 1)

No DLT occurred during the first 2 cycles (4 weeks) at level 1 or level 2. At dose level 3, three patients were

Table 3 Patient characteristics

Characteristic	Step 1				Step 2	Total at the recommended dose (level 3)
	Level 1	Level 2	Level 3	Level 4	Level 3	
No. of patients	3	3	6	3	34	40
Age, years						
Median	66	58	64	62	63.5	64
Range	55–69	51–58	48–71	52–70	40–80	40–80
Sex, *n* (%)						
Male	1 (33)	3 (100)	4 (67)	1 (33)	19 (56)	23 (58)
Female	2 (67)	0	2 (33)	2 (67)	15 (44)	17 (48)
ECOG performance status, *n* (%)						
0	2 (67)	2 (67)	5 (83)	2 (67)	22 (65)	27 (68)
1	1 (33)	1 (33)	1 (17)	1 (33)	12 (35)	13 (33)
Primary tumor, *n* (%)						
Head	1 (33)	2 (67)	2 (33)	2 (67)	17 (50)	19 (48)
Body/tail	2 (67)	1 (33)	4 (67)	1 (33)	17 (50)	21 (53)
Metastatic site, *n* (%)						
Liver	3 (100)	3 (100)	6 (100)	1 (33)	25 (74)	31 (78)
Lung	1 (33)	0	0	2 (67)	7 (21)	7 (18)
Peritoneum	1 (33)	1 (33)	0	1 (33)	11 (32)	11 (28)
Lymph node	0	2 (67)	0	0	11 (32)	11 (28)
Tumor stage at the start of prior treatment, *n* (%)						
Locally advanced	0	0	0	1 (33)	7 (21)	7 (18)
Metastatic	3 (100)	3 (100)	6 (100)	2 (67)	27 (79)	33 (83)
Prior treatment, *n* (%)						
Gemcitabine alone	3 (100)	3 (100)	5 (83)	3 (100)	26 (76)	31 (78)
Gem + Axitinib	0	0	0	0	2 (6)	2 (5)
Gem + Erlotinib	0	0	1 (17)	0	6 (18)	7 (18)

evaluated first, and none developed DLT. Since all 3 patients experienced DLT at dose level 4 (grade 4 neutropenia in two patients, grade 3 stomatitis in one patient), 3 additional patients were evaluated at dose level 3. A DLT (grade 4 neutropenia) was experienced by 2 of the 3 patients in this additional cohort in dose level 3, and dose level 3 was determined to be the MTD. Based on these results, the RD was determined to be level 3.

Phase II (efficacy and safety profile in the 40 patients treated at dose level 3)

In step 2, the RD of FDR-Gem and S-1 was administered to an additional 34 patients, and a total 40 patients were treated at dose level 3 to evaluate the objective tumor response to this combination therapy. As of the date of the analysis, the protocol treatment had been concluded in 39 of the 40 patients, and a total of 286 courses (median: 5 courses; range 1–31 courses) had been administered at level 3. The actual mean weekly dose administered were gemcitabine 545 mg/m^2/week (90.8% of planned dosage) and 90.1% of planned dosage of S-1. Dose reduction was required in 10 patients because of grade 4 neutropenia (five patients), grade 3 fatigue (1 patient), grade 2 fatigue with grade 2 appetite loss (one patient), grade 2 nausea (two patients) and grade 3 rash (1). The reasons for treatment discontinuation in phase II were radiological disease progression (33 patients), clinical disease progression (two patients), recurrent grade 4 neutropenia despite dose reduction due to grade 4 neutropenia (two patients), grade 4 myocardial infarction (one patients) and patient request to return to his distant hometown (one patient). All patients who discontinued treatment because of adverse events recovered from the toxicities after discontinuation. Twelve patients received third-line chemotherapy after discontinuation of FGS: S-1 monotherapy in four patients, gemcitabine + S-1 combination therapy on another treatment schedule in three patients, chemoradiotherapy with S-1 in one patient and new molecularly targeted agents in four patients who participated in a different clinical trial. Twenty-two patients received best supportive care, the other five patients transferred to another hospital, and no

information is available about their treatment after discontinuation of FGS.

Toxicity

All patients in steps 1 and 2 were evaluated for toxicity. In step 1, grade 3/4 non-hematological toxicity was observed in two patients (grade 3 fatigue during the third course in one patient, grade 3 stomatitis during the second course in one patient). No grade 4 leukocytopenia was observed at any dose level, but grade 4 neutropenia was observed in one out of three patients at dose level 1, none of the three patients at dose level 2, two of the six patients at dose level 3 and all three of the patients at dose level 4. Grade 3 thrombocytopenia was observed in one patient at dose level 2.

Table 4 summarizes the toxicities in the 40 patients who received the RD (level 3). All 40 eligible patients were assessable for toxicities, and FGS combination therapy at the RD was generally well tolerated. The most common toxicities were leukocytopenia (60%) and neutropenia (60%), but most of these toxicities were tolerable and reversible. Grade 4 neutropenia was noted as hematological toxicity in five patients (13%). Grade 3 non-hematological toxicities consisted of fatigue (one patient), vomiting (one patient), rash (one patient) and liver abscess (one patient). The patient who developed the grade 3 liver abscesses recovered after appropriate treatment with intravenous antibiotic alone. One female patient, who had hypercholesterolemia and history of smoking of 30 cigarettes/day, experienced a grade 4 acute myocardial infarction on day 1 of the third course of treatment, after gemcitabine had been administered but before the start of oral S-1. Emergency coronary angiography showed total occlusion of the left anterior descending coronary artery. The patient recovered from the cardiogenic shock due to myocardial infarction after coronary stent implantation and appropriate supportive treatment. S-1 monotherapy for the pancreatic cancer was started about 1 month after the infarction. No other severe or unexpected toxicities were noted in any of the patients.

Table 4 Treatment-related adverse events among the 40 patients who received the recommended dosages: highest grade reported during the treatment period

	Grade				Grade 1–4	Grade 3–4
	n					
	1	2	3	4	*n* (%)	*n* (%)
Hematological toxicities						
Leukocytes	11	4	9	0	24 (60)	9 (23)
Neutrophils	10	1	8	5	24 (60)	13 (33)
Hemoglobin	5	11	1	0	17 (43)	1 (3)
Platelets	11	2	1	0	14 (35)	1 (3)
Non-hematological toxicities					(0)	
Aspartate aminotransferase	8	1	0	0	9 (23)	0 (0)
Alanine aminotransferase	8	3	0	0	11 (28)	0 (0)
Alkaline phosphatase	5	2	0	0	7 (18)	0 (0)
Total bilirubin	3	0	0	0	3 (8)	0 (0)
Fatigue	15	2	1	0	18 (45)	1 (3)
Nausea	13	4	0	0	17 (43)	0 (0)
Vomiting	8	1	1	0	10 (25)	1 (3)
Anorexia	19	6	0	0	27 (68)	0 (0)
Stomatitis	4	0	0	0	4 (10)	0 (0)
Alopecia	8	0	–	–	8 (20)	–
Diarrhea	7	2	0	0	9 (23)	0 (0)
Rash	3	4	1	0	8 (20)	1 (3)
Hyperpigmentation	9	1	–	–	10 (25)	–
Hand-foot skin reaction	1	2	0	0	3 (8)	0 (0)
Watery eye	2	0	0	–	2 (5)	0 (0)
Hoarseness	1	0	0	0	1 (3)	0 (0)
Infection liver abscess	0	0	1	0	1 (3)	1 (3)
Myocardial infarction	0	0	0	1	1 (3)	1 (3)

Three patients died within 30 days after the final dose of the study drug. All 3 of the deaths were attributed to disease progression, and there were no treatment-related deaths.

Efficacy

It was possible to assess all 40 eligible patients who received the RD for response. Thirty-four patients had died by the completion of the follow-up period. There were no complete responses, but a partial response was achieved in seven patients (18, 95% confidence interval, 7.3–32.8%). Stable disease was noted in 19 patients (48%) and progressive disease in 14 patients (35%). Tumor responses to second-line FGS therapy are classified according to the tumor responses to first-line gemcitabine in Table 5. Three of 10 patients whose best response was progression disease in first-line chemotherapy achieved partial response in FGS therapy. The median progression-free survival time was 2.8 months. The median overall survival time after the start of second-line therapy was 7.0 months (range 1.3–18.9+), and the 1-year survival rate was 18% (Fig. 1). The median overall survival time after the start of first-line therapy was 13.9 months (range 5.2–31.4).

Table 5 Objective tumor response

Response (2nd line)	*n* (%)	Response (1st line)		
		PR	SD	PD
PR	7 (18)	1	3	3
SD	19 (48)	3	12	4
PD	14 (35)	2	9	3
Total	40 (100)	6	24	10

Response rate: 18% (95% CI: 7.3–32.8)

RECIST criteria

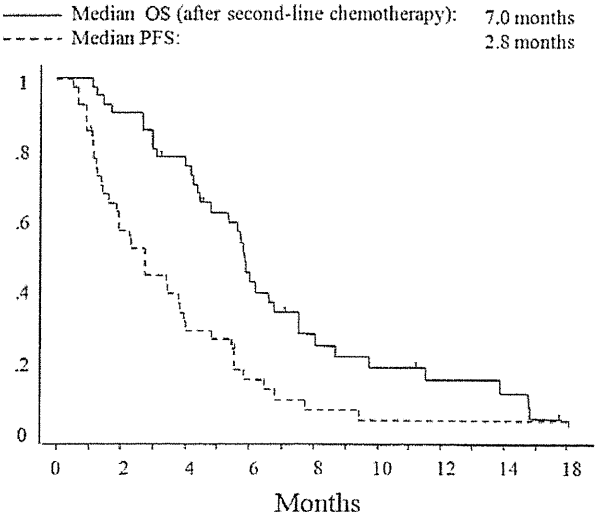

Fig. 1 Survival curves. Survival ($n = 40$). Progression-free survival (*dashed line*) and overall survival time (*solid line*) curves of patients with gemcitabine-refractory pancreatic cancer receiving systemic chemotherapy with FGS

Discussion

In the last decade, several clinical trials (mainly phase II) have been conducted in patients with advanced pancreatic cancer after failure of first-line gemcitabine or a gemcitabine-based combination regimen. The results of a randomized trial ($n = 168$) comparing fluorouracil and folinic acid versus oxaliplatin, fluorouracil and folinic acid (OFF) indicated that OFF improved progression-free survival and overall survival as a second-line chemotherapy. The median progression-free survival time and median survival time of OFF were 3 and 6 months, respectively [22]. In the present study, FGS yielded a median progression-free survival time of 2.8 months and a median overall survival time of 7.0 months, similar to the data mentioned above. Furthermore, the response rate of 18% in the present study was above the pre-established boundary (objective response in five or more of the 40 patients) required for the regimen to be considered effective. However, the gap between the median overall survival time and the median progression-free survival time in the present study was relatively large. Although the reason for this gap is unknown, a bias arising from the selection of patients with a good general condition or with a small tumor burden may explain these findings.

Whether gemcitabine as an FDR infusion is active even after progression during treatment with the standard 30-min administration of gemcitabine was the critical clinical question examined in this study. Differentiating between the relative roles of gemcitabine and S-1 in overcoming tumor resistance is difficult. The efficacy and survival data obtained in the present study seem to be better than those of previous studies for oral fluoropyrimidine monotherapy as a salvage chemotherapy for advanced pancreatic carcinoma (Table 6) [1, 2, 17, 28, 29]. However, since all the data were obtained in single-arm studies, a randomized study is needed to make these suggestions reliable. Furthermore, whether the combined regimen in the present study is superior to other regimens, such as the OFF regimen, remains an essential clinical question.

Safety and convenience as well as antitumor efficacy are critically important issues with regard to second-line chemotherapy. One patient experienced an acute myocardial infarction. Although she had other risk factors, such as a smoking habit and hyperlipidemia, a relation between gemcitabine and the acute myocardial infarction cannot be ruled out because gemcitabine had been administered on the day of the infarction. The toxicity profile of FGS

Table 6 Comparison between the current study and previous studies of oral fluoropyrimidine monotherapy as salvage chemotherapy for advanced pancreatic carcinoma

Study	References	Phase	Regimen	*n*	PR + CR (%)	Median PFS (months)	Median OS (months)
Morizane et al.	[12]	II	S-1	40	15	2.0	4.5
Abbruzzese et al.	[29]	II	S-1	45	0	1.4	3.1
Sudo et al.	[31]	II	S-1	21	9.5	4.1	6.3
Todaka et al.	[32]	Retrospective	S-1	52	4	2.1	5.8
Boeck et al.	[30]	II	Capecitabine	39	0	2.3	7.6
Morizane et al.	Current study	II	FGS	40	18	2.8	7.0

therapy in the other patients was acceptable, and the most common grade 1–4 adverse reactions were anorexia (68%), leukocytopenia (60%) and neutropenia (60%), although most episodes were tolerable and reversible. The safety profile in this study suggests that FGS can be safely administered to pancreatic cancer patients even in a second-line setting, at least in select populations. The biweekly schedule allows enough time to recover from myelosuppression and non-hematological toxicities before the following cycle, enabling patients to receive treatment as scheduled. Actually, the relative dose intensities of gemcitabine and S-1 in our study were high (90.8 and 90.1%, respectively). Furthermore, because of the biweekly schedule, patients do not need to come to the hospital for treatment as often compared with the first-line standard schedule of gemcitabine therapy. Our new treatment schedule may therefore improve the patients' quality of life during anticancer treatment.

We concluded that combination therapy consisting of gemcitabine as a fixed dose rate infusion and S-1 (FGS) provided a promising antitumor activity and tolerable toxicity in patients with gemcitabine-refractory metastatic pancreatic cancer. A larger randomized controlled trial is needed to confirm the clinical benefits of FGS following gemcitabine failure.

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Possibility of immunotherapy for biliary tract cancer: how do we prove efficacy? Introduction to a current ongoing phase I and randomized phase II study to evaluate the efficacy and safety of adding Wilms tumor 1 peptide vaccine to gemcitabine and cisplatin for the treatment of advanced biliary tract cancer (WT-BT trial)

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Abstract
Background/purpose In biliary tract cancer, few clinical studies evaluating immunotherapy have been reported. A phase I and randomized phase II study with Wilms tumor 1 (WT1) peptide vaccine plus gemcitabine and cisplatin (GC) for chemo-naïve patients with unresectable or recurrent biliary tract cancer was started, because the overexpression of WT1 is seen in the majority of patients with this disease, encouraging the potential of WT1-based immunotherapy. This trial was registered at the UMIN Clinical Trials Registry as UMIN 000004886.
Methods and results The aim of this trial is to evaluate the efficacy and safety of the regimen and to determine whether the regimen should be compared with the current standard regimen, GC, in a subsequent phase III trial for patients with unresectable or recurrent biliary tract cancer. Six patients in the phase I study and a total of 100 patients in the phase II study will be accrued over a 2-year period. The patients in the phase II study will be randomized at a 2:1 ratio to receive GC either with or without WT1 peptide vaccine. The primary endpoint of the phase II study is the 1-year overall survival rate.
Conclusions This is the first randomized trial to evaluate the use of immunotherapy in patients with advanced biliary tract cancer.

Keywords Biliary tract cancer · Immunotherapy · Chemotherapy · Wilms tumor 1 (WT1) peptide vaccine · Randomized trial

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Introduction

Systemic chemotherapy is usually indicated for patients with unresectable advanced biliary tract cancer or for those who have relapsed after operation; however, no standard treatments with solid evidence of a survival benefit have been established for such patients [1]. Although gemcitabine (GEM) alone was regarded as the de-facto standard regimen for advanced biliary cancer until recently, gemcitabine plus cisplatin (GC) has become the new standard regimen, based on the results of the ABC-02 trial [2], which showed a significant survival advantage for the GC combination over GEM alone. Even with the establishment of a standard therapy for this disease, the prognosis of these patients remains dismal: their median survival period is only around 10 months [2, 3]. Therefore, a clear need exists for new, effective, treatments for the management of biliary tract cancer (Fig. 1).

Recent progress in understanding the basic aspects of immunology has led to the development of immune-based therapies for various types of cancers. The identification of

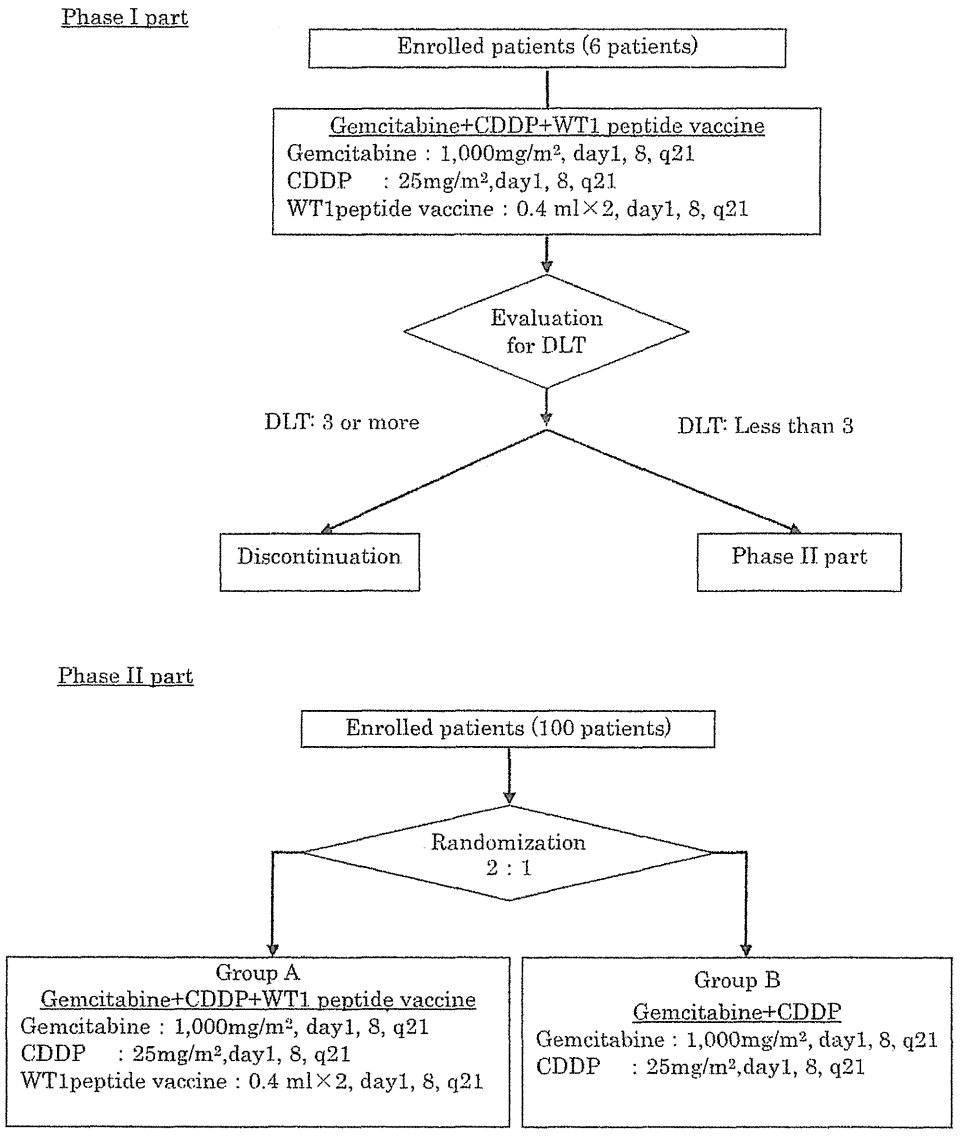

Fig. 1 Study design. *DLT* dose-limiting toxicity, *CDDP* cisplatin, *WT 1* Wilms tumor 1

various cancer antigens has facilitated many clinical trials of cancer vaccines that are expected to become new treatment strategies. Recently, sipuleucel-T immunotherapy for metastatic, asymptomatic hormone-refractory prostate cancer [4] and immunotherapy with ipilimumab for metastatic melanoma [5] have produced statistically significant improvements in survival, and both of these treatments have been approved by the United States Food and Drug Administration. Sipuleucel-T stimulates T-cell immunity against prostatic acid phosphatase, and ipilimumab blocks the potentiation of cytotoxic T-lymphocyte-associated antigen 4 and the antitumor T-cell response. Unfortunately, few preclinical studies examining biliary tract cancer have shown promising immune responses similar to those induced by sipuleucel-T against prostate cancer or those induced by ipilimumab against melanoma, and few clinical studies of immunotherapy for biliary tract cancer have been reported because of the rarity of this disease and the poor physical conditions of most patients at the time of the initial diagnosis. However, GEM has been reported not to suppress immunological cells, but to increase the population of dendritic cells that serve as antigen-presenting cells [6, 7]. Therefore, we conducted a phase I trial of Wilms tumor 1 (WT1) peptide vaccine and GEM combination therapy in patients with advanced pancreatic or biliary tract cancer [8]. Although the aim of that study was to assess the safety of the combination of WT1 peptide vaccine and GEM in a small population, it also showed that the WT1 peptide vaccine was safe enough to be employed in patients with advanced pancreatic or biliary tract cancer in combination with GEM, and

that the efficacy of the combination therapy seemed to be promising, as outlined below.

We recently initiated a phase I and randomized phase II study to evaluate the efficacy and safety of adding the WT1 peptide vaccine to GC in advanced biliary tract cancer (WT-BT trial), since GC has become the new standard and because the WT1 peptide vaccine is an attractive candidate as a partner for chemotherapy to improve survival in patients with advanced biliary tract cancer. WT1 protein is overexpressed in various types of cancer cells, including biliary tract cancer cells [9], and it was ranked as the No. 1 antigen in the cancer antigen prioritization project of the National Cancer Institute [10].

To our knowledge, this is the first randomized clinical trial to evaluate immunotherapy for biliary tract cancer. The study complied with the Declaration of Helsinki. Informed consent was obtained from all the patients, and the protocol was approved by the ethics committees at all participating institutions. This trial was registered at the UMIN Clinical Trials Registry as UMIN 000004886 (http://www.umin.ac.jp/ctr/index.htm). The study was initiated in January 2011.

1. The results of a phase I trial of WT1 peptide vaccine and GEM combination therapy in patients with advanced pancreatic or biliary tract cancer

An open-labeled, dose-escalation phase 1 trial of WT1 vaccine and GEM combination therapy for patients with advanced pancreatic cancer or biliary tract cancer was performed. The primary endpoint was the evaluation of the toxicity, safety, and optimal immunological dose of the vaccine. Human leukocyte antigen (HLA)-A 0201, HLA-A 0206, and/or HLA-A 2402-positive patients with inoperable advanced pancreatic or biliary tract cancer who had not previously been treated with GEM were eligible for this study. Six doses of GEM and 4 doses of WT1 peptide (1 or 3 mg) emulsified in Montanide adjuvant were administered over 2 months. Twenty-five patients (13 male and 12 female) were enrolled. Nine patients had inoperable advanced pancreatic cancer, 8 had gallbladder cancer, 4 had intrahepatic, and 4 had extrahepatic bile duct cancer. The adverse events were comparable to those seen with GEM alone. Delayed-type hypersensitivity test was positive after vaccination in 2 patients, and WT1-specific T cells in peptide-stimulated culture were detected by tetramer assay in 59% (13 of 22) of the patients. The disease control rate at 2 months was 89% for pancreatic cancer and 50% for biliary tract cancer. With a median follow-up time of 259 days, the median survival time for patients with biliary tract cancer was 288 days, and that for patients with pancreatic cancer was 259 days. Although objective clinical efficacy was not apparent, the safety of the WT1 vaccine and GEM combination therapy was confirmed in this study.

2. An ongoing phase I and randomized phase II study to evaluate the efficacy and safety of adding WT1 peptide vaccine to GC in advanced biliary tract cancer (WT-BT trial).

Protocol summary of the WT-BT trial

Study setting

The study is a multi-institutional open-label phase I and randomized phase II trial.

Objectives and endpoints

The aim of this phase I/II study is to determine the recommended dosage of WT1 peptide vaccine when used in combination with GC chemotherapy and to clarify the safety and efficacy of GC plus WT1 peptide vaccine when administered at the recommended dose, in comparison with GC alone.

In the phase I study, we will investigate the frequency of the dose-limiting toxicity (DLT). The criteria for a DLT will include: Grade 4 neutropenia for 8 or more consecutive days, Grade 3 neutropenia accompanied by a fever ($\geq$37.5°C), Grade 4 thrombocytopenia or the need for a transfusion, a Grade 4 aspartate transaminase (AST)/alanine transaminase (ALT) elevation or a Grade 3 AST/ALT elevation for 8 or more consecutive days, Grade 3 or 4 nonhematological toxicity (except for rash, hyperglycemia, gamma-GTP elevation, and any temporary events not affecting the protocol treatment), Grade 3 or 4 local skin inflammation at the vaccine injection sites, or Grade 1 or greater interstitial pneumonia.

In the phase II study, the primary endpoint will be the 1-year overall survival rate for all eligible patients. Overall survival will be defined as the number of days from randomization until death from any cause, and the data will be censored as of the last follow-up day on which the patient was alive. The secondary endpoints will be progression-free survival, response rate, median survival time, 2-year overall survival rate, percentage of adverse events, percentage of serious adverse events, and immunological responses (multimer assay and delayed-type hypersensitivity).

Eligibility criteria

Inclusion criteria

For inclusion in the study, patients are required to fulfill all the following criteria:

1. Clinically diagnosed with biliary tract cancer, including intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma, gallbladder cancer, and ampulla of Vater cancer.
2. Recurrent or unresectable biliary tract cancer.
3. Histologically proven papillary adenocarcinoma, tubular adenocarcinoma, or adenosquamous carcinoma for patients with extrahepatic cholangiocarcinoma, gallbladder cancer, and ampulla of Vater cancer; histologically proven adenocarcinoma for patients with intrahepatic cholangiocarcinoma.
4. Without central nervous system metastasis.
5. Without moderate or greater ascites/pleural effusion.
6. No previous therapy for biliary tract cancer.
7. No previous operation, chemotherapy, or radiotherapy for any other malignancies within the past 5 years.
8. No previous chemotherapy containing gemcitabine or cisplatin for any other malignancies.
9. An Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1.
10. Sufficient oral intake.
11. Age of 20–80 years.
12. Adequate organ functions.
13. HLA of A2402, A0201, or A0206.
14. Written informed consent.

Exclusion criteria

Patients will be excluded if they meet any of the following criteria:

1. Simultaneous or metachronous (within the past 5 years) double cancers, with the exception of intramucosal tumors curable with local therapy.
2. Pregnant or lactating women or women of childbearing potential and men who wish to father children.
3. Psychosis.
4. Patients requiring systemic steroid medication.
5. Interstitial pneumonia or fibroid lung disease.
6. Active bacterial or fungous infection.
7. Severe complications.
8. Drug allergies to drugs containing iodine compounds and/or gadolinium.
9. Inadequate physical condition, as diagnosed by the primary physician.

Randomization in the phase II study

After the fulfillment of the eligibility criteria has been confirmed, patient registration for both the phase I and II studies will be made by faxing the Data Center. Eligible patients in the phase II study will be stratified according to HLA (A2402/A02XX) and then randomized at the Data Center at a 2:1 ratio, using a minimization method and balancing the study arms according to institution, primary tumor (gallbladder cancer/other than gallbladder cancer), and history of surgical resection for the primary tumor (recurrent/advanced) to receive GC either with or without the WT1 peptide vaccine.

Treatment methods

For the patients in the phase I study, the GC and WT1 vaccine will be administered according to the following schedule: cisplatin (25 mg per m^2 of body-surface area) followed by gemcitabine (1000 mg per m^2) administered intravenously on days 1 and 8 every 3 weeks, with the vaccine (3 mg per body) injected subcutaneously alternating between 2 areas on the unilateral axillary fossa and inguen on days 1 and 8.

For both arms in the phase II study, GC will be administered according to the same dose and schedule as those used in the phase I study, but the vaccine will be administered only for the GC plus WT1 peptide vaccine arm.

The protocol treatments will be continued until disease progression, unacceptable toxicity, or patient refusal, although cisplatin will be continued for only a maximum of 24 weeks.

Follow-up

Enhanced abdominal computed tomography (CT)/magnetic resonance imaging, chest CT/X-rays, and tumor marker levels (carcinoembryonic antigen [CEA] and carbohydrate antigen [CA] 19-9) will be evaluated at least every 6 weeks during the protocol treatment. Patients will be seen on days 1 and 8 of every cycle for a physical examination to monitor their symptoms and the possible toxic effects of treatment. Adverse events will be graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0.

Study design and statistical analysis

In the phase I study, six patients will be recruited to determine whether a WT1 peptide vaccine dose of 3 mg per body can be recommended for use in combination with GC. A dose of 3 mg per body is the recommended dose for the WT1 peptide vaccine when used in combination with GEM alone, as determined in the previous phase I study. If treatment-related DLTs occur in no more than two of the six patients, transition to the phase II study will be