Table 1 Patient characteristics

	LigaSure ($n = 81$)	Conventional ($n = 79$)	P
Age (years)			
Median (range)	72 (40–85)	69 (33–86)	0.178*
Sex			
Male	55	57	0.607**
Female	26	22	
Body mass index (kg/m^2)			
Median (range)	22.3 (15.3–29.9)	21.9 (12.8–30.5)	0.824*
Tumor size (cm)			
Median (range)	3.5 (0.5–17.5)	4.0 (0.5–13.0)	0.718*
Depth of tumor invasion			
T1	36	44	0.252**
T2 or higher	45	35	
TNM Stage			
I	48	48	0.838***
II	9	10	
III	17	17	
IV	7	4	
Anticoagulant use			
Yes	6	6	1.000**
No	75	73	

P values were calculated using the *Mann–Whitney U test for age, body mass index, and tumor size; **Fisher's exact test for sex, depth of tumor invasion, and history of anticoagulant use; and the ***Chi square test for TNM stage

Table 2 Details of the surgical procedures

	LigaSure ($n = 80$)	Conventional ($n = 78$)	P
Type of resection			
Total gastrectomy	25	19	0.377
Distal subtotal gastrectomy	55	59	
Reconstruction			
Billroth I	31	38	0.319
Roux-en-Y	49	40	
Lymph node dissection			
D0, D1	29	32	0.624
D2	51	46	
Cholecystectomy			
Yes	35	30	0.624
No	45	48	
Splenectomy			
Yes	11	7	0.454
No	69	71	
Omentectomy			
Yes	36	30	0.424
No	44	48	

All P values were calculated using Fisher's exact test

There were no significant differences between the groups in baseline characteristics that may affect surgical outcomes, such as body mass index, tumor size, tumor stage, and history of anticoagulant therapy (Table 1). Table 2 summarizes the details on the surgical procedures. There were 25 patients in the LigaSure group and 19 patients in the conventional group, who underwent total gastrectomy. The proportion of patients who underwent D2 lymphadenectomy, cholecystectomy, and splenectomy was comparable in the two groups. Omentum-preserving gastrectomy was performed in patients whose tumors did not reach the serosal surface of the stomach, based on macroscopic inspection. In these patients, the greater omentum was divided 3 cm away from the course of the gastroepiploic artery and the distal part of the greater omentum was preserved, whereas in patients with serosal infiltration, total omentectomy or bursectomy was performed. Omentectomy was performed in 36 patients in the LigaSure group and 30 in the conventional group ($P = 0.424$).

The primary end point of this study, median operative blood loss, was 288 ml in the LigaSure group and 260 ml in the conventional group ($P = 0.748$) (Table 3). The median operative time was similar in the two groups, at 223 min in the LigaSure group and 225 min in the conventional group ($P = 0.368$). The median daily volume of abdominal drainage fluid was 132 ml in the LigaSure group and 114 ml in the conventional group ($P = 0.418$), and the median duration of drainage was 7 and 6 days, respectively. When we examined these endpoints in a subgroup analysis of the patients who underwent omentum-preserving procedures, the blood loss was significantly lower in the LigaSure group than in the conventional group (179 vs. 245 ml, $P = 0.033$). The operative time was shorter in the LigaSure group, at 195 vs. 221 min ($P = 0.039$; Table 4).

Postoperative complications developed in 40 (25.3 %) patients: 24 from the LigaSure group and 16 from the conventional group ($P = 0.259$; Table 5). There were no significant differences between the groups in the eight major complications associated with gastrectomy. However, the incidences of pancreatic leakage and wound infection were slightly higher in the LigaSure group ($P = 0.094$). One patient in the LigaSure group underwent reoperation for residual tumor in the cut end of the stomach and five patients in the conventional group underwent reoperation, for postoperative bleeding, anastomotic leakage, anastomotic stenosis, bowel obstruction, and wound complications in the abdominal wall in one each. Two patients from the conventional group died during hospitalization, of tumor metastasis to the liver and peritoneum, and sepsis secondary to intestinal necrosis, respectively.

Table 3 Surgical outcomes

	LigaSure ($n = 80$)	Conventional ($n = 78$)	P
Blood loss (ml)			
Median (range)	288 (25–1430)	260 (40–1258)	0.748*
Operative time (min)			
Median (range)	223 (130–405)	225 (111–415)	0.368*
Blood transfusion			
Yes	6	8	0.586**
No	74	70	
Volume of drainage (ml/day)			
Median (range)	132 (8–1120)	114 (18–730)	0.418*
Duration of drainage (days)			
Median (range)	7 (1–36)	6 (1–108)	0.245*

P values were calculated using the *Mann–Whitney U test for operative time and blood loss, and **Fisher's exact test for blood transfusion

Table 4 Subset analysis of patients who underwent omentum-preserving gastrectomy and omentectomy

	LigaSure	Conventional	P
Omentum-preserving gastrectomy	($n = 44$)	($n = 48$)	
Blood loss (ml)			
Median (range)	179 (25–637)	245 (40–1105)	0.033
Operative time (min)			
Median (range)	195 (130–290)	221 (142–415)	0.039
Omentectomy	($n = 36$)	($n = 30$)	
Blood loss (ml)			
Median (range)	400 (75–1430)	305 (80–1258)	0.197
Operative time (min)			
Median (range)	244 (145–405)	236 (111–329)	0.122

P values were calculated using the Mann–Whitney U test

There were no postoperative deaths in the LigaSure group. The median hospital stay was 16 days (range 8–67) for the LigaSure group and 15 days (range 7–122) for the conventional group ($P = 0.333$; Table 5). There was no significant difference in the total cost associated with hospitalization between the LigaSure and conventional groups (median \$17,060 vs. \$16,520, $P = 0.702$).

Discussion

Several clinical studies have demonstrated that the LigaSure vessel sealing system decreases both the blood loss and operative time associated with hemorrhoidectomy [5, 17], hysterectomy [18, 19], splenectomy, [8] and hepatectomy [9]. In the present randomized controlled trial we hypothesized that the use of LigaSure might also improve the surgical outcomes of gastrectomy for gastric cancer, but surgery using the LigaSure technique was not shown to be significantly superior to conventional surgery.

Table 5 Postoperative complications and hospital mortality

	LigaSure ($n = 80$)	Conventional ($n = 78$)	P
Total complications	24	16	0.259
Postoperative bleeding	0	1	0.494
Anastomotic leakage	0	1	0.494
Pancreatic leakage	7	1	0.094
Abdominal abscess	2	2	1.000
Wound infection	7	1	0.094
Bowel obstruction	4	3	1.000
Pneumonia	3	1	0.620
Anastomotic stenosis	2	1	1.000
Other	3	7	0.207
Re-operation	1	5	0.114
Hospital death	0	2	0.242
Postoperative hospital stay (days)	16 (8–67)	15 (7–122)	0.333

P values were calculated using Fisher's exact test

These findings can be explained by the varying utility of LigaSure from one type of surgery to another. Operations where LigaSure was shown to provide an advantage involve the excision of an organ where the vessel-sealing device can play a prominent role. In contrast, radical gastrectomy for gastric cancer consists of three different phases: resection of the stomach, lymphadenectomy, and reconstruction. Certainly, the vessel sealing system may be most useful during the resection phase, but it is not suitable for the meticulous manipulation involved in lymphadenectomy and bursectomy because the tip of the device is blunt. Moreover, LigaSure has almost no role in reconstruction and anastomosis, which are as time-consuming as resection of the stomach. It is likely that individual manual ligation and dissection take more time and incur more blood loss than mechanical sealing by LigaSure, but using LigaSure did not result in an overall reduction in the operative time or total blood loss from the beginning of laparotomy until skin closure. This explanation is supported by the results of the subgroup analysis of patients who underwent omentum-preserving gastrectomy. For these patients with relatively early stage cancers, the omentum was segmented in the middle and prophylactic lymphadenectomy and bursectomy were omitted, leading to a relatively dominant advantage of LigaSure in the course of the entire operation, with a 66 ml reduction in blood loss ($P = 0.033$) and a 26 min reduction in operative time ($P = 0.039$) compared with conventional surgery.

Contrary to our results, Lee et al. [20] reported that the use of LigaSure shortened the operative time and decreased blood loss in gastric cancer resection with D2 lymphadenectomy in a randomized controlled trial. This discrepancy may be due to differences in the surgical procedures defined by the protocols of the two trials. In the LigaSure group, they sealed all of the lymphatic ducts and blood vessels using LigaSure without any suture ligation, whereas in the present study we used hand ligation in addition to LigaSure to occlude the major arteries and vessels. We also used manual manipulation in the delicate parts of lymphadenectomy, even in the LigaSure group. A similar discrepancy was demonstrated between two randomized trials evaluating LigaSure in hepatic resection [9, 21], and those authors also attributed the discordant results to differences in surgical techniques between the two studies [21].

Although the overall incidence of operative complications did not differ significantly between the two groups in this study, it is noteworthy that the incidence of pancreatic leakage was slightly higher in the LigaSure group ($P = 0.094$). The reason for this is not clear, but we speculate that the mechanical compression of tissues adjacent to the pancreas with LigaSure may cause a minor leakage of pancreatic juice. Although our present study did not show a significant difference in the drainage volume between the two groups, a risk of pancreatic leakage and lymphorrhea with manipulation around the pancreas during lymphadenectomy with the use of LigaSure should be borne in mind. This is further supported by the previous report that the use of LigaSure increased the volume of the drainage fluid after radical gastrectomy compared with conventional surgery [20],

Multicenter randomized controlled trials of surgical procedures are affected by the experience of and variations among surgeons [22]. In the present study, the median blood loss and operative time (260 ml and 225 min), as well as the operative morbidity (20.5 %), in the conventional group was consistent with the results from our previous studies [15, 16]. Furthermore, we found no significant differences in the results of surgical outcomes between the participating hospitals (data not shown), indicating that the quality of surgery in our study is consistent.

In conclusion, the application of LigaSure to open gastrectomy for gastric cancer was not associated with a reduction in blood loss or operative time compared with conventional techniques. The effectiveness of the device is limited, and its use should be based on the individual operator's decision, depending on the type of surgery.

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Conflict of interest None declared.

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Prognostic Value of CEA and CK20 mRNA in the Peritoneal Lavage Fluid of Patients Undergoing Curative Surgery for Gastric Cancer

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Abstract

Background Peritoneal recurrence is the most common type of recurrence in gastric cancer. Although cytological examination of peritoneal lavage fluid has been used to predict peritoneal spread, peritoneal recurrences often occur even in patients with negative cytology. Our previous retrospective study suggested that reverse transcriptase-polymerase chain reaction (RT-PCR) using peritoneal lavage fluid may be useful for predicting peritoneal recurrence in patients with negative cytology. This prospective study was conducted to validate the clinical impact of this RT-PCR method.

Methods From July 2009 to June 2012, a total of 118 cT2-4 gastric cancer patients underwent surgery. Since 14 patients were ineligible because they had incurable factors, the remaining 104 eligible patients were evaluated for carcinoembryonic antigen (CEA) and cytokeratin 20 (CK20) messenger RNA (mRNA) using RT-PCR. If either CEA or CK20 mRNA was detected by RT-PCR, the patient was defined as PCR-positive as in our previous study. The association between recurrence-free survival (RFS) and background factors was analyzed using Cox proportional hazards models.

Results Of 104 patients, 16 (15.4 %) were positive for either CEA or CK20. PCR-positive patients had significantly worse RFS than PCR-negative patients (log-rank $p = 0.007$). Regarding the pattern of recurrence, 4 of 16 (25 %) PCR-positive patients and 2 of 88 (2 %) PCR-negative patients had peritoneal recurrence ($p < 0.001$), but there were no significant differences in recurrence at other sites. Cox multivariate analysis indicated only PCR-positivity as a significant predictor of poor RFS ($p = 0.029$).

Conclusion This prospective study demonstrated that CEA and CK20 PCR results could predict peritoneal recurrence after curative surgery.

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Introduction

The prognosis of advanced gastric cancer remains poor, even after curative surgery. Peritoneal dissemination, mainly caused by the seeding of free cancer cells from the primary lesion, is the most common type of recurrence [1]. Cytological examination of peritoneal lavage fluid collected during surgery is used to predict peritoneal spread since positive peritoneal cytology (CY1) has been found to be an independent predictor of disease recurrence and poor overall survival [2–4]. However, peritoneal recurrences often occur even in patients with negative cytology, which indicates that cytological examination is not sensitive enough for the detection of residual cancer cells in the peritoneum.

Molecular diagnosis using reverse transcriptase-polymerase chain reaction (RT-PCR) has been used to detect cancer micrometastases [5–7]. Carcinoembryonic antigen (CEA) and cytokeratine-20 (CK20) are the most common targets for detecting isolated tumor cells using PCR [8–10],

and we previously reported that, among 36 gastric cancer patients, PCR-positive patients had significantly worse survival than PCR-negative patients [11]. However, our previous study was retrospective in nature and included a small number of patients, so we conducted a prospective study to validate the prognostic value of molecular detection in over 100 patients undergoing curative surgery for gastric cancer.

Patients and methods

Patients

Peritoneal lavage fluid was prospectively collected during surgery from 118 consecutive patients with cT2-4 gastric cancer at Osaka University Hospital between July 2009 and June 2012. All patients were histologically diagnosed with adenocarcinoma of the stomach. Patients with incurable factors, such as peritoneal metastases (P1), CY1, or other distant metastases (M1) were excluded from this study.

Peritoneal lavage fluid was collected as described in our previous report [12]. In brief, peritoneal lavage fluid was immediately obtained from the pouch of Douglas and the left subdiaphragmatic space after laparotomy or the insertion of trocars. We injected 100 mL of normal saline and suctioned again. Approximately half of the sample was examined cytologically and the remainder was centrifuged at $300\times g$ for 5 min. Cells were then suspended in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and stored at −80 °C. Pathological staging of the tumor was based on the seventh edition of the International Union Against Cancer (UICC) tumor–node–metastasis classification guidelines [13]. The study protocol was approved by the institutional review board of Osaka University Hospital. All patients provided written informed consent for their samples to be used in research.

Quantitative RT-PCR

RNA isolation and RT-PCR were performed using a method similar to those in our previous studies [11, 12]. Frozen samples in TRIzol reagent were thawed and total RNA was extracted using the acid guanidinium thiocyanate-phenol–chloroform method [14]. Its concentration was determined spectrophotometrically by measuring the absorbance of RNA at 260 nm. First strand complementary DNA (cDNA) was synthesized from total RNA (1 μg), mixed with RT reaction reagents, including oligo-(dT)15 primer, using the protocol recommended by the manufacturer (Promega, Madison, WI, USA). CEA-specific oligonucleotide primers for RT-PCR were 5′-TCTGGAACTTCTCCTGGTCTCTC AGCTGG-3′ (forward) and 5′-TGTAGCTGTTGCAAATG CTTTAAGGAAGAAGC-3′ (reverse) to amplify a 160 bp PCR product. CK20-specific oligonucleotide primers for RT-PCR were 5′-GGTCGCGACTACAGTGCATATTACA -3′ (forward) and 5′-CCTCAGCAGCCAGTTTAGCAT TATC-3′ (reverse) to amplify a 121 bp PCR product. The integrity of extracted RNA was confirmed by RT-PCR analysis of a housekeeping gene, porphobilinogen deaminase (*PBGD*). Primer sequences for *PBGD* were 5′-TGTCT GGTAACGGCAATGCGGCTGCAAC-3′ (forward) and 5′-TCAATGTTGCCACCACACTGTCCGTCT-3′ (reverse). The integrity of all RNA samples was verified by quantitative RT-PCR for *PBGD* in each sample. The emission intensity of SYBR Green was detected in real time with the LightCycler 3.5 instrument (Roche Diagnostics, Mannheim, Germany). The external standards were prepared by serial dilution (1:1–1:10,000) of cDNA from the MKN45 cell line. CEA messenger RNA (mRNA) was detected to 10,000 times attenuation (1:10,000), and CK20 mRNA was detected to 500 times attenuation (1:500). If either CEA or CK20 mRNA was detected by RT-PCR analysis, the patient was defined as PCR-positive, similar to our previous study [11].

Statistical analysis

Patient clinicopathological data were prospectively recorded. The relationship between RT-PCR results and various background factors was assessed using the χ^2 test. Recurrence-free survival (RFS) was defined as the time from surgery to first recurrence. RFS was censored at the time of the last follow-up or death without recurrence. Survival curves were estimated using the Kaplan–Meier method and compared using the log-rank test. The impact of background factors (age, sex, histology, neoadjuvant chemotherapy, and pathological T and N stages) on survival was analyzed with univariate and multivariate Cox proportional hazards models. p values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS Statistics software, version 20 (IBM Corp., Armonk, NY, USA).

Results

PCR results

From July 2009 to June 2012, a total of 118 patients with cT2-4 gastric cancer underwent surgery; 14 were ineligible due to incurable factors such as P1, CY1, or M1. The remaining 104 eligible patients were evaluated for CEA and CK20 mRNA using RT-PCR. Among the 104 patients, 11 patients (10.6 %) were positive for CEA and ten patients (9.6 %) were positive for CK20 (Table 1). In total,

Table 1 RT-PCR positive rate for each marker

CK20	CEA, *n* (%)	
	Positive	Negative
Positive	5 (4.8 %)	5 (4.8 %)
Negative	6 (5.8 %)	88 (84.6 %)

CEA carcinoembryonic antigen, *CK* cytokeratin, *RT-PCR* reverse transcriptase-polymerase chain reaction

16 patients (15.4 %) were positive for either CEA or CK20, and we defined these patients as PCR-positive.

We examined the relationship between the PCR results and background factors (Table 2). The PCR-positive group included more female patients and higher pathological N-stage patients than the PCR-negative group ($p = 0.032$, $p = 0.029$). No significant relationship was observed with other background factors, including age, histology, surgical approach, neoadjuvant chemotherapy, clinical T or N stage, and pathological T stage. Regarding the neoadjuvant chemotherapy, we used three types of regimens; S-1 plus cisplatin ($n = 6$), S-1 plus docetaxel ($n = 2$), and S-1 plus cisplatin plus docetaxel ($n = 12$). There was no significant difference in neoadjuvant regimens between PCR-positive and negative patients ($p = 0.25$).

Prognostic value of CEA and CK20 mRNA

The median follow-up in this prospective study was 18.2 months, during which 7 of 16 (44 %) PCR-positive patients and 13 of 88 (15 %) PCR-negative patients had recurrences ($p = 0.007$). PCR-positive patients had significantly worse RFS than PCR-negative patients (log-rank $p = 0.007$) (Fig. 1), and the hazard ratio for recurrence in PCR-positive patients was 3.28 (95 % confidence interval [CI] 1.31–8.24). The 2-year RFS rate in PCR-positive patients was 50.3 %, while that of PCR-negative patients was 83.0 %. Regarding the pattern of recurrence, 4 of 16 (25 %) PCR-positive patients and 2 of 88 (2 %) PCR-negative patients had peritoneal recurrence ($p < 0.001$), while PCR-positive and -negative patients were similar with respect to other sites of recurrence (Table 3).

We conducted Cox univariate and multivariate analyses to find independent prognostic factors of RFS. The multivariate analysis indicated that PCR-positivity was a significant predictor of poor RFS ($p = 0.029$) (Table 4).

Table 2 Relationship between PCR results and background factors

Factors	PCR-positive ($n = 16$)	PCR-negative ($n = 88$)	*p* value*
Age, years			0.30
≤65	4 (25 %)	34 (39 %)	
>65	12 (75 %)	54 (61 %)	
Sex			0.032
Male	8 (50 %)	67 (76 %)	
Female	8 (50 %)	21 (24 %)	
Histology			0.74
Differentiated	8 (50 %)	48 (55 %)	
Undifferentiated	8 (50 %)	40 (45 %)	
Surgical approach			0.71
Open	15 (94 %)	80 (91 %)	
Laparoscopic	1 (6 %)	8 (9 %)	
Neoadjuvant chemotherapy			0.52
Yes	4 (25 %)	16 (18 %)	
No	12 (75 %)	72 (82 %)	
cT			0.86
T2	3 (19 %)	18 (20 %)	
T3	6 (38 %)	27 (31 %)	
T4	7 (44 %)	43 (49 %)	
cN			0.50
N0	6 (38 %)	47 (53 %)	
N1	4 (25 %)	17 (19 %)	
N2–3	6 (38 %)	24 (27 %)	
pT			0.14
T1–2	2 (13 %)	33 (38 %)	
T3	10(63 %)	42 (48 %)	
T4	4 (25 %)	13 (15 %)	
pN			0.029
N0	5 (31 %)	52 (59 %)	
N1	4 (25 %)	22 (25 %)	
N2–3	7 (44 %)	14 (16 %)	

Data are presented as *n* (%)

PCR polymerase chain reaction

* χ^2 test

Discussion

Peritoneal recurrence of gastric cancer occurs often, even in patients who have undergone curative resection. Although peritoneal lavage cytology has been widely used for the detection of isolated tumor cells and prediction of peritoneal recurrence, the sensitivity is relatively low. Our previous retrospective study involving 36 gastric cancer patients suggested that RT-PCR of peritoneal lavage fluid may be useful in predicting peritoneal recurrence in patients with negative cytology (CY0) [11]. This prospective study involving over 100 patients undergoing curative surgery for cT2-4 gastric cancer revealed that PCR results were a significant and independent prognostic factor of RFS. Indeed, 25 % of PCR-positive patients experienced peritoneal recurrence, compared with only 2 % of PCR-

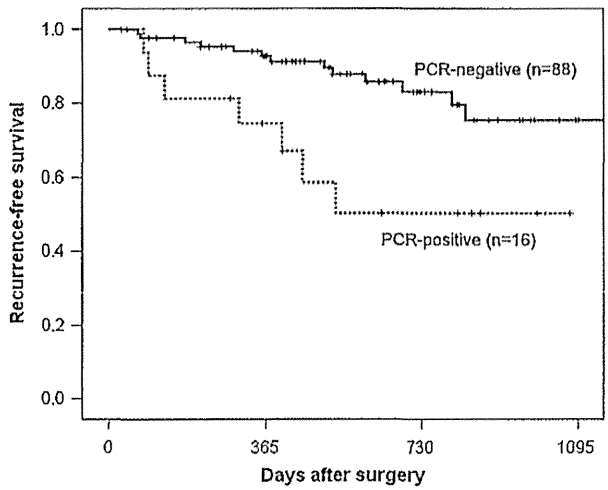

Fig. 1 Recurrence-free survival of PCR-positive patients ($n = 16$) versus PCR-negative patients ($n = 88$). *PCR* polymerase chain reaction

Table 3 Sites of tumor recurrence

Site	PCR-positive ($n = 16$)	PCR-negative ($n = 88$)	p value*
Peritoneum	4 (25 %)	2 (2 %)	<0.001
Liver	2 (13 %)	8 (9 %)	0.67
Lymph nodes	1 (6 %)	2 (2 %)	0.38
Others	1 (6 %)	2 (2 %)	0.38

Data are presented as n (%). Both groups had one duplicate site of recurrence

PCR polymerase chain reaction

* χ^2 test

negative patients. Therefore, this study demonstrated the clinical usefulness of PCR of peritoneal lavage fluid.

The RT-PCR technique has become popular as a highly sensitive method for detecting cancer cells. CEA is the most common tumor marker, and has been reported to be a reliable target for the detection of isolated tumor cells [10, 15, 16]. Ito et al. [17] reported that survival in patients with positive CEA mRNA was significantly worse than in patients with negative CEA mRNA in their retrospective study. However, another study reported that CEA frequently resulted in false positives [18], because the expression level of CEA mRNA was heterogeneous in gastric tumors [16] and there is weak expression in non-cancerous cells, such as mesothelial cells [10]. Thus, in order to more precisely predict recurrence, it may be necessary to use multiple markers [16, 19, 20]. Since CK20 is usually expressed in adenocarcinomas, it is one of the candidates for improving the sensitivity of gastric cancer cell detection [21]. Tamura et al. [22] reported that detection of CEA and CK20 mRNA by RT-PCR with peritoneal lavage fluid was useful for identifying patients at high risk of peritoneal recurrence. However, their study included many patients with incurable factors such as P1, CY1, or M1. Such incurable patients should be treated with intensive chemotherapy, regardless of PCR results. Therefore, we only included patients without incurable factors in this study in order to identify patients who need intensive adjuvant chemotherapy.

Although we successfully demonstrated associations between peritoneal recurrence and CEA and CK20 PCR results in our preliminary reports, one limitation of this study was the relatively small number of patients and the short follow-up period. Although our study could not evaluate overall survival due to the low number of events, RFS could be evaluated. We think a multicenter study with a larger cohort and a longer follow-up period is required to evaluate the generalizability of this method.

In conclusion, our prospective study confirmed our preliminary findings that CEA and CK20 RT-PCR results could predict peritoneal recurrence after curative surgery. This sensitive system can be used to identify high-risk patients who require intensive adjuvant chemotherapy and close follow-up. When this system is used as a preoperative screening tool, with peritoneal lavage fluid collected by staging laparoscopy, we can also do neoadjuvant chemotherapy for PCR-positive patients before surgery.

Table 4 Univariate and multivariate Cox analysis of recurrence-free survivals

	Univariate		Multivariate	
	HR(95 % CI)	p value	HR (95 % CI)	p value
Age (≤65 years)	1.06 (0.43–2.59)	0.90	1.07 (0.37–3.11)	0.90
Sex (male)	1.72 (0.57–5.13)	0.33	3.02 (0.89–10.3)	0.077
Histology (undifferentiated)	1.95 (0.75–5.08)	0.17	1.95 (0.71–5.38)	0.20
Neoadjuvant chemotherapy (yes)	2.27 (0.87–5.94)	0.094	2.02 (0.72–5.63)	0.18
pT (T3–4)	3.51 (1.02–12.0)	0.046	2.17 (0.59–8.01)	0.24
pN (N1–3)	1.88 (0.78–4.55)	0.16	1.44 (0.55–3.73)	0.46
PCR (positive)	3.28 (1.31–8.24)	0.011	3.49 (1.14–10.7)	0.029

CI confidence interval, *HR* hazard ratio, *PCR* polymerase chain reaction

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Conflicts of interest The authors have no conflicts of interest to declare.

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Effect of ghrelin on autonomic activity in healthy volunteers

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ABSTRACT

Ghrelin is a novel growth hormone (GH)-releasing peptide originally isolated from the stomach. Recently, we have shown that ghrelin suppresses cardiac sympathetic activity and prevents early left ventricular remodeling in rats with myocardial infarction. In the present study, we evaluated the effect of ghrelin on autonomic nerve activity in healthy human subjects. An intravenous bolus of human synthetic ghrelin (10 μg/kg) was administered to 10 healthy men (mean age, 33 years). Holter monitoring assessment was performed before and during 2 h after the ghrelin therapy. The standard deviation of normal RR intervals (SDNN), square root of the mean of the sum of the squares of differences between adjacent RR intervals (rMSSD), high-frequency power (HF), and low-frequency power (LF) were analyzed. Blood samples were also obtained before and after the therapy. A single administration of ghrelin decreased both heart rate and blood pressure. Interestingly, ghrelin significantly decreased the LF and LF/HF ratio of heart rate variability and increased the SDNN, rMSSD, and HF. Ghrelin also elicited a marked increase in circulating GH, but not insulin-like growth factor-1. These data suggest that ghrelin might suppress cardiac sympathetic nerve activity and stimulate cardiac parasympathetic nerve activity.

Introduction

Ghrelin is a growth hormone (GH)-releasing peptide originally isolated from the stomach that serves as an endogenous ligand for the GH secretagogue receptor (GHS-R) [6]. Beside its potent GH-releasing activity, ghrelin induce a positive energy balance by stimulating food intake and inducing adiposity through growth hormone independent mechanisms [15]. Ghrelin also has several cardiovascular effects, as suggested by the presence of the receptor on blood vessels and in the cardiac ventricles [5]. We previously demonstrated that ghrelin suppresses cardiac sympathetic nervous activity and prevents early left ventricular remodeling in rats after myocardial infarction [19]; administration of ghrelin immediately after myocardial infarction prevents the early increases in cardiac sympathetic nerve activity, resulting in improved survival [17]. Furthermore, we have recently reported that ghrelin administration reduced ventricular arrhythmias concomitant with prevention of the loss of connexin 43 during acute myocardial ischemia at least in part by modulation of cardiac autonomic nerve activity [20].

In human, Nagaya et al. investigated the effects of intravenous bolus ghrelin administration to patients with congestive heart failure for 3 weeks and found that repeated ghrelin injection improves left ventricular (LV) function in these patients [14]. In fact, ghrelin increased the LV ejection fraction, which was associated with an increase in LV mass and a decrease in LV end-systolic volume. Although these results suggest that ghrelin has therapeutic potential in cardiac dysfunction, how ghrelin exerts its beneficial action on cardiac function remained to be elucidated.

Abbreviations: GH, growth hormone; GHS-R, growth hormone secretagogue receptor; LV, left ventricular; ECG, electrocardiography; SDNN, standard deviation of normal RR intervals; rMSSD, square root of the mean of the sum of the squares of differences between adjacent R-R intervals; CVRR, coefficient of variance of R-R interval; HF, high-frequency power; LF, low-frequency power; IGF-1, insulin like growth factor-1.

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At present, the effect of ghrelin on human autonomic activity remains unknown. Therefore, in the present study, we evaluated the effect of ghrelin on cardiac autonomic activity in healthy human subjects.

Methods

Preparation of human ghrelin

Human synthetic ghrelin was obtained from the Peptide Institute Inc. (Osaka, Japan). This peptide is not commercially available. Ghrelin was dissolved in distilled water with 4% D-mannitol and sterilized by passage through a 0.22-μm filter (Millipore Corp., Bedford, MA). Ghrelin was stored in 2-mL volumes, each containing 700 μg ghrelin. The chemical nature and content of the human ghrelin in vials were verified by high-performance liquid chromatography and radioimmunoassay. All vials were stored frozen at −80 °C from the time of dispensing until the time of preparation for administration.

Study subjects

This study included 16 healthy men (mean age 33 years) who had no history of any mental or medical disorders. All the subjects gave written informed consent to participate to the study, which had been approved by the Institutional Review Board of The University of Tokushima Clinical Research Center. The study protocol was in accordance with the Declaration of Helsinki Principles. The subjects randomly received either ghrelin (ghrelin group, $n = 10$) or placebo (placebo group, $n = 6$).

Study protocol

After overnight fasting, the study was begun in the morning at 0830–0930. An intravenous bolus of 0.9% saline, with or without human synthetic ghrelin (10 μg/kg), was administered. Holter monitoring assessment was performed before and during 2 h after the ghrelin therapy using two-channel Holter electrocardiography (ECG) recorder (SM-50; Fukuda Denshi Co., Ltd., Tokyo, Japan). The standard deviation of normal R-R intervals (SDNN), square root of the mean of the sum of the squares of differences between adjacent RR intervals (rMSSD), coefficient of variance of R-R interval (CVRR), high-frequency power (HF, 0.15–0.40 Hz), and low-frequency power (LF, 0.04–0.15 Hz) were analyzed using a time-series data-analysis software (Fukuda Denshi Co., Ltd., Tokyo, Japan). Blood samples were also obtained before and after the therapy (at 30 min and at 120 min). The blood was immediately transferred into a chilled glass tube containing disodium ethylenediaminetetraacetic acid (1 g/L) and aprotinin (500 U/mL) and centrifuged immediately at 4 C. The plasma samples were frozen and stored at −80 C until the measurements. Serum growth hormone (GH) and insulin like growth factor-1 (IGF-1) concentrations were measured by enzyme immunoassay kits (R&D Systems, Inc. Quantikine Human IGF-I Immunoassay, Quantikine Human Growth Hormone Immunoassay).

Statistical analysis

Data are expressed as the mean ± S.D. Differences among the groups were evaluated by two-way analysis of variance for repeated measurements. When a statistical difference was detected by analysis of variance, the Bonferroni method of adjusting for multiple pairwise comparisons was used. A value of $P < 0.05$ was considered statistically significant.

Results

Sixteen healthy men were randomly divided into 2 groups who receive ghrelin or placebo. There were no significant difference in age (33 ± 5 vs 34 ± 8 years). A single administration of ghrelin decreased both heart rate and blood pressure (Fig. 1). The hypotensive effect of ghrelin lasted for 30 min and the bradycardia effect of ghrelin lasted for 60 min after the ghrelin infusion. On the other hand, the administration of placebo did not change heart rate and blood pressure (Fig. 1). Interestingly, the administration of ghrelin increased the HF, reflecting the parasympathetic activity. Ghrelin also decreased LF and LF/HF ratio, reflecting the sympathetic activity while placebo did not (Fig. 2). The effect of ghrelin on HF lasted longer than 60 min and the effect of ghrelin on LF/HF ratio lasted longer than 120 min after the infusion of ghrelin (Fig. 2). In addition, ghrelin increased the SDNN, rMSSD, and CVRR, which effect lasted for 60 min after the ghrelin administration (Fig. 3). These effects of ghrelin on the autonomic parameters were not found in placebo group. Furthermore, ghrelin also elicited a marked increase in circulating GH 30 min after the infusion (Fig. 4) whereas placebo did not. On the other hand, no significant change in serum IGF-1 level was observed throughout the study period after the infusion of ghrelin (Fig. 4). In this study, we saw some side effects of ghrelin such as perspiration (8/10), drowsiness (5/10), hunger (3/10), general fatigue (1/10), and mild diarrhea (1/10). However, these side effects were transient and not severe.

Discussion

The main novel findings of the present study are that ghrelin significantly decreased the LF and LF/HF ratio of heart rate variability and increased the SDNN, rMSSD, CVRR, and HF in human subjects. Although some previous studies showed that ghrelin decreased blood pressure or heart rate in human subjects [7,13], there were no studies investigating the effect of ghrelin on the autonomic activities in human subjects. Then, the present study is the first research to demonstrate that ghrelin might suppress cardiac sympathetic

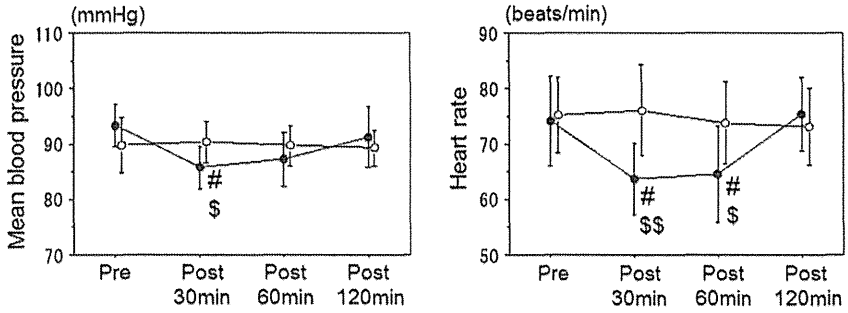

Fig. 1. Changes in mean arterial pressure and heart rate during infusion of ghrelin (closed circle) or placebo (open circle). Intravenous infusion of ghrelin but not placebo significantly decreased both mean blood pressure and heart rate. $^{\#}P < 0.05$ vs pre-infusion; $^{\$\$}P < 0.01$/$^{\$}P < 0.05$ vs respective placebo group.

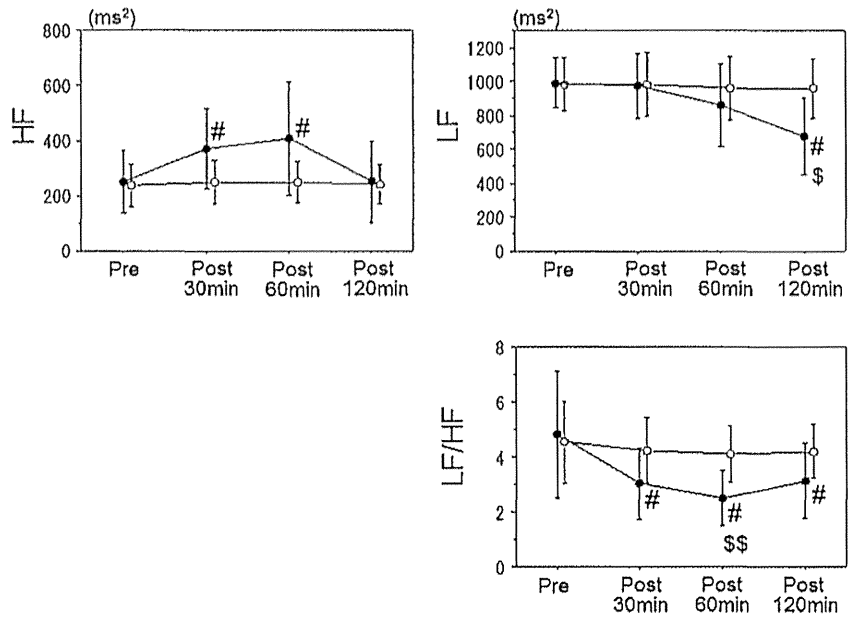

Fig. 2. Changes in power spectral analysis of heart rate variability during infusion of ghrelin (closed circle) or placebo (open circle). The administration of ghrelin increased the high-frequency power (HF) and decreased low-frequency power (LF) and LF/HF ratio. The effect of ghrelin on HF lasted longer than 60 min and the effect of ghrelin on LF/HF ratio lasted longer than 120 min after the infusion of ghrelin. On the other hand, placebo did not change these parameters at any time points. $^{\#}P<0.05$ vs pre-infusion; $^{\$\$}P<0.01/^{\$}P<0.05$ vs respective placebo group.

nerve activity and stimulate cardiac parasympathetic nerve activity in human subjects.

These results were compatible with our previous studies in animal model showing that ghrelin suppressed cardiac sympathetic nervous activity and increased parasympathetic nervous activity in rats after myocardial ischemia [17,19,20]. Since these effects of ghrelin led to the prevention of left ventricular remodeling, reduced ventricular arrhythmia, and improved survival in the animal model of myocardial ischemia, the effect of ghrelin on the autonomic activity in human subjects may be applicable to the anti-arrhythmic and anti-remodeling therapy in patients with cardiovascular disease.

In fact, aberrant sympathetic activation has been implicated as part of the sequelae consistent with the development of cardiovascular disease [11], and β-blockers have been efficacious and beneficial in the treatment of various cardiovascular disease states, including angina, heart failure, myocardial infarction, and ventricular arrhythmias [3]. Vagal nerve stimulation also has been reported to prevent ventricular fibrillation in dogs [21] and improve the long-term survival of rats with chronic heart failure after

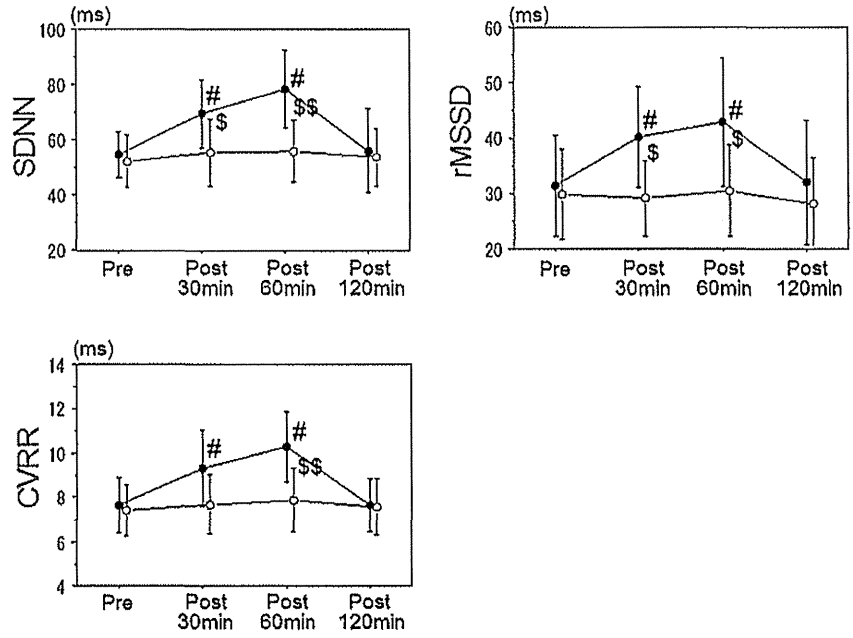

Fig. 3. Changes in time domain analysis of heart rate variability during infusion of ghrelin (closed circle) or placebo (open circle). Ghrelin increased the standard deviation of normal RR intervals (SDNN), square root of the mean of the sum of the squares of differences between adjacent RR intervals (rMSSD), and coefficient of variance of R-R interval (CVRR), which effect lasted for 60 min after the ghrelin administration. These effects of ghrelin on the autonomic parameters were not found in placebo group. $^{\#}P<0.05$ vs pre-infusion; $^{\$\$}P<0.01/^{\$}P<0.05$ vs respective placebo group.

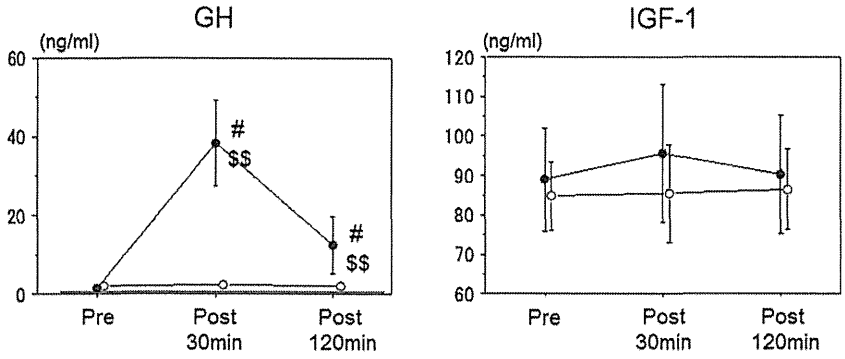

Fig. 4. Changes in serum levels of growth hormone (GH) and insulin like growth factor-1 (IGF-1). Ghrelin (closed circle) elicited a marked increase in circulating GH 30 min after the infusion whereas placebo (open circle) did not. On the other hand, no significant change in serum IGF-1 level was observed throughout the study period after the infusion of ghrelin. $^{\#}P<0.05$ vs pre-infusion; $^{\$\$}P<0.01/^{\$}P<0.05$ vs respective placebo group.

myocardial infarction [9]. The exact mechanisms by which vagal nerve stimulation alters the outcomes of myocardial infarction, however, have not been elucidated. Antiarrhythmogenic effects by vagal nerve stimulation might be due, at least in part, to the prevention of the loss of phosphorylated connexin 43, which might improve electrical instability during acute myocardial ischemia [20].

Several studies have demonstrated a suppressive effect of ghrelin on cardiac sympathetic activity in animal models [10,12,17,19,20] and one recent study has shown the activated cardiac vagal nerve by centrally administered ghrelin in rabbits [18]. However, the mechanism by which peripheral administration of ghrelin modulates autonomic nerve activity is not well understood. Previous studies demonstrated that microinjection of ghrelin into the nucleus of the solitary tract elicited dose-related decreases in heart rate and mean arterial pressure; in the central nervous system, the GHS-R is predominantly distributed in the nucleus of the solitary tract [10]. In addition, ghrelin produced in the stomach stimulates gastric vagal afferent nerves to influence neuronal activity in the nucleus of the solitary tract [2], resulting in increases in feeding behavior. In our previous study, the effects of ghrelin on sympathetic and parasympathetic activities and ventricular arrhythmias were abrogated by co-administration of atropine or by blockade of vagal afferents in rats with myocardial ischemia [20]. These data suggest that peripheral ghrelin might act on cardiac vagal afferent nerves to send signals to the vasomotor center of the medulla through the nucleus of the solitary tract, resulting in increases in parasympathetic activity and decreases in sympathetic activity.

In the present study, the effect of ghrelin on the parasympathetic parameters including HF peaked at 60 min and decreased back to basal level by 120 min while the effect of ghrelin on LF/HF ratio or LF lasted longer than 120 min after the infusion of ghrelin. On the other hand, heart rate bottomed at 30–60 min and blood pressure bottomed at 30 min and increased thereafter, which indicate the reverse change of the parasympathetic parameters. From these results, the effect of ghrelin on hemodynamic changes might be more attributable to an increase in the parasympathetic activity rather than a decrease in the sympathetic activity.

The hypotensive effects of ghrelin in the present study might be mediated in part by its direct action. GHS-R mRNA was detectable in the heart and blood vessels in rats and humans [4] and ghrelin was shown to increase forearm blood flow in a dose-dependent manner [16]. In addition, ghrelin was reported to be a potent physiological antagonist of endothelin-1 [22]. These results suggest that ghrelin has direct vasodilatory effects. However, the hypotensive effects of ghrelin might be mainly attributed to the suppression of the sympathetic nerve activity because of the concomitant reduction of heart rate but not compensatory response to lowering blood pressure (increase in heart rate) in the present study. In agreement with our study, Krapalis et al. demonstrated that ghrelin infusion induced the suppression of central nervous sympathetic activity combined with increased baroreflex sensitivity [7]. They also suggest the existence of an immediate phase of direct peripheral vasodilation with basoreflex-mediated transient sympathoactivation by ghrelin infusion.

On the other hand, ghrelin has potent GH releasing activity [6], which might influence the cardiac autonomic activity. In fact, in the present study, ghrelin elicited a marked increase in circulating GH. However, in a previous study, mean blood pressure and heart rate were reported to be unaffected by GH administration in male volunteers [1]. In addition, in patients with GH deficiency, reduced but not increased sympathetic tone was found [8]. These previous data support our speculation that the effects of ghrelin on cardiac autonomic activity might be independent of the effects of GH. This speculation is supported by our findings in the present study that serum level of IGF-1, the mediator of GH, did not change significantly after the administration of ghrelin.

To our best knowledge, symptomatic side effects of ghrelin were not reported in any previous reports. In the present study, we saw some side effects of ghrelin such as perspiration, drowsiness, hunger, general fatigue, and mild diarrhea. These symptoms might be associated with the increased parasympathetic activity by ghrelin. However, these side effects were transient and not severe. Therefore, they do not disturb clinical application of ghrelin.

Some limitations are present in this study. First, our sample size was small. Although we believe that the changes of heart rate variability are too distinct to be accidental, further large studies are needed to confirm our results. Second, it remains unknown whether the results of the present study using healthy volunteers can be directly applicable for patients with cardiovascular disease. Further clinical studies are needed to investigate the potentially beneficial effects of ghrelin on autonomic activity under pathological conditions associated with permanently increased sympathetic and decreased parasympathetic activities, like chronic heart failure.

Conclusion

These data suggest that ghrelin might suppress cardiac sympathetic nerve activity and stimulate cardiac parasympathetic nerve activity.

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Pulmonary, gastrointestinal and urogenital pharmacology

Ghrelin relieves cancer cachexia associated with the development of lung *adenocarcinoma in mice*

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ABSTRACT

Cancer cachexia is a multifactorial, critical illness syndrome characterized by an ongoing loss of skeletal muscle and adipose tissue. The reductions in body weight and skeletal muscle mass are important prognostic indicators for cancer patients that are refractory to current therapies. Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, is produced in the stomach, stimulates food intake and growth hormone secretion, suppresses inflammation, and prevents muscle catabolism. We investigated the pharmacological potential of ghrelin in the treatment of cancer cachexia by using urethane-treated, bronchioalveolar epithelium-specific *Pten*-deficient mice that developed lung adenocarcinomas. Ghrelin or phosphate-buffered saline was given to mice daily for four weeks beginning at five months after urethane injection, which corresponded to the time point of lung adenocarcinoma formation. Ghrelin inhibited the inductions of C-reactive protein, tumor necrosis factor-α, interleukin-1β, and interleukin-6, mitigated the reduction of food intake and fat mass, and consequently ameliorated body weight loss in the mouse model of lung adenocarcinoma. We also demonstrated that skeletal muscle mass and muscle contraction force in both fast-twitch muscle and slow-twitch muscle were retained in ghrelin-treated mice in conjunction with an upregulation of local insulin-like growth factor 1/Akt signaling. In addition, ghrelin administration reduced the expressions of phosphorylated-p38 mitogen-activated protein kinase, phosphorylated-nuclear factor-kappa B, Forkhead box protein O1, muscle RING-finger protein-1, and F-Box protein 32 in the lysates of skeletal muscle in the tumor-bearing state. Our results indicate that ghrelin administration exerts a protective effect against cancer cachexia by ameliorating skeletal muscle wasting and regulating systemic inflammation.

1. Introduction

Cancer cachexia affects up to 80% of patients with advanced cancers and accounts for nearly 30% of cancer-related deaths (Acharyya et al., 2005; Fearon, 2008). A key feature of cachexia is significant reduction in body weight resulting predominantly from progressive depletion of skeletal muscle mass (Fearon et al., 2012). Muscle atrophy leads to general muscle weakness, impairment of activity of daily life, and eventually death through respiratory failure. The mechanisms of cancer cachexia are multifactorial, and cannot be fully reversed by nutritional support alone. Various hormones, proinflammatory cytokines, and tumor-derived factors have been shown to influence muscle protein synthesis/degradation balance through several major intracellular signal-transduction systems, including the insulin-like growth factor 1 (IGF1)/insulin receptor substrate 1/Akt pathway and the Forkhead box protein O1 (FoxO1)/muscle RING-finger protein-1 (MuRF1)/F-Box protein-32 (Atrogin1) pathway (Zhou et al., 2010). Ideal interventions against cancer cachexia should exert their effects both upstream (antagonizing key mediators of systemic inflammation) and downstream (blocking catabolic pathways or stimulating anabolic pathways in skeletal muscle) (Fearon et al., 2012). Despite recent advances in understanding the pathological mechanisms of cancer cachexia, few therapeutic options are currently available. In addition, there is no ideal rodent cancer cachexia model for replicating the condition in humans.

Ghrelin is a 28-amino-acid peptide initially isolated from the human and rat stomach as an endogenous ligand for the growth hormone secretagogue (GHS)-receptor (Kojima et al., 1999). While ghrelin has a potent orexigenic effect independently of growth hormone (GH) secretion, ghrelin is also known to have multifaceted effects on energy metabolism, including decrease of energy expenditure (Yasuda et al., 2003), stimulation of adiposity (Tschop et al., 2000),

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and prevention of muscle catabolism (Koshinaka et al., 2011; Sugiyama et al., 2012). In addition, ghrelin inhibits the expression of proinflammatory anorectic cytokines in human monocytes and T cells (Dixit et al., 2004). These observations suggest that ghrelin might improve cachectic conditions. Previous studies using tumor implantation models have reported that ghrelin administration resulted in significant increases in food intake and body weight (DeBoer et al., 2007; Hanada et al., 2003). However, the therapeutic effect and molecular mechanisms of ghrelin treatment against cancer cachexia, including muscle wasting, remain unknown, especially in the context of a model of cancer development.

We previously reported that almost all mice with a bronchioalveolar epithelium-specific null mutation of *Pten*, a tumor suppressor gene mutated in many human cancers, including lung adenocarcinoma (Marsit et al., 2005; Tang et al., 2006), spontaneously developed lung adenocarcinomas (Yanagi et al., 2007). This animal model of lung adenocarcinoma is highly reproducible and accelerates cancer formation over a relatively short time course by administration of urethane, a well-known initiator of lung carcinomas (Malkinson and Beer, 1983). In this study, we showed that there were marked upregulations of markers of muscle atrophy and proinflammatory cytokines as well as significant reduction in body weight and loss of skeletal muscle mass in urethane-treated, bronchioalveolar epithelium-specific *Pten*-deficient mice. To investigate the efficacy of ghrelin treatment against the syndrome of cancer cachexia in the present study, we used this mouse model of lung adenocarcinoma.

2. Materials and methods

2.1. Animals and administration of doxycycline

We generated bronchioalveolar epithelial cell-specific *Pten*-deficient mice as previously described (Yanagi et al., 2007). Briefly, $Pten^{flox/flox}$ mice (129Ola × C57BL6/J F6) generated as described in an earlier study (Suzuki et al., 2001) were mated to *SP-C-rtTA* mice (Perl et al., 2002) that express the reverse tetracycline-controlled transactivator (rtTA) gene (which can be activated by doxycycline) under the control of the 3.7-kb human surfactant protein-C (SP-C) promoter (Perl et al., 2002). The human SP-C promoter selectively directs expression of the transgene to the developing and the mature pulmonary epithelium of the primordial lung buds, and to bronchiolar epithelial cells and type II alveolar epithelial cells after birth (Wert et al., 1993). Triple transgenic mice were generated by mating *SP-C-rtTA/*$Pten^{flox/flox}$ mice with $(tetO)_7$*-Cre* mice (Sauer, 1998) that express Cre recombinase under the control of rtTA. Unless otherwise noted, offspring carrying *SP-C-rtTA/*$(tetO)_7$*-Cre/*$Pten^{flox/flox}$ ($SOPten^{f/f}$) and their littermates carrying $(tetO)_7$*-Cre/*$Pten^{flox/flox}$ ($OPten^{f/f}$) were used in the experiments as homozygous mutant and wild-type mice, respectively. To induce expression of the *Cre* transgene in bronchioalveolar epithelial cells postnatally, 3-week-old mice (P21) were administered doxycycline (Sigma-Aldrich Japan, Tokyo) in their drinking water (1 mg/ml) for 1 week. After administration of doxycycline, $SOPten^{f/f}$ mice, which were fed doxycycline from P21 to P27, were designated as $SOPten^{\Delta/\Delta}$ mice. Mice were housed in a temperature-controlled room (23 ± 1 °C) on a 12-h light (08:00–20:00 h)/12-h dark cycle and fed a standard laboratory chow with ad libitum access to food. All experimental procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care and were approved by the Ethics Committee on Animal Experimentation of the University of Miyazaki.

2.2. Lung carcinogenesis

The 8-week-old $SOPten^{\Delta/\Delta}$ and $OPten^{f/f}$ mice were intraperitoneally administered 1 g/kg of urethane (Sigma-Aldrich Japan, Tokyo) which was dissolved in 200 μl phosphate buffered saline (PBS). For the survival study ($SOPten^{\Delta/\Delta}$ mice, $n=28$; and $OPten^{f/f}$ mice, $n=25$), the measurements of body weights ($SOPten^{\Delta/\Delta}$ mice, $n=15$; and $OPten^{f/f}$ mice, $n=24$), and the histological assay ($SOPten^{\Delta/\Delta}$ mice, $n=10$; and $OPten^{f/f}$ mice, $n=10$), we used male 8-week-old $SOPten^{\Delta/\Delta}$ and $OPten^{f/f}$ mice, and monitored them for 30 weeks.

2.3. Administration of ghrelin

At the age of 38 weeks, the urethane-injected $SOPten^{\Delta/\Delta}$ mice were intraperitoneally administered 10 nmol/mouse of human ghrelin (Asubio Pharma Co., Kobe, Japan) dissolved in 200 μl PBS or PBS alone at 07:00 and 19:00 over a 28-day interval. The 38-week-old, urethane-injected $OPten^{f/f}$ mice were given PBS intraperitoneally during the 28-day study interval. On 28 days after administration of ghrelin or PBS, mice were anesthetized by intraperitoneal injection of pentobarbital sodium and sacrificed. We then designated the ghrelin-treated, urethane-injected $SOPten^{\Delta/\Delta}$ mice as the $SOPten^{\Delta/\Delta}$/ghrelin group, the PBS-treated, urethane-injected $SOPten^{\Delta/\Delta}$ mice as the $SOPten^{\Delta/\Delta}$/PBS group, and the PBS-treated, urethane-injected $OPten^{f/f}$ mice as the $OPten^{f/f}$/PBS group. Body weights and food intake were measured daily from 1 day before ghrelin or PBS administration to day 28. We used the animals from the $SOPten^{\Delta/\Delta}$/ghrelin group, $SOPten^{\Delta/\Delta}$/PBS group and $OPten^{f/f}$/PBS group for the measurements of body weights and food intake ($n=12$–13 per group). For the measurements of skeletal muscle mass (gastrocnemius: $n=12$–23 per group; tibialis anterior muscle: $n=12$–13 per group; and soleus muscle: $n=12$–13 per group), the measurements of muscle contraction force ($n=6$–8 per group), the measurements of intra-abdominal fat mass ($n=12$–23 per group), the enzyme-linked immunosorbent assay (ELISA) ($n=8$–15 per group), the quantitative real-time PCR ($n=5$–7 per group), the immunohistochemical analyses ($n=5$ per group), and the Western blotting ($n=3$ per group), we used three

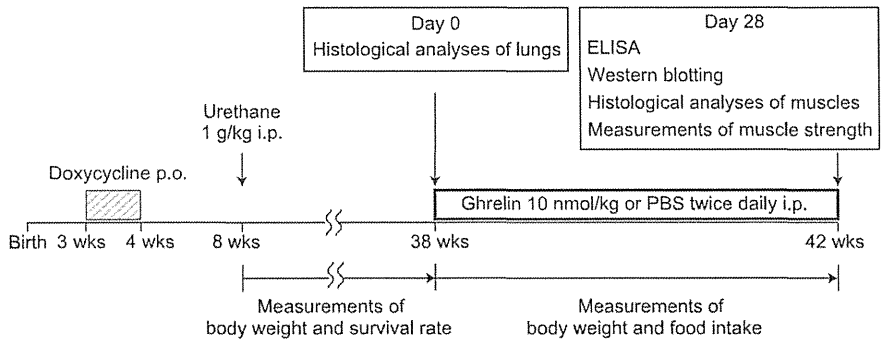

Fig. 1. Timeline of doxycycline, urethane, and ghrelin treatment in $OPten^{f/f}$ and $SOPten^{\Delta/\Delta}$ mice. PBS, phosphate buffered saline; ELSIA, enzyme-linked immunosorbent assay.

additional groups and examined the animals on day 28. The experimental protocol of the study is outlined in Fig. 1.

2.4. Measurement of plasma ghrelin levels

The 10-week-old *OPten*$^{f/f}$ mice were intraperitoneally administered 1 or 10 nmol/mouse of human ghrelin (Asubio Pharma Co., Kobe, Japan) dissolved in 200 μl PBS or PBS alone. At 2, 3, or 12 h after the ghrelin or PBS injection, blood was taken from the mice by heart puncture and collected in a tube which contained aprotinin and ethylenediaminetetraacetic acid (Wako, Osaka, Japan). The contents were mixed well and immediately centrifuged at 4 °C. After plasma collection, a 1/10 volume of 1 mol/L HCl was added. The prepared plasma was stored at −80 °C until the measurement of ghrelin. The plasma ghrelin assay is a two-site immunoenzymometric assay requiring 100 μl of plasma sample, which is performed automatically by an AIA-600II immunoassay analyzer (Tosoh Corp., Tokyo, Japan).

2.5. Measurement of plasma cytokine levels, C-reactive protein, and IGF1

An ELISA was run using the plasma samples to measure the concentrations of C-reactive protein (Life Diagnostics Inc., West Chester, NY), interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and IGF1 by using the commercially available ELISA kits specifically designed for each protein (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.6. Histological analyses

The lungs and gastrocnemius muscles were fixed in 10% buffered formalin solution and embedded in optimum cutting temperature compound (Sakura Finetek Japan, Tokyo). Lung sections (4 μm thickness) and muscles (6 μm thickness) were mounted on slides for hematoxylin-eosin (HE) staining or immunostaining with an antibody recognizing laminin (Sigma-Aldrich Japan, Tokyo). For each muscle section, the whole muscle cross section was analyzed to calculate the average fiber size (cross-section area) by using the program Image J 1.46 (freeware developed by Dr. W. Rasband at the Research Services Branch, National Institute of Mental Health, and available at http://rsb.info.nih.gov/ij). The cross section areas of muscle sections from 5 mice per group were quantified by using the Image J software. The mean of the cross section area was calculated based on measurements of 229–370 myofibers per mouse.

2.7. Measurements of the contractile force of skeletal muscles

For measurements of the contractile force of skeletal muscles in the *SOPten*$^{\Delta/\Delta}$/ghrelin group, *SOPten*$^{\Delta/\Delta}$/PBS group and *OPten*$^{f/f}$/PBS group, mice were anesthetized at day 28 of ghrelin or PBS treatment. After exteriorization of the right soleus muscle or right tibialis anterior muscle and the right sciatic nerve, the Achilles tendon or tendon of the tibialis anterior was cut and connected to a muscle force-measuring device, the MLTF500/ST Teaching force transducer (ADInstruments Japan, Nagoya, Japan). For the measurements of the twitching contractile force or tetanic contractile force, the dominant nerve of muscles was electrically stimulated with 5 mA/1 Hz (duration: 10 s; pulse width: 0.1 ms) or 5 mA/75 Hz (duration: 5 s; pulse width: 0.1 ms), respectively, by an FE180 Stimulus isolator (ADInstruments Japan). The maximal contractile forces under fast-twitching stimulation or tetanic stimulation were analyzed by a ML846 PowerLab 4/26 system (ADInstruments Japan).

2.8. Extraction of mRNA and quantitative real-time PCR

We measured the mRNA expressions of atrogin-1, MuRF-1, and IGF1 in the gastrocnemius muscles of the *SOPten*$^{\Delta/\Delta}$/ghrelin group, *SOPten*$^{\Delta/\Delta}$/PBS group and *OPten*$^{f/f}$/PBS group at day 28 of ghrelin or PBS treatment. The mRNA was extracted from the whole gastrocnemius muscle using a Ribopure™ Kit (Life Technologies Japan Inc., Tokyo, Japan). First-strand cDNA was generated by reverse transcription using a High Capacity RNA-to-cDNA Kit (Life Technologies Japan Inc.). Quantitative real-time PCR was performed using Taqman Fast Universal PCR Master Mix (Life Technologies Japan Inc.) and a Thermal Cycler Dice Real Time System II (Takara Bio Inc., Tokyo, Japan). The levels of mRNA were determined by using cataloged primers (Applied Biosystems, Foster City, CA) for mice (Atrogin1: Mm00499523_m1; MuRF1: Mm01185221_m1; and IGF1: Mm00439560_m1). Expression of these genes was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (Gapdh Mm99999915_g1), and the results were expressed as relative fold differences.

2.9. Western blotting

Western blot analysis on whole gastrocnemius muscle was performed as described previously (Kristensen et al., 2014; Tsubouchi et al., 2014). Briefly, the gastrocnemius muscles were homogenized using a Tissuelyser II (Qiagen, Hilden, Germany). The muscle homogenates were centrifuged at 15,000 rpm for 15 min and the supernatants were stored at −70 °C until used. Total protein contents in supernatants were determined using a Bradford assay. Equal amounts of proteins were fractionated by 10% SDS-PAGE and transferred to Immobilon Transfer Membranes (Merck, Tokyo, Japan). We measured the expression levels of proteins in the lysates of gastrocnemius muscle by Western blotting, using antibodies recognizing the following proteins: β-actin (Sigma-Aldrich Japan), phosphorylated-Akt (Ser473), Akt, phosphorylated-FoxO1 (Ser256), FoxO1, phosphorylated-p38 mitogen-activated protein kinase (MAPK) (Thr180/Thy182), p38 MAPK, and phosphorylated-nuclear factor-kappa B (NF-κB) (Cell Signaling Technology Japan Inc., Tokyo, Japan). To quantify the protein expression, densitometry was performed on the lanes using Gene Tools software (Syngene, Frederick, MD).

2.10. Statistical analysis

All results were expressed as the mean ± S.E.M. Data were analyzed by a Tukey–Kramer honestly significant difference test. A Student's *t*-test analysis was used for the single-parameter comparisons between two groups. Statistical analyses were done with JMP 10 (SAS Institute Japan, Ltd., Tokyo, Japan) and values of $P < 0.05$ were considered statistically significant. Kaplan–Meier survival analysis was performed using Prism 5 (GraphPad Software, La Jolla, CA) and statistical significance was determined by a log-rank test.

3. Results

3.1. Urethane-induced lung carcinogenesis and loss of body weights in lung epithelium-specific Pten-deficient mice

The overall survival rate of *SOPten*$^{\Delta/\Delta}$ mice was significantly lower than that of *OPten*$^{f/f}$ mice 30 weeks after urethane injection (Fig. 2A). The body weights in *SOPten*$^{\Delta/\Delta}$ mice were significantly lower than those of *OPten*$^{f/f}$ mice at 30 weeks after injection (Fig. 2B). As shown in Fig. 2C, all of the *SOPten*$^{\Delta/\Delta}$ mice developed macroscopic lung tumors. Histological examination demonstrated that 9 of the 10 *SOPten*$^{\Delta/\Delta}$ mice developed lung adenocarcinomas

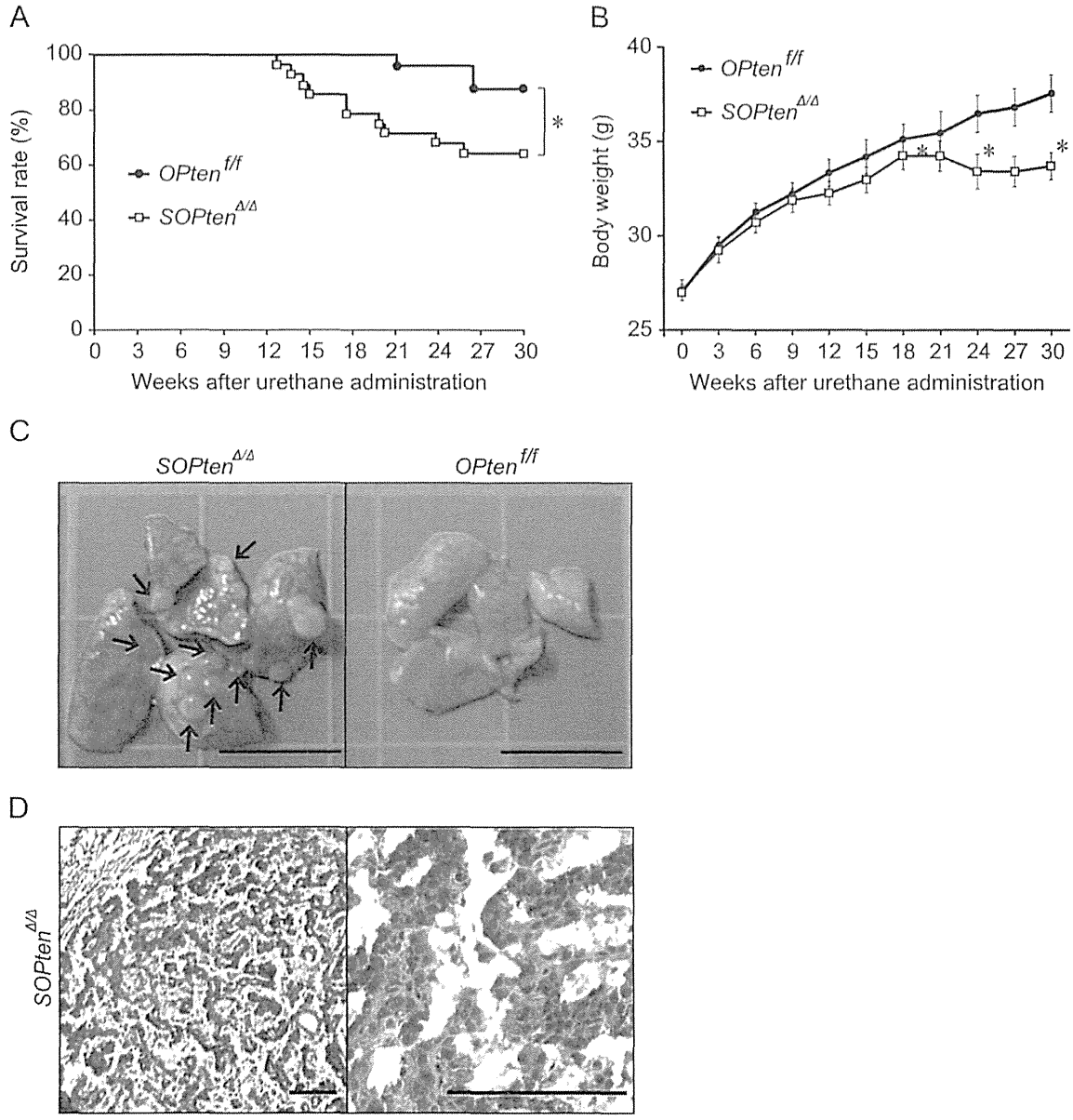

Fig. 2. Urethane-induced lung carcinogenesis and loss of body weights in lung epithelium-specific Pten-deficient mice. (A) Kaplan–Meier survival curves for the *SOPten*$^{\Delta/\Delta}$ mice ($n=28$) and *OPten*$^{f/f}$ mice ($n=25$) for 30 weeks after urethane administration. *$P<0.05$. (B) Alterations in body weights of *SOPten*$^{\Delta/\Delta}$ mice ($n=15$) and *OPten*$^{f/f}$ mice ($n=24$) over 30 weeks after administration of urethane. Data are shown as the mean ± S.E.M. *$P<0.05$. (C) Gross appearance of urethane-induced lung tumors (arrows) in *SOPten*$^{\Delta/\Delta}$ mice. The images shown are representative lung sections of 10 mice per group. Scale bars: 1 cm. (D) Histology of lung adenocarcinoma in the lungs of urethane-injected *SOPten*$^{\Delta/\Delta}$ mice, representative of adenocarcinomas observed in 9 of 10 of these mice. Scale bars: 100 μm.

(Fig. 2D), whereas all other tumors that formed in both *OPten*$^{f/f}$ and *SOPten*$^{\Delta/\Delta}$ lungs were lung adenomas.

3.2. The plasma ghrelin levels after ghrelin treatment

To determine the experimental dose and the administration interval of ghrelin, we measured the plasma ghrelin levels in 10-week-old *OPten*$^{f/f}$ mice at 2, 3, or 12 h after 1 or 10 nmol/mouse of ghrelin or PBS administration. At 12 h after ghrelin or PBS administration, the plasma ghrelin levels were comparable between the mice that were treated with 1 nmol/mouse of ghrelin and the mice that were treated with PBS (Fig. 3A). On the other hand, the plasma ghrelin levels of mice that were treated with 10 nmol/mouse of ghrelin were significantly higher than those in the PBS-treated mice or the mice treated with 1 nmol/mouse of ghrelin. In addition, the plasma ghrelin levels of mice that were administered 10 nmol/mouse of ghrelin decreased to approximately one tenth of the plasma ghrelin levels of mice at 2 h after the same dose of ghrelin treatment (Fig. 3B). Thus, we administered the 10 nmol/mouse of ghrelin or PBS alone at 12-h administration intervals in the following experiments.

3.3. Effects of ghrelin on body weight, food intake, and fat mass in urethane injected-SOPten$^{\Delta/\Delta}$ mice

To confirm the effects of ghrelin administration on cachectic conditions in urethane-injected *SOPten*$^{\Delta/\Delta}$ mice, we started daily administration of ghrelin or PBS at 30 weeks after urethane injection. *SOPten*$^{\Delta/\Delta}$/PBS mice showed significant reduction of body weight, food intake, and intra-abdominal fat mass compared to *OPten*$^{f/f}$/PBS mice (Fig. 4A–C). Ghrelin administration significantly attenuated the weight loss, reduction of food intake and loss of intra-abdominal fat mass in urethane-injected *SOPten*$^{\Delta/\Delta}$ mice.

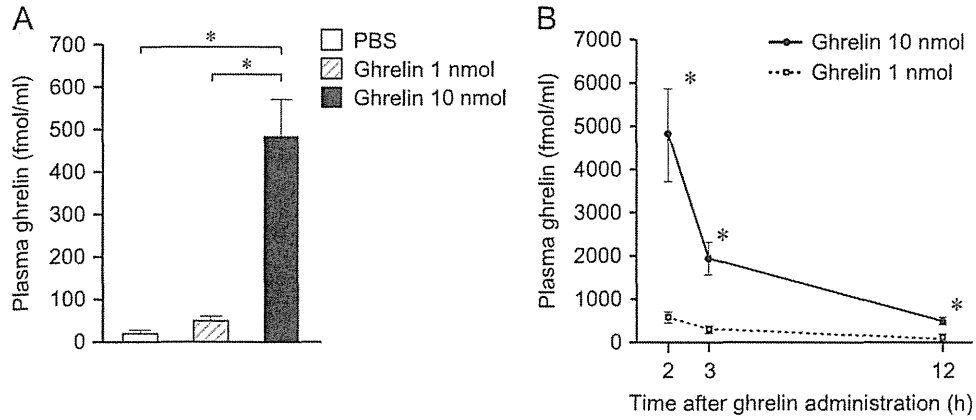

Fig. 3. The plasma ghrelin levels after ghrelin treatment. (A) The plasma ghrelin levels at 12 h after ghrelin administration (1 or 10 nmol/mouse) or phosphate buffered saline (PBS) are shown. Plasma ghrelin levels of mice that received ghrelin or PBS treatment were 53.0 ± 10.7, 489.4 ± 86.1, and 23.3 ± 3.9 fmol/ml, corresponding to 1 nmol/mouse ghrelin, 10 nmol/mouse ghrelin and PBS, respectively. Data are shown as the mean ± S.E.M. of 3 mice per group. $^*P < 0.01$. (B) The plasma ghrelin levels at 2, 3, and 12 h after ghrelin administration (1 or 10 nmol/mouse) are shown. Data are shown as the mean ± S.E.M. of 3 mice per group. $^*P < 0.05$.

3.4. *Effects of ghrelin on plasma levels of proinflammatory cytokines, C-reactive protein, and IGF1 in urethane injected-SOPten$^{\Delta/\Delta}$ mice*

The plasma levels of IL-1β, IL-6, TNF-α, and C-reactive protein in *SOPten$^{\Delta/\Delta}$*/PBS mice were significantly higher than those of *OPten$^{f/f}$*/PBS mice. Ghrelin administration significantly reduced the levels of these cytokines and C-reactive protein in urethane-injected *SOPten$^{\Delta/\Delta}$* mice (Fig. 5). Meanwhile, the plasma level of IGF1 was similar among the three groups.

3.5. *Effects of ghrelin on the skeletal muscle mass, contractile force of skeletal muscle and levels of catabolic factors in urethane-injected SOPten$^{\Delta/\Delta}$ mice*

We next explored whether ghrelin treatment mitigates the skeletal muscle wasting in urethane-injected *SOPten$^{\Delta/\Delta}$* mice. The gastrocnemius muscles of *SOPten$^{\Delta/\Delta}$*/PBS mice exhibited excessive shrinkage of muscle fibers and reduction of muscle weights compared with the *OPten$^{f/f}$*/PBS muscles (Fig. 6A–C). Ghrelin administration suppressed the reduction of muscle fiber size and loss of muscle weights, including those of the gastrocnemius muscle, soleus muscle and tibialis anterior muscle in urethane-injected *SOPten$^{\Delta/\Delta}$* mice (Fig. 6A–C). Ghrelin treatment also retained the muscle contraction force of both soleus muscle and tibialis anterior muscle (Fig. 6D and E). We next examined whether ghrelin administration affected the levels of catabolic factors in the skeletal muscles by measuring the mRNA levels of E3 ubiquitin ligases. We found that the mRNA levels of Atrogin1 and MuRF1 in the gastrocnemius muscles of urethane-injected *SOPten$^{\Delta/\Delta}$*/PBS mice were significantly higher than those of *OPten$^{f/f}$*/PBS mice (Fig. 7A). Ghrelin administration decreased these parameters in urethane-injected *SOPten$^{\Delta/\Delta}$* mice. In addition, ghrelin restored the reduced level of IGF1mRNA in urethane-injected *SOPten$^{\Delta/\Delta}$* mice. The gastrocnemius muscle lysates from *SOPten$^{\Delta/\Delta}$*/PBS mice showed decreased expressions of phosphorylated-Akt and phosphorylated-FoxO1 and increased expressions of phosphorylated-p38 MAPK and phosphorylated-NF-κB compared with their counterparts in *OPten$^{f/f}$*/PBS mice (Fig. 7B and C). Ghrelin administration restored the expressions of the phosphorylated-Akt and phosphorylated-FoxO1, and decreased the expressions of phosphorylated-p38 MAPK and phosphorylated-NF-κB in the lysates of gastrocnemius muscle isolated from the urethane-injected *SOPten$^{\Delta/\Delta}$* mice.

4. Discussion

In this study, we reported for the first time a rodent model of cancer cachexia associated with the development of lung adenocarcinoma, and the protective effect of ghrelin against this inexorable condition. Ghrelin inhibited the induction of proinflammatory cytokines, mitigated the reduction of food intake, and consequently ameliorated body weight loss in the mouse model of lung adenocarcinoma. We also demonstrated that the skeletal muscle mass and muscle contraction force were retained in tumor-bearing, ghrelin-treated mice with upregulation of local IGF1 signaling and downregulation of the FoxO1/MuRF1/Atrogin1 pathway. Muscle atrophy in cancer patients is a life-threatening condition because of the impairment of normal activity and respiratory failure. The most essential thing for control of the cachectic status is prevention of skeletal muscle wasting; however, current therapeutic strategies against muscle atrophy are quite limited. Ghrelin and its mimetic drugs have been tested in patients with cachexia associated with chronic heart failure (Nagaya et al., 2004), chronic obstructive pulmonary disease (Nagaya et al., 2005), and cancer (Garcia et al., 2013; Strasser et al., 2008). Although these studies demonstrated that ghrelin administration increased food intake (Nagaya et al., 2005, 2004), body weight (Garcia et al., 2013; Nagaya et al., 2005), and lean body mass (Nagaya et al., 2005, 2004), the effect of ghrelin on muscle atrophy, including its influence on both skeletal muscle mass and skeletal muscle function, and its molecular mechanisms have not been well documented. Our results suggest that ghrelin could provide a hopeful therapeutic strategy for cancer cachexia patients by exerting a protective effect against muscle wasting.

In the present study, the cachectic condition of urethane-injected *SOPten$^{\Delta/\Delta}$* mice was demonstrated not only through the development of lung adenocarcinoma, but also through decreases in body weight gain, food intake, fat and skeletal muscle mass and through increases in circulating proinflammatory cytokine levels and in mRNA expression levels of muscle-specific E3 ubiquitin ligases. Since these conditions are also the main characteristics of cancer cachexia observed in patients with various cancers (Fearon et al., 2012), the present results suggested that lung adenocarcinoma-bearing mice could be an ideal cancer cachexia model for estimating effects in humans. Relative to their wild-type counterparts, the body weights of *SOPten$^{\Delta/\Delta}$* mice began to decrease at five months after urethane injection, which corresponds to the time point of lung adenocarcinoma formation (Yanagi et al., 2007). We therefore started the daily administration of ghrelin to *SOPten$^{\Delta/\Delta}$* mice five months after urethane injection.

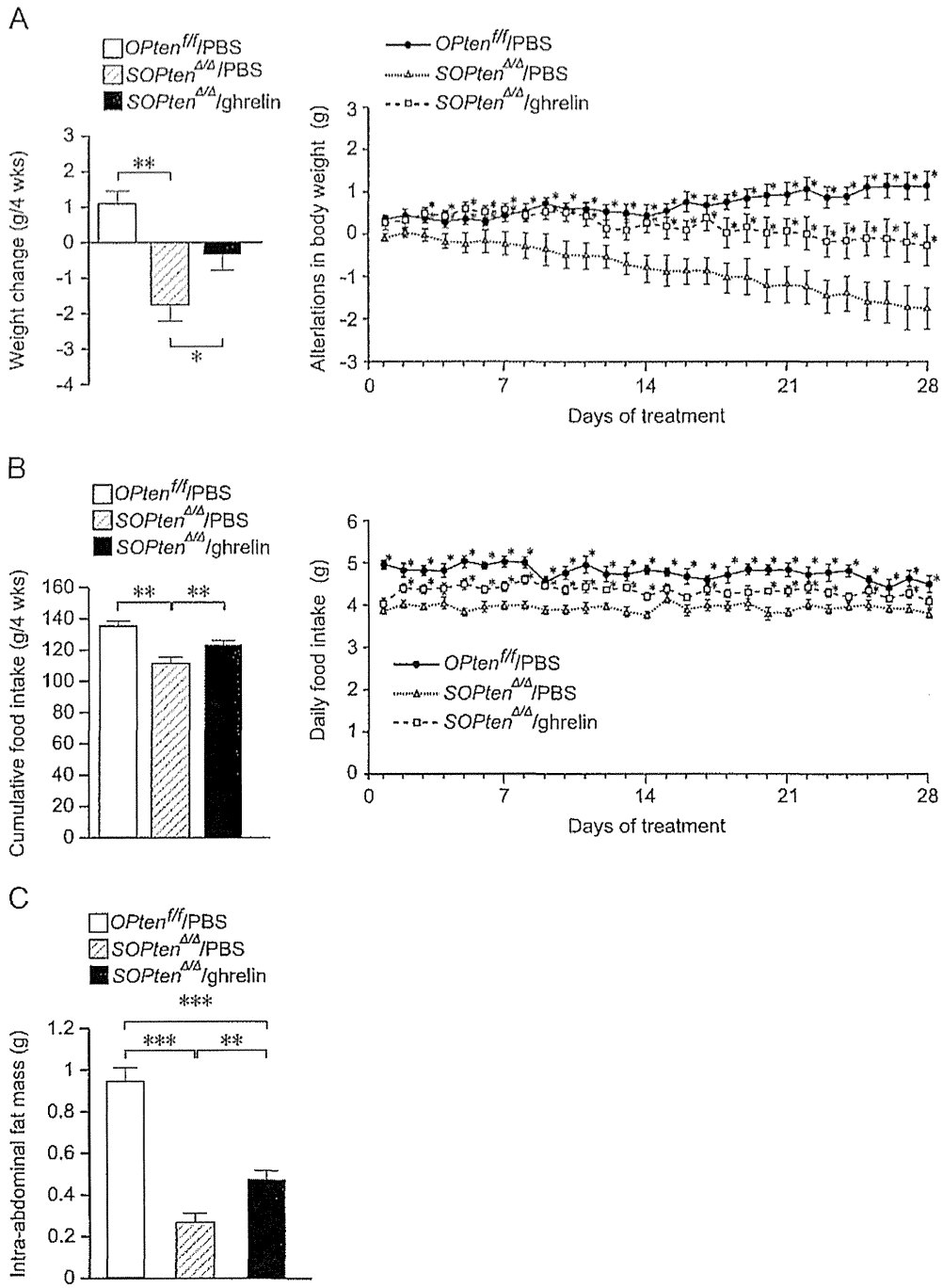

Fig. 4. Effects of ghrelin administration on weight change, food intake, and fat mass in urethane-injected SOPten$^{\Delta/\Delta}$ mice. (A) Overall changes of body weight over the 28-day study interval (left panel) and daily body weight change from the baseline (body weight at day 0, right panel) in the *SOPten*$^{\Delta/\Delta}$/phosphate-buffered saline (PBS) group, *SOPten*$^{\Delta/\Delta}$/ghrelin group and *OPten*$^{f/f}$/PBS group are shown (*SOPten*$^{\Delta/\Delta}$/PBS group, $n=12$; *SOPten*$^{\Delta/\Delta}$/ghrelin group, $n=13$; *OPten*$^{f/f}$/PBS group, $n=12$). Data are shown as the mean ± S.E.M. Differences between groups were evaluated using the Tukey–Kramer honestly significant difference test (left panel) and Student's *t*-test (right panel). $^{*}P<0.05$, $^{**}P<0.01$. vs. the *SOPten*$^{\Delta/\Delta}$/PBS group. (B) Cumulative food intake (left panel) and daily food intake (right panel) of the *SOPten*$^{\Delta/\Delta}$/ PBS group, *SOPten*$^{\Delta/\Delta}$/ghrelin group and *OPten*$^{f/f}$/PBS group over the 28-day study interval are shown (*SOPten*$^{\Delta/\Delta}$/PBS group, $n=12$; *SOPten*$^{\Delta/\Delta}$/ghrelin group, $n=13$; *OPten*$^{f/f}$/PBS group, $n=12$). Data are shown as the mean ± S.E.M. Differences between groups were evaluated using the Tukey–Kramer honestly significant difference test (left panel) and Student's *t*-test (right panel). $^{*}P<0.05$, $^{**}P<0.01$. vs. the *SOPten*$^{\Delta/\Delta}$/PBS group. (C) Intra-abdominal fat mass at 28 days after administration of ghrelin or PBS is shown (*SOPten*$^{\Delta/\Delta}$/PBS group, $n=22$; *SOPten*$^{\Delta/\Delta}$/ghrelin group, $n=23$; *OPten*$^{f/f}$/PBS group, $n=12$). Data are shown as the mean ± S.E.M. Differences between groups were evaluated using the Tukey–Kramer honestly significant difference test. $^{**}P<0.01$, $^{***}P<0.001$.

Ghrelin administration to the urethane-injected *SOPten*$^{\Delta/\Delta}$ mice suppressed the expressions of Atrogin1 and MuRF1 in skeletal muscle to levels comparable to those seen in urethane-injected *OPten*$^{f/f}$ mice, in which cancer did not occur. Expression of these two muscle-specific E3 ligases is essential for ubiquitination and subsequent degradation of myofiber proteins, and many studies have shown that the ubiquitin-proteasome proteolytic pathway plays a major role in the degradation of muscle proteins during cachexia. With regard to the mechanisms of downregulation of E3 ligases in skeletal muscle in ghrelin-treated mice, we demonstrated significant reductions of IL-1β, IL-6, and TNF-α in plasma as well as decreased expressions of p38 MAPK and NF-κB in skeletal muscle. One prominent subset of the procachectic mechanism is induction of proinflammatory cytokines which signal activation of the NF-κB