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Randomized, double-blind, placebo-controlled trial of bovine lactoferrin in patients with chronic hepatitis C

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Several studies have suggested that lactoferrin administration may decrease the serum level of hepatitis C virus (HCV) RNA in patients with chronic hepatitis C. The aim of the present study was to confirm the efficacy of orally administered bovine lactoferrin (bLF) in patients with chronic hepatitis C. The patients with chronic hepatitis C randomly received either oral bLF at a dose of 1.8 g daily for 12 weeks, or an oral placebo. The primary endpoint was the virologic response, defined as a 50% or greater decrease in serum HCV RNA level at 12 weeks compared with the baseline. The secondary endpoint was the biochemical response, which was defined as a 50% or greater decrease in the serum alanine aminotransferase (ALT) level at 12 weeks compared with the baseline. One hundred and ninety-eight of 199 patients were evaluable for efficacy and safety. bLF treatment was well tolerated and no serious toxicities were observed. A virologic response was achieved in 14 of 97 patients (14.4%) in the bLF group, and 19 of 101 (18.8%) in the placebo group. There was no significant difference in virologic response rates between the two groups (–4.4%, 95% confidence interval –14.8, 6.1). In addition, bLF intake did not have any favorable effect on the serum ALT level. The virologic responses were not different between two groups in any subgroup analysis. In conclusion, orally administered bLF does not demonstrate any significant efficacy in patients with chronic hepatitis C. (*Cancer Sci* 2006; 97: 1105–1110)

Hepatitis C virus is a leading cause of chronic liver disease in Japan, and nearly two million people are estimated to be infected.[1] It is well known that HCV infection frequently causes chronic hepatitis, and that chronic hepatitis eventually progresses to liver cirrhosis and HCC approximately 30 years after HCV infection.[2] In Japan, more than 30 000 people die of HCC annually, and approximately 80% of HCC patients are infected with HCV.[3] Therefore, effective anti-HCV therapy is necessary to reduce the number of patients suffering from cirrhosis or HCC. To date, interferon-based therapy is the only effective treatment used clinically for chronic hepatitis C. A sustained complete virologic response (loss of detectable serum HCV RNA) occurs in 15–20% of patients with chronic hepatitis C after interferon therapy.[4] Moreover, recent studies have demonstrated that interferon with ribavirin or peginterferon with ribavirin improves the sustained complete virologic response rate by up to 40–50%.[5,6] However, because more than half of patients do not respond to interferon therapy, and because interferon therapy sometimes induces strong adverse effects, further developments in the treatment of chronic hepatitis C are required.

Lactoferrin, a member of the transferrin family of iron-binding glycoproteins, is present mainly in breast milk and other exocrine secretions. Several biological activities of lactoferrin have been demonstrated, including regulation of iron absorption in the intestine and modulation of immunoreactions.[7] Lactoferrin also plays an important role in human innate defense mechanisms against bacteria, fungi and viruses.[8] *In vitro* studies to date have shown that lactoferrin has antiviral effects against human immunodeficiency virus-1 and human cytomegalovirus.[9] Recent experimental studies have suggested that lactoferrin has antiviral effect against HCV.[10–12] Yi *et al.* have reported that lactoferrin binds to HCV envelope proteins *in vitro*.[10] Ikeda *et al.* have reported that lactoferrin prevents HCV infection in cultured human hepatocytes, and suggested that the anti-HCV activity of lactoferrin might be related to its direct binding to viral surfaces.[11,12] In addition, recent clinical studies have demonstrated the potential efficacy of lactoferrin against chronic hepatitis C.[13,14] Tanaka *et al.* reported that 8-week oral administration of bLF at a dose of 1.8 or 3.6 g/day decreased the serum level of HCV RNA markedly in three of four patients with a low pre-treatment HCV RNA level (<100 Kcopy/mL).[13] Iwasa *et al.* administered bLF (3.6 g/day) orally to 15 patients with high viral loads (≥100 KIU/mL), and reported that the mean serum HCV RNA level decreased significantly from 1106 KIU/mL at entry to 612 KIU/mL after 6 months of treatment ($P < 0.01$).[14] Based on these promising findings, we planned to investigate the efficacy of orally administered bLF in patients with chronic hepatitis C. First, we conducted a dose-finding study in 45 patients with chronic hepatitis C.[15] In that study, three dose levels of bLF (1.8, 3.6 and 7.2 g/day) were scheduled, and 15 patients at each dose level received the determined dose of bLF for 8 weeks. bLF treatment was well tolerated up to 7.2 g/day, and no serious adverse events were observed. Although no relationship between bLF dose and efficacy was recognized, a 50% or greater decrease in the serum HCV RNA level was seen in four of 45 patients (8.9%). Furthermore, the HCV RNA level was decreased by 50% or more in eight patients (17.8%) at week 8 after the end of treatment. These results encouraged us to conduct further investigations, and the present randomized

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Abbreviations: ALT, alanine aminotransferase; bLF, bovine lactoferrin; CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IL, interleukin; NK, natural killer.

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trial was designed to clarify the anti-HCV activity of bLF in patients with chronic hepatitis C.

Patients and Methods

Patients. Each patient was required to meet the following eligibility criteria: 20–74 years of age; positivity for anti-HCV antibody; an HCV RNA level of 0.5–850 KIU/mL evaluated within 1 month before entry; a sustained elevation of serum ALT level for at least 6 months; a serum ALT level of at least twice the upper normal limit evaluated within 1 month before entry; no evidence of HCC on the basis of ultrasonography or computed tomography carried out within 3 months before entry; and adequate bone marrow function (white blood cell count $\geq 4000/mm^3$, platelet count $\geq 100\,000/mm^3$, and hemoglobin level ≥ 11 g/dL), liver function (total bilirubin level ≥ 2.0 mg/dL, serum albumin level ≥ 3.5 g/dL, and serum aspartate aminotransferase and ALT level ≥ 200 IU/L) and renal function (normal serum creatinine and blood urea nitrogen levels).

The exclusion criteria were: positivity for hepatitis B surface antigen; interferon therapy within 6 months before entry; immunomodulatory or corticosteroid therapy within 3 months before entry; intravenous glycyrrhizin therapy within 1 month before entry; past or present history of bLF tablet intake; pregnant or lactating females; severe hepatic disease (e.g. autoimmune hepatitis and primary biliary cirrhosis); other serious medical conditions (e.g. gastrointestinal bleeding, active infection, severe pulmonary disease and psychiatric disorders).

Methods. This double-blind, placebo-controlled phase III trial was conducted at 11 centers in Japan. The study was approved by the institutional review board at each center, and all the participants provided written informed consent. Eligible participants were assigned randomly to one of two treatment groups in equal proportions using permutation blocks stratified by centers. A randomization list was drawn up using the SAS random number generator at the data center (Quintiles Transnational Japan K. K. Tokyo, Japan). The treatments consisted of bLF at a dose of 1.8 g/day or a placebo, administered orally twice daily for 12 weeks. In the current study, bLF at 1.8 g/day was selected on the basis of the previous dose-finding study, which indicated that there was no significant relationship between bLF dose (range, 1.8–7.2 g/day) and anti-HCV activity.[15] After the treatment allocation, the data center sent a numbered container of bLF or placebo tablets to a participant. During treatment, combined use of interferon, immunomodulatory therapy, corticosteroid and intravenous glycyrrhizin was prohibited. bLF (450 mg/tablet) and placebo tablets were provided by Morinaga Milk Industries (Tokyo, Japan).

In the current study, we tested the hypothesis that oral administration of bLF would: (1) reduce the serum HCV RNA level; and (2) reduce the serum ALT level in patients with chronic hepatitis C. In addition, we investigated the influence of orally administered bLF on systemic immune response in a small group of participants. The participants were evaluated every 4 weeks as outpatients until 4 weeks after completion of treatment. Serum HCV RNA level and serum ALT level were measured before treatment, during treatment at weeks 4, 8 and 12, and at 4 weeks after treatment. Serum HCV RNA level was determined by reverse transcription–polymerase chain reaction using the Amplicor-HCV monitor V 2.0 kit with a sensitivity of 0.5 KIU/mL (Roche Diagnostics, Tokyo, Japan). Anti-HCV antibody was determined by chemiluminescent enzyme immunoassay (Ortho-Clinical Diagnostics, Tokyo, Japan). HCV serotyping was carried out as described previously.[16] HCV serotype 1 corresponds to genotypes 1a and 1b of the Simmonds classification, and HCV serotype 2 corresponds to genotypes 2a and 2b.[17] Serum concentration of IL-18 was measured in participants at two institutions (National Cancer Center Hospital and Osaka Red Cross Hospital), and the percentage of $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ peripheral blood lymphocytes was measured in participants at the National Cancer Center Hospital. IL-18 and all lymphocytes were measured before treatment, during treatment at weeks 4, 8 and 12, and at 4 weeks after completion of treatment. Serum concentration of IL-18 was assayed with a human IL-18 enzyme-linked immunosorbent assay kit (Medical and Biological Laboratories, Nagoya, Japan). Lymphocyte surface phenotypes of CD4, CD8, CD16 and CD56 were determined by flow cytometry.

Adverse events were graded for severity according to the Japan Society for Cancer Therapy criteria,[18] which are similar to the National Cancer Institute Common Toxicity criteria. During treatment, participants were asked to record in a daily journal both compliance and any adverse events they experienced.

Assessment of efficacy and statistical analysis. Analyses were carried out on an intention to treat basis. The primary endpoint was a virologic response. In the current study, we defined a virologic response as a 50% or greater decrease in the serum HCV RNA level at 12 weeks compared with the baseline. Secondary endpoints were a biochemical response, as were changes in serum HCV RNA level and serum ALT level. If the serum ALT level at 12 weeks showed both a ≥50% decrease compared with the baseline and was ≤ twice the upper normal limit, we considered it a biochemical response. Response rate was calculated as the number of responders divided by the total number in each group. Participants whose HCV RNA (or ALT) data at 12 weeks were missing were included only in the denominator. Change in HCV RNA level (or ALT level) was calculated as the logarithm of the HCV RNA level (or ALT level) at 12 weeks minus the logarithm of these at the baseline. Differences in the virologic or biochemical response rates between two groups were analyzed using a test for the difference between two proportions. Differences in the change in HCV RNA level or ALT level between two groups were analyzed using a test for the difference between two means. In addition to the above planned analyses, subgroup analyses for virologic response were carried out based on pretreatment variables including age, serum HCV RNA level and HCV serotype. In a small group of participants, change in the serum concentration of IL-18 and changes in the percentage of $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ peripheral blood lymphocytes during the study period were investigated. Analyses were carried out using JMP4.0 and PC SAS Release v.8.02 (SAS Institute Japan Ltd, Tokyo, Japan). All P-values are two-tailed, and differences at $P < 0.05$ were regarded as statistically significant.

We estimated that a total of 250 participants would be the maximum to enroll for a 2-year enrollment period. Subsequent power analysis revealed that 125 participants per group would have 75% power to detect a 10% difference in the virologic response rate (15 vs 5%) at the 5% level of significance. An interim analysis by the independent data monitoring committee was planned after the first 125 participants had been enrolled. All trial personnel and participants were blinded to treatment assignment for the duration of the trial. Only the trial statistician and the independent data monitoring committee saw unblinded data. In the interim analysis of the primary endpoint, the O'Brien–Fleming method was used.[19]

Results

Patients. Enrollment began at seven institutions in April 2001. Because 250 participants were not enrolled for the 2 years planned originally, we extended the registration period for one more year and increased the number of participating institutions from seven to 11. An interim analysis was carried out in March 2004 with the data from the first 125 participants. Because the results of the interim analysis indicated that it was highly unlikely that a significant difference in treatment efficacy between

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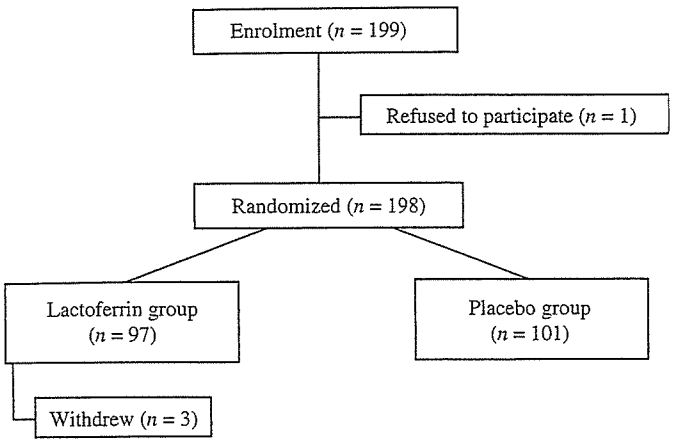

Fig. 1. Flow diagram of participant enrolment.

Table 1. Baseline characteristics of the patients

Characteristic	Bovine lactoferrin	Placebo
No. patients	97	101
Age (years)†	61 (29–74)	58 (31–74)
Sex (male/female)	53/44	55/46
History of interferon therapy	25	29
ALT level (IU/L)†	91 (41–340)	98 (27–250)
HCV RNA level (KIU/mL)†	378 (8.8–960)	452 (8.0–1560)
HCV serotype (1/2/ND)	78/17/1	76/22/3

†Median (range). ALT, alanine aminotransferase; HCV, hepatitis C virus; ND, not determined.

the two groups would be observed with the planned full enrollment of 250 participants, the data monitoring committee recommended discontinuation of further enrollment. Therefore, enrollment was stopped on 31 March 2004, at which point 199 participants had been enrolled. Because one patient refused to participate in the study before randomization, efficacy and safety were analyzed in the remaining 198 participants (97 bLF and 101 placebo) (Fig. 1). Although three participants in the bLF group discontinued treatment for reasons other than an adverse event, the remaining 195 participants completed the scheduled 12 weeks of treatment. The baseline characteristics of the 198 participants are shown in Table 1. There was no significant difference between the bLF and placebo groups regarding the pretreatment characteristics including age, sex, serum ALT level and serum HCV RNA level.

Virologic efficacy. Virologic response, the primary endpoint, was assessed in all 198 participants who received at least one dose of treatment. Virologic response was observed in 14 of 97 participants (14.4%) in the bLF group, and in 19 of 101 (18.8%) in the placebo group (Table 2). No complete virologic response (loss of detectable serum HCV RNA) was seen in either of the groups. There was no significant difference in the virologic response rate with bLF treatment in comparison with the placebo (–4.4%, 95% CI –14.8, 6.1). Change in the HCV RNA level at 12 weeks compared with the baseline was assessed in 190 participants (93 bLF group, 97 placebo group), excluding eight participants for whom HCV RNA data at 12 weeks were lacking. The change in the mean logarithm of the HCV RNA level was –0.09 in the bLF group and –0.09 in the placebo group, indicating no significant difference between the groups ($P = 1.00$).

Biochemical efficacy. Biochemical response was assessed in 198 participants. Biochemical response was seen in six of 97 participants (6.2%) in the bLF group, and in four of 101 participants (4.0%) in the placebo group (Table 2). No significant difference in the biochemical response rate was seen between the groups (2.2%, 95% CI –3.9, 8.3). Change in the serum AST level was assessed in 192 participants (93 bLF group, 99 placebo group), excluding six participants for whom ALT data at 12 weeks were lacking. The change in the mean logarithm of the ALT level was –0.085 in the bLF group and –0.080 in the placebo group, indicating no significant difference ($P = 0.93$).

Subgroup analysis. The rates of virologic response with respect to pretreatment variables are presented in Table 3. Among participants with a low HCV RNA level (<100 KIU/mL), the virologic response rate was 29.4% in the bLF group and 15.4% in the placebo group, indicating no significant difference between the groups (14.0%, 95% CI –15.2, 43.2). The virologic responses were also not different between two groups in other subgroup analyses such as age, sex and HCV serotype.

Analysis of IL-18 and lymphocytes. The serum concentration of IL-18 was measured in 73 participants enrolled at the National Cancer Center Hospital and Osaka Red Cross Hospital (36 bLF, 37 placebo). Figure 2 shows the changes in the mean IL-18 levels in the bLF group and placebo group. The mean IL-18 levels in the bLF and placebo groups were 293.9 pg/mL and 309.9 pg/dL at the baseline and 280.7 pg/mL and 291.5 pg/mL at 12 weeks, respectively. The corresponding changes in the mean IL-18 level at 12 weeks were –14.5 pg/mL and –15.9 pg/mL, respectively, indicating no significant difference between the groups ($P = 0.91$). Similarly, there were no significant differences between the groups at any other points during the study period. The percentage of lymphocyte was measured in 46 participants at the National Cancer Center Hospital (bLF 23, placebo 23), and the results are shown in Fig. 3. The percentage of $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ peripheral blood lymphocytes remained almost unchanged throughout the study in both groups, and the differences between them were not significant.

Safety. Safety was assessed in 198 participants who received at least one dose of bLF or placebo during the study. The bLF treatment was well tolerated, and no serious complications occurred during the treatment. Although minor adverse events including neutropenia, γ-GTP elevation and hyperglycemia were observed in participants treated with bLF, their frequency and intensity did not differ from those in the placebo group. HCC was detected in one participant in the bLF group and in one participant in the placebo group during the study period.

Discussion

The present study was carried out to confirm the anti-HCV activity of orally administered bLF in patients with chronic hepatitis C. A virologic response (a 50% or greater decrease in the serum level of HCV RNA at 12 weeks compared with the baseline) was observed in 14 of 97 participants (14.4%) in the bLF group, and 19 of 101 (18.8%) in the placebo group, the difference between the groups being non-significant. The virologic responses were not different between two groups in any subgroup analysis. Furthermore, bLF intake did not have any favorable effect on the serum ALT level. On the basis of these results, we concluded that orally administered bLF did not have any efficacy, including anti-HCV activity, in patients with chronic hepatitis C.

The virologic response rate of 14.4% observed in the bLF group was somewhat higher than that reported in the previous dose-finding study,[15] in which four of 45 patients (8.9%) showed a virologic response at the end of bLF treatment. Nevertheless, the current study failed to demonstrate any anti-HCV activity of bLF, because a similar virologic response rate to that in the bLF group was seen in the placebo group. Having designed this randomized study, we assumed that a virologic

Table 2. Virologic and biochemical efficacy

Characteristic	Bovine Lactoferrin	Placebo	Difference (95% CI)	*P*-value
Virologic efficacy				
Response rate (%)	14.4	18.8	−4.4 (−14.8, 6.1)	
Change in HCV RNA level†	−0.09	−0.09		1.00
Biochemical efficacy				
Response rate (%)	6.2	4.0	2.2 (−3.9, 8.3)	
Change in ALT level†	−0.085	−0.080		0.93

†Mean logarithm. ALT, alanine aminotransferase; CI, confidence interval; HCV, hepatitis C virus.

Table 3. Virologic response rate as a function of baseline variables

Variable	Bovine lactoferrin (*n* = 97)		Placebo (*n* = 101)		Difference	
	Response/total	%	Response/total	%	%	95% CI
Age						
<65 years	12/62	19.4	14/77	18.2	1.2	−11.9, 14.2
≥65 years	2/35	5.7	5/24	20.8	−15.1	−33.1, 2.9
Sex						
Male	10/53	18.9	10/55	18.2	0.7	−14.0, 15.3
Female	4/44	9.1	9/46	19.6	−10.5	−24.7, 3.8
ALT level						
<100 IU/L	6/57	10.5	7/51	13.7	−3.2	−15.6, 9.2
≥100 IU/L	8/40	20.0	12/50	24.0	−4.0	−21.1, 13.1
HCV RNA level						
<100 KIU/mL	5/17	29.4	2/13	15.4	14.0	−15.2, 43.2
≥100 KIU/mL	9/80	11.3	17/88	19.3	−8.0	−18.8, 2.7
HCV serotype†						
1	11/78	14.1	16/76	21.1	−7.0	−18.9, 5.0
2	3/18	16.7	2/22	9.1	7.6	−31.4, 28.6

†Hepatitis C virus serotype was not measured in four patients. ALT, alanine aminotransferase; CI, confidence interval; HCV, hepatitis C virus.

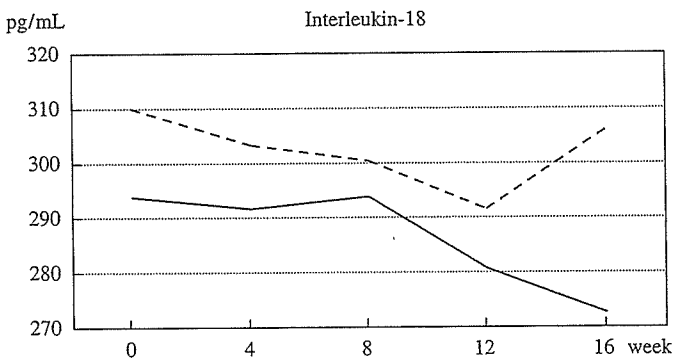

Fig. 2. Changes in the mean serum concentration of interleukin-18 in the bovine lactoferrin group (straight line, $n = 36$) and the placebo group (dotted line, $n = 37$).

response rate of around 5% would be seen in the placebo group due to spontaneous remission of viral activity. However, contrary to our expectation, 19 of 101 participants (18.8%) in the placebo group showed a ≥50% decrease in the HCV RNA level at 12 weeks, indicating that our assumption was inappropriate. Our results suggested that in order to assess the reduction of the HCV RNA level, periodic evaluation would be necessary to exclude the influence of spontaneous fluctuation of HCV RNA.

Several experimental studies have suggested that lactoferrin has some activity against HCV. Yi *et al.*[10] reported that lactoferrin binds to the HCV E1 and E2 envelope proteins *in vitro*, and Ikeda *et al.*[11,12] reported that lactoferrin prevents HCV infection in cultured human hepatocytes. They suggested that the anti-HCV activity of lactoferrin might be due to a neutralizing efficacy, in which the administered lactoferrin became bound directly to the HCV virion, thus inhibiting adsorption of the HCV-lactoferrin complex into human hepatocytes. Therefore, intravenous administration of lactoferrin might improve the viremic state in patients with chronic hepatitis C. However, for practical application, administration of lactoferrin directly into blood does not seem to be a suitable approach because lactoferrin is a large glycoprotein molecule (80 kDa) that may cause allergic reactions. Therefore, oral administration of bLF was selected for the present study, even though the metabolism and mechanism of ingested lactoferrin are yet to be clarified. As to absorption, it has been reported that intact lactoferrin and its fragments are present in the urine of human milk-fed preterm infants.[20] However, in adult rats, lactoferrin and its fragments are not detectable in portal blood after bLF ingestion,[21] and in adult humans, the serum lactoferrin level does not increase after oral administration of recombinant human lactoferrin.[22] However, several studies have suggested that orally administered lactoferrin might enhance immune responses via cytokine production.[23,24] It has been reported that oral administration of bLF to mice enhances the production of IL-18 and interferon-γ in the mucosa of the small intestine, and increases the number of $CD4^+$, $CD8^+$ and NK cells in the small-intestinal epithelium.[25,26] Varadhachary *et al.* reported that oral administration of recombinant human lactoferrin to mice stimulates IL-18 production from gut enterocytes, and augments the NK activity of spleen cells and production of blood $CD8^+$ cells.[27] Furthermore, a recent clinical study has demonstrated that oral administration of bLF (0.6 g/day) for 3 months in 36 patients with chronic hepatitis C increased the serum IL-18 level significantly compared with the

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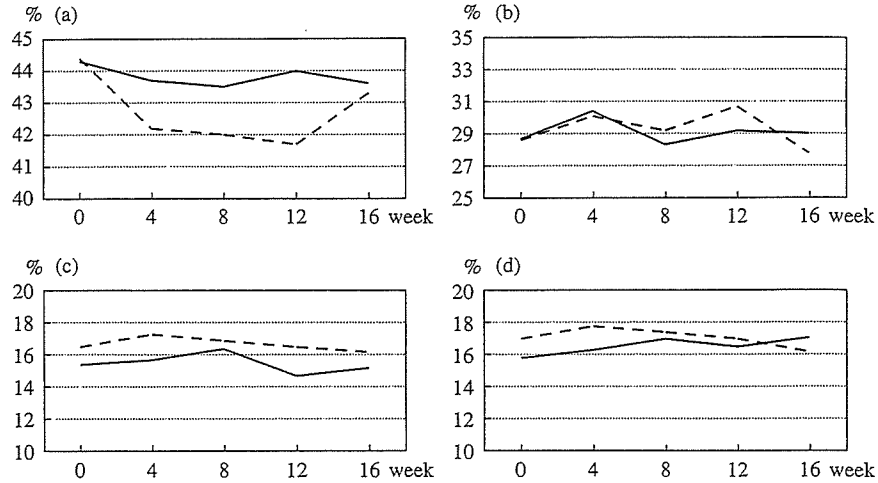

Fig. 3. Changes in the mean percentages of (a) $CD4^+$, (b) $CD8^+$, (c) $CD16^+$ and (d) $CD56^+$ peripheral blood lymphocytes in the bovine lactoferrin group (straight line, $n = 23$) and the placebo group (dotted line, $n = 23$).

baseline.[28] However, our study found no evidence that oral administration of bLF influences the serum concentration of IL-18 or the percentage of $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ lymphocytes. Further investigations are required to clarify the peripheral and systemic effects of orally administered lactoferrin. In addition, as many *in vitro* studies have suggested that lactoferrin has direct binding neutralizing efficacy against HCV,[29–31] further investigations are needed to devise a means of delivering lactoferrin or its fragment into the bloodstream safely and effectively.

Recently, several studies have investigated the value of adding lactoferrin to interferon therapy for chronic hepatitis C. Hirashima *et al.* randomly assigned 21 patients with chronic hepatitis C to either a consensus interferon plus oral lactoferrin (3.0 g/day) group or a consensus interferon monotherapy group.[32] Three of 10 patients in the consensus interferon plus lactoferrin group showed a sustained complete virologic response, as did four of 11 patients in the consensus interferon group, indicating no statistically significant difference between the groups. Ishibashi *et al.* conducted a randomized controlled trial to investigate the efficacy of interferon α-2b and ribavirin plus oral lactoferrin (0.6 g/day) compared with interferon α-2b and ribavirin plus placebo in 36 patients with chronic hepatitis C.[33] A sustained complete virologic response was seen in six of 18 patients in the lactoferrin group and in five of 18 patients in the placebo group, there being no statistically significant difference between the groups ($P = 0.7$). Although the numbers of patients recruited in the two randomized trials were small, these results suggested that the additional value of oral lactoferrin combined with interferon therapy would be negative for the treatment of chronic hepatitis C.

In summary, oral administration of bLF at a dose of 1.8 g/day for 12 weeks showed an acceptable safety profile in patients with chronic hepatitis C. However, there was no significant difference in the virologic responses between patients who received oral bLF and those receiving placebo. In addition, bLF intake did not have any favorable effect on the serum ALT level. These findings do not support the practical use of oral bLF in patients with chronic hepatitis C.

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Evaluation of Acute Intestinal Toxicity in Relation to the Volume of Irradiated Small Bowel in Patients Treated with Concurrent Weekly Gemcitabine and Radiotherapy for Locally Advanced Pancreatic Cancer

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Abstract. *Background: Treatment of concurrent gemcitabine and radiotherapy for pancreatic cancer was reported to have a higher rate of severe acute intestinal toxicity. This study evaluated the acute intestinal toxicity in relation to the volume of irradiated small bowel and other factors using dosimetric analyses in pancreatic cancer patients treated with gemcitabine-based chemoradiotherapy. Materials and Methods: The patient population was derived from a phase II trial of concurrent weekly gemcitabine and radiotherapy for locally advanced pancreatic cancer. Gemcitabine was administered weekly at a dose of 250 mg/m^2. The total dose was 50.4 Gy in 28 fractions using a four-field conformal technique. A dose-volume histogram was generated for the small bowel, colon and planning target volume (PTV) and dosimetric parameters were recorded. Correlations between the acute intestinal toxicity and the volume of irradiated small bowel and other factors were evaluated. Results: Forty-two patients enrolled between July 2001 and July 2002 were analyzed. Grade 3+ acute intestinal toxicities were observed in twenty-four (62%) patients. There was no correlation between the acute intestinal toxicity and the volume of irradiated small bowel. However, the total volume of PTV was shown to be significantly correlated with the development of Grade 3+ acute intestinal toxicity ($p=0.021$). Conclusion: The volume of irradiated small bowel did not directly influence the acute intestinal toxicity, but only the volume of PTV significantly correlated with severe acute intestinal toxicity.*

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Pancreatic cancer is usually diagnosed as an unresectable locally advanced or metastatic disease in most patients. In patients with locally advanced pancreatic cancer, chemoradiotherapy has been commonly used as a standard treatment since it was recognized that radiotherapy with concurrent 5-fluorouracil (5-FU) prolonged survival when compared to radiotherapy or chemotherapy alone (1-3). Various novel agents and radiation schedules have been examined in clinical trials to improve the efficacy of the treatment (4).

Gemcitabine is a novel deoxycitidine analog with a broad spectrum of antitumor activity against a variety of solid tumors, including pancreatic cancer, which has demonstrated greater clinical benefit and survival compared with 5-FU in patients with advanced pancreatic cancer (5). Gemcitabine has also been shown to be a potent radiosensitizer in human pancreatic cancer (6-8). Therefore, concurrent gemcitabine and radiotherapy are currently being examined in clinical trials, suggesting that the combination of radiotherapy and gemcitabine may improve survival in patients with locally advanced pancreatic cancer (9-13).

However, significant acute intestinal toxicity (AIT) in the treatment of concurrent gemcitabine and radiotherapy was reported compared with concurrent 5-FU and radiotherapy (9, 10, 14). In rectal cancer treated with concurrent chemoradiotherapy, a significant relationship between the intestinal toxicity and the volume of irradiated small bowel is well recognized from the results of examinations using small bowel contrast and orthogonal radiographs to calculate the volume of small bowel in the high-dose volume (15-17) and more accurately three-dimensional (3D) treatment-planning tools (18). However, it has not been reported whether the volume of irradiated small intestine is related to the degree of AIT in patients treated with concurrent chemoradiotherapy for pancreatic cancer. The purpose of this study was to evaluate the AIT in relation to

the volume of irradiated small bowel and to other factors using dosimetric analyses in patients treated with concurrent weekly gemcitabine and radiotherapy for locally advanced pancreatic cancer.

Materials and Methods

Patient population. The patient population for this study was derived from a phase II trial of concurrent weekly gemcitabine and radiotherapy for unresectable locally advanced pancreatic cancer at the National Cancer Center Hospital (19). Eligibility criteria for this phase II trial included histologically or cytologically confirmed nonresectable adenocarcinoma, 20-74 years of age, an Eastern Cooperative Oncology Group (ECOG) performance status of 0-2, no evidence of distant metastasis, adequate hematological function (hemoglobin ≥10 g/dl, leukocytes ≥4000 mm^3, neutrophils >2000 mm^3, and platelets ≥100000 mm^3), adequate hepatic function (serum total bilirubin ≤2.0 mg/dl, and serum transaminase (aspartate aminotransferase (AST)/ alanine aminotransferase (ALT) < 2.5 times the upper normal limit (UNL), adequate renal function (serum creatinine within normal limit) and written informed consent.

Treatment details and dosimetric analysis. Gemcitabine was administered intravenously over 30 min starting 2 h before radiotherapy, weekly for 6 weeks, at a dose of 250 mg/m^2, which had been previously determined in a phase I trial in our hospital (20). When grade 3 hematological toxicity, serum creatinine of 1.5-2.0 times UNL, total bilirubin level of 3.0-5.0 times UNL, serum AST/APT of 5.0-10 times UNL and/or grade 2 non-hematological toxicity (excluding nausea, vomiting, anorexia, fatigue, constipation, alopecia and dehydration) were observed, gemcitabine administration was omitted and postponed to the next scheduled treatment days.

Radiotherapy was delivered *via* a racetrack microtron (MM50, Scanditronix, Uppsala, Sweden) with a 25 MV X-rays. All patients had treatment planning computed tomography (CT) scans (X-vision, Toshiba, Tokyo, Japan), 5 mm thickness with a 5 mm slice interval, with oral small bowel contrast. The clinical target volume (CTV) included the primary tumor, nodal involvement detected by CT scan, and draining and para-aortic lymph nodes. The planning target volume (PTV) was defined as CTV plus a 10 mm margin in the lateral direction and a 10-20 mm margin in the cranio-caudal direction. Four-field techniques (anterior, posterior and opposed lateral fields) were used. The spinal cord dose was maintained below 45 Gy and ≥50% of the liver was limited to ≤30 Gy, ≥50% of both kidneys were limited to ≤20 Gy. The prescription dose was 50.4 Gy, delivered in 1.8 Gy daily fractions. FOCUS (version 3.2.1, CMS, St. Louis, MO, USA) was used as a radiotherapy treatment planning system. The individual loops of small bowel and colon were delineated on each slice of the planning CT scan from the upper end level of the liver to the lower end level of the kidneys. The volumes of small bowel receiving doses between 5 and 45 Gy were recorded from DVH at 5-Gy intervals.

Toxicity assessment. Patients were evaluated at least weekly during radiotherapy, prospectively. National Cancer Institute common toxicity criteria, version 2.0, were used for toxicity assessment. AIT was defined as any toxicity that could be related to the small bowel, which included nausea, vomiting, anorexia and diarrhea, according to the previous report for rectal cancer (17) and ≥grade 3 was considered severe toxicity.

Table I. *Patient characteristics.*

Characteristic	No. of patients (N=42)
Gender	
male	19
female	23
Age, years	
range	43-73
median	59
Performance status	
0	12
1	30
Tumor size, cm	
range	2.0-10.0
median	4.0
Tumor site	
head	20
body-tail	22

Statistical analysis. For each 5-Gy dose level from 5 to 45 Gy, an association between the volume of small bowel irradiated and grade 3+ AIT was analyzed. The differences in mean small bowel volume irradiated to each 5-Gy dose level from 5 to 45 Gy were assessed using the *t*-test for the equality of means. Univariate analysis comparing the clinical and treatment factors and grade 3+ AIT was performed using the Fisher's exact test. *P*-values less than 0.05 were considered to be statistically significant.

Results

Forty-two patients were enrolled in a phase II trial between July 2001 and July 2002, and all patients were entered in this study. The patient characteristics are shown in Table I. Forty patients completed the planned radiotherapy (50.4 Gy). Two patients discontinued radiotherapy. One patient stopped at 30.6 Gy because of duodenal bleeding and another patient stopped at 45.0 Gy because of refusal due to general fatigue. The number of times gemcitabine was administered was 6 times in 17 patients, 5 times in 15 patients, 4 times in 6 patients, 3 times in 2 patients and 2 times in 2 patients. Grade 3 and grade 4 non-hematological toxicities were observed in 31% and 33% of patients, respectively. Overall, the maximum AIT encountered during radiotherapy was grade 0 in 4 patients (9.5%), grade 1 in 9 patients (21.4%), grade 2 in 3 patients (7.2%), grade 3 in 12 patients (28.6%) and grade 4 in 14 patients (33.3%). Median and range values of the dosimetric parameters of small bowel, colon and PTV are shown in Table II. The volume of irradiated small bowel ranged from 43 cm^3 to 552 cm^3, with a median value of 251 cm^3 and the volume of

Table II. *Median and range values of dosimetric parameters.*

Parameter	Median	Range
Small bowel		
total volume, cm^3	274	47-663
irradiated volume, cm^3	251	43-552
max dose, cGy	5072	3079-5229
mean dose, cGy	1485	376-2915
Colon		
total volume, cm^3	403	120-714
irradiated volume, cm^3	397	117-686
max dose, cGy	5028	1975-5221
mean dose, cGy	1516	633-2848
Planning target volume		
total volume, cm^3	555	357-1215
max dose, cGy	5120	3106-5275
mean dose, cGy	4948	3002-5045

Table III. *Volume of irradiated small intestine at each 5-Gy dose level between 5 and 45 Gy vs. the degree of acute intestinal toxicity (mean±SE, cm^3).*

RT dose level (Gy)	Grade 0-2 toxicity	Grade 3-4 toxicity	*p*-value
5	169±99	182±99	0.669
10	150±94	161±92	0.707
15	140±90	148±90	0.787
20	64±41	66±50	0.873
25	53±36	55±42	0.879
30	49±33	50±40	0.910
35	43±27	45±36	0.864
40	38±23	41±32	0.786
45	32±20	35±28	0.715

PTV ranged from 357 cm^3 to 1215 cm^3, with a median value of 555 cm^3, corresponding to a cube of 8.2 cm on a side. The average volume of small bowel irradiated at each 5-Gy dose level between 5 and 45 Gy are shown in Table III.

The average volume of small bowel irradiated at each dose level was not significantly different between the group of grade 3+ AIT and the group of grade 0-2 AIT by the *t*-test for equality of means. The relationships between grade 3+ AIT and clinical factors are shown in Table IVa. No significant correlation was seen between grade 3+ AIT and clinical factors, including age, performance status, tumor size, tumor site, and number of times gemcitabine was administered. The relationships between grade 3+ AIT and the calculated parameters are shown in Table IVb. No significant correlation was seen between grade 3+ AIT and the volume of small bowel irradiated or other parameters regarding the small bowel and the colon. However, the total volume of PTV was shown to be significantly

Table IVa. *Univariate analysis of clinical and treatment factors related to the development of ≥ grade 3 acute intestinal toxicity.*

Characteristic	n	% toxicity	*p*-value*
Gender			
male	19	63.2%	
female	23	60.9%	>0.999
Age, years			
<60	22	54.5%	
≥60	20	70.0%	0.355
PS			
0	12	41.7%	
1	30	70.0%	0.158
Tumor size, cm			
≤4	22	54.5%	
>4	20	70.0%	0.355
Tumor Site			
head	20	65.0%	
body-tail	22	59.1%	0.758
Number of times gemcitabine was administered			
<5	10	80.0%	
≥5	32	56.3%	0.270

*Fisher's exact test.

Table IVb. *Univariate analysis of calculated parameters related to the development of ≥ grade 3 acute intestinal toxicity.*

Characteristic	n	% toxicity	*p*-value*
Small bowel			
irradiated volume, cm^3			
<250	18	66.7%	
≥250	24	58.3%	0.750
max dose, cGy			
<5100	30	60.0%	
≥5100	12	66.7%	0.740
mean dose, cGy			
<1500	22	63.6%	
≥1500	20	60.0%	>0.999
Colon			
irradiated volume, cm^3			
<400	22	59.1%	
≥400	20	65.0%	0.758
max dose, cGy			
<5000	16	68.8%	
≥5000	26	57.7%	0.530
mean dose, cGy			
<1500	21	66.7%	
≥1500	21	57.1%	0.751
Planning target volume			
total volume, cm^3			
<500	16	37.5%	
≥500	26	76.9%	0.021

*Fisher's exact test.

correlated with the development of grade 3+ AIT (p=0.021). The highest incidence of grade 3+ AIT was in patients with the volume of PTV ≥500 cm^3, corresponding to a cube of 7.9 cm on a side.

Discussion

We evaluated the relationship between the AIT and the volume of irradiated small bowel in patients treated with concurrent gemcitabine and radiotherapy for pancreatic cancer and univariate analysis revealed that the volume of irradiated small bowel, which was significantly related to AIT in the treatment of rectal cancer, did not correlate to the AIT here. Minsky *et al.* reported a significant relationship between AIT and the volume of irradiated small bowel in patients with rectal cancer treated with concurrent 5-FU-based chemotherapy and pelvic radiotherapy (17). Orthogonal radiographs were used to calculate the volume of small bowel within the treated volume, using the sum of the anterior-posterior film volume and the lateral film volume. The volume of small bowel in the pelvic radiation field was greater for patients who experienced grade 3+ AIT (441±153 cm^3) compared with those who experienced grade 0-2 acute intestinal toxicity (230±43 cm^3). Baglan *et al.* reported a strong dose-relationship for the development of grade 3+ AIT in patients treated with concurrent 5-FU based chemoradiotherapy for rectal cancer using three-dimensional (3D) treatment planning tools, the same as our method (18). A highly significant association was found between the development of grade 3+ AIT and the average volume of small bowel irradiated to each 5-Gy dose level between 5 and 40 Gy (p<0.001). The volume of small bowel that received at least 15 Gy (V15) was strongly associated with the degree of AIT.

The present report represents the first analysis of AIT using dosimetric analysis in pancreatic cancer treated with chemoradiotherapy. In this study, the patient population and treatment schedule was more homogeneous compared with previous reports for rectal cancer and toxicities were evaluated prospectively, because all patients entered in this analysis were previously enrolled in a clinical trial. The reasons for the different results regarding AIT and the volume of irradiated small bowel between rectal cancer and pancreatic cancer could be several. First, the agent of chemotherapy in the combination of radiotherapy was different between the two groups. In previous reports for rectal cancer, 5-FU based chemotherapy was used, while in our study for pancreatic cancer, gemcitabine was used. An *in vivo* study showed that there was markedly increased normal tissue toxicity, such as jejunal mucosa, when gemcitabine was given more than once a week in combination with radiotherapy (21). Second, the volume of irradiated stomach and duodenum may be related to the AIT in part, since in the treatment of pancreatic cancer the upper abdomen is irradiated and the stomach and duodenum are usually included in the treated volume. However, in this study we did not evaluate the volume of irradiated stomach since it was difficult to evaluate the volume of stomach, exactly, due to the great variation in volume depending on the time of day compared with the small bowel and colon. We also did not evaluate the volume of irradiated duodenum. Because most of the duodenum was included in the radiation field with prophylactic regional lymph node area, the volume of irradiated duodenum was considered similar among the patients.

We found that the PTV was significantly associated with severe AIT. This result indicates that a larger treated volume affects a large volume of normal tissue, not just the small bowel. Recently, in an attempt to decrease the toxicity in the treatment of gemcitabine-based chemoradiotherapy, researchers at the University of Michigan and M.D. Anderson Cancer Center performed and recommended radiation treatment planning, which set only the gross tumor in the target volume without a prophylactic regional lymph node area (11, 14, 22). These authors reported that the PTV ranged from 134 cm^3 to 465 cm^3, with a median value of 255 cm^3, corresponding to a cube of only 6.3 cm on a side, which was much smaller compared with conventional radiotherapy and patients were able to tolerate the treatment (22). Our result that the smaller PTV (<500 cm^3, corresponding to a cube of 7.9 cm on a side) had less acute intestinal toxicity supports their recommendation. However, the efficacy of treatment without prophylactic regional lymph node irradiation should be evaluated in clinical trials and a longer follow-up is needed.

In conclusion, the volume of irradiated small bowel did not directly influence the AIT in patients treated with concurrent weekly gemcitabine and radiotherapy for locally advanced pancreatic cancer. However, only the PTV significantly correlated with severe AIT. Reducing the treated volume, *e.g.*, by omitting prophylactic regional lymph node irradiation, seemed to result in decreased AIT when patients were treated concurrently with gemcitabine-based chemoradiotherapy.

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ORIGINAL REPORT

Pharmacokinetics of Gemcitabine in Japanese Cancer Patients: The Impact of a Cytidine Deaminase Polymorphism

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ABSTRACT

Purpose
Gemcitabine is rapidly metabolized to its inactive metabolite, 2′,2′-difluorodeoxyuridine (dFdU), by cytidine deaminase (CDA). We previously reported that a patient with homozygous 208A alleles of *CDA* showed severe adverse reactions with an increase in gemcitabine plasma level. This study extended the investigation of the effects of *CDA* genetic polymorphisms on gemcitabine pharmacokinetics and toxicities.

Patients and Methods
Genotyping of *CDA* was performed by a direct sequencing of DNA obtained from the peripheral blood of Japanese gemcitabine-naïve cancer patients (n = 256). The patients recruited to the association study received a 30-minute intravenous infusion of gemcitabine at a dose of either 800 or 1,000 mg/m^2, and eight blood samples were periodically collected (n = 250). Plasma levels of gemcitabine and dFdU were measured by high-performance liquid chromatography. Plasma CDA activities toward cytidine and gemcitabine were also measured (n = 121).

Results
Twenty-six genetic variations, including 14 novel ones and two known nonsynonymous single nucleotide polymorphisms (SNPs), were detected. Haplotypes harboring the nonsynonymous SNPs 79A>C (Lys27Gln) and 208G>A (Ala70Thr) were designated **2* and **3*, respectively. The allelic frequencies of the two SNPs were 0.207 and 0.037, respectively. Pharmacokinetic parameters of gemcitabine and plasma CDA activities significantly depended on the number of haplotype **3*. Haplotype **3* was also associated with increased incidences of grade 3 or higher neutropenia in the patients who were coadministered fluorouracil, cisplatin, or carboplatin. Haplotype **2* showed no significant effect on gemcitabine pharmacokinetics.

Conclusion
Haplotype **3* harboring a nonsynonymous SNP, 208G>A (Ala70Thr), decreased clearance of gemcitabine, and increased incidences of neutropenia when patients were coadministered platinum-containing drugs or fluorouracil.

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INTRODUCTION

Gemcitabine (2′,2′-difluorodeoxycytidine) is a nucleoside anticancer drug that has a broad spectrum of antitumor activity against various solid tumors, such as non–small-cell lung cancer and pancreatic cancer.[1] In a randomized clinical trial, gemcitabine was confirmed to provide a survival advantage over fluorouracil in addition to symptom-relieving benefits in patients with advanced pancreatic cancer.[2] On the basis of these results, gemcitabine has generally been accepted as a standard chemotherapeutic agent for advanced pancreatic cancer.

Gemcitabine is transported into cells by concentrative and equilibrative nucleoside transporters,[3-8] where it is phosphorylated to its monophosphate form by deoxycytidine kinase. Gemcitabine triphosphate, an active form of gemcitabine, is incorporated into an elongating DNA strand, and is followed by the addition of another deoxynucleotide that leads to the halt of DNA synthesis.[9,10] Another mode of action in solid tumors, associated with the inhibition of ribonucleotide reductase, has also been suggested.[11]

Gemcitabine is rapidly metabolized to an inactive metabolite, 2′,2′-difluorodeoxyuridine (dFdU)

Table 1. *CDA* Haplotypes Estimated in This Study

Region		5'-Flanking			Exon 1 (5'-UTR)			Exon 1	Intron 1	Exon 2		Intron 2		
SNP ID		CDA001	CDA002	CDA003	CDA004	CDA005	CDA007	CDA009	CDA010	CDA011	CDA012	CDA014	CDA016	CDA017
Nucleotide change		−451C>T	−205C>G	−182G>A	−116G>A	−92A>G	−33_−31 delC	79A>C	IVSI+37 G>A	208G>A	210T>C	IVS2 +87_+88 insTCAT	IVS2+242 A>G	IVS2+296 T>A
Amino acid change								Lys27Gln		Ala70Thr	Ala70Ala			
Haplotypes														
*1	*1a													
	*1b													
	*1c													
	*1d													
	*1e													
	*1f													
	*1g													
	*1h													
	*1i													
	*1j													
	*1k													
	*1l													
	*1m													
	*1n													
	Other *1													
*2	*2a													
	*2b													
	*2c													
	*2d													
	Other *2													
*3	*3a													
	*3b													

(continued on next page)

NOTE. The haplotypes were described as a number plus a small alphabetical letter. Four single nucleotide polymorphisms (SNPs) (CDA006, 008, 013, 015) were found only in the very rare ambiguous *1 haplotypes. Since these ambiguous haplotypes were grouped and described as "Other *1" in this table, the four SNPs are not shown in the row of nuclotide change. White, major allele; gray, minor allele.

by cytidine deaminase (CDA),[9] and most of an administered dose is recovered as dFdU in the urine.[12] CDA is expressed at varying levels in the human tissues,[13] and the rapid clearance of gemcitabine can be attributed to its plentiful occurrence in the liver.[14] Two single nucleotide polymorphisms (SNPs), 79A>C (Lys27Gln) and 435T>C (Thr145Thr), have been discovered in *CDA*, the CDA-encoding gene in humans.[15,16] The 79A>C SNP reportedly reduces the deamination activity (maximum velocity/Km) toward 1-beta-D-arabinofuranosyl cytosine (cytarabine),[15] and increases Km toward gemcitabine,[17] in vitro. A recently discovered third SNP, 208G>A (Ala70Thr) displayed a decrease in deamination activity of 60% for cytidine and 68% for cytarabine when introduced into a *CDA*-null yeast strain.[18]

Toxicity of gemcitabine is generally mild,[19,20] but unpredictable severe toxicities such as myelosuppression are occasionally experienced.[21,22] Our previous case report described a patient with homozygous 208A alleles of the *CDA* gene who showed severe adverse reactions with increased plasma gemcitabine levels.[23] In addition, there has been controversy over the relationship between cellular CDA activity and the clinical effects of cytarabine.[24-27] This study examined the relationship between *CDA* polymorphisms, and the pharmacokinetics of gemcitabine, plasma CDA activity, or adverse reactions in Japanese cancer patients.

PATIENTS AND METHODS

Gemcitabine and dFdU for analytic standards were supplied by Eli Lilly Japan K.K. (Kobe, Japan). Tetrahydrouridine, 3'-deoxy-3'-fluoro-thymidine (3'-dFT), cytidine and uridine (Sigma-Aldrich Chemical Co, St Louis, MO) were purchased. All other chemicals were of highest grade available.

Patients

The participants in this study consisted of 256 Japanese patients with carcinoma, including six patients described in a previous report,[23] at the National Cancer Center Hospital (Tokyo, Japan) and National Cancer Center Hospital East (Kashiwa, Japan). Two hundred fifty-one patients received a 30-minute intravenous infusion of gemcitabine at a dose of either 800 or 1,000 mg/m^2, and five patients received a fixed dose-rate (10 mg/m^2/min) infusion at a dose between 1,000 and 1,500 mg/m^2. The eligibility criteria for the study were as previously reported.[23] The ethics committees of the National Cancer Center and the National Institutes of Health Sciences approved this study. Written informed consent was obtained from each participant.

Table 1. *CDA* Haplotypes Estimated in This Study (continued)

Intron 3					Exon 4	Exon 4 (3'-UTR)					
CDA018	CDA019	CDA020	CDA021	CDA022	CDA023	CDA024	CDA025	CDA026	No.	Frequency	
IVS3+71 T>C	IVS3 −194_−193 insAlu	IVS3-56 G>A	IVS3-36 G>A	IVS3-23 C>T	435C>T	510 (*69) G>T	637_638 (*196_*197) insC	676 (*235) A>G			
					Thr145Thr						
									175	0.342	
									63	0.123	
									52	0.102	
									17	0.033	
									13	0.025	
									12	0.023	
									12	0.023	
									11	0.021	0.756
									8	0.016	
									5	0.010	
									4	0.008	
									4	0.008	
									2	0.004	
									1	0.002	
									8	0.016	
									84	0.164	
									11	0.021	
									5	0.010	0.207
									3	0.006	
									3	0.006	
									18	0.035	0.037
									1	0.002	
									512	1.000	1.000

Monitoring and Toxicities

A complete medical history and data on physical examinations were recorded before the gemcitabine therapy. CBC and platelet counts, as well as blood chemistry, were measured once a week during the first 2 months of gemcitabine treatment. Toxicities were graded according to the National Cancer Institute Common Toxicity Criteria, version 2.

DNA Sequencing

All four exons and the 5'-upstream region (approximately 800 base pairs [bp] from the translation initiation codon) of *CDA* were amplified from 100 ng of DNA extracted from peripheral blood, and sequenced along both strands. Polymerase chain reaction (PCR) primers[23] and sequencing and PCR conditions[28] were described previously. For detection of an approximately 300-bp Alu insertion (IVS3-194_-193insAlu), PCR was performed using a specific primer set (5'- TTGTCATAGCAGAAGGAGGTT-3' and 5'- TCAG CTCTCCACACCATAAGG-3') and 100 ng of DNA as a template. Then, sizes of the amplified fragments were determined by 1% agarose gel electrophoresis. NT_004610.17 (GenBank, National Center for Biotechnology Information, Bethesda, MD) was used as the reference sequence.

Linkage Disequilibrium and Haplotype Analyses

Hardy-Weinberg equilibrium and linkage disequilibrium (LD) analyses were performed by SNPAlyze software (Dynacom Co, Yokohama, Japan). All of the detected variations were found to be in Hardy-Weinberg equilibrium ($P \geq .05$), except for the SNP IVS1+37G>A ($P = .002$). Some of the haplotypes were unambiguously assigned from subjects with homozygous variations at all sites or a heterozygous variation at only one site. The diplotype configurations (a combination of haplotypes) were separately inferred by LDSUPPORT software,[29] which determines the posterior probability distribution of the diplotype configuration for each subject based on the estimated haplotype frequencies. The diplotype configurations of all but 11 subjects were inferred with probability of more than 0.93. All haplotypes inferred in single subjects were gathered as the groups "Other *1" and "Other *2" in Table 1.

Pharmacokinetic Study

Five patients with fixed dose-rate infusion and one patient with interruption of infusion for more than 15 minutes were excluded from the pharmacokinetic analysis described herein. Heparinized blood was collected before administration of gemcitabine and used to measure plasma CDA activity. Five milliliters of heparinized blood was also sampled for pharmacokinetic analysis before the first gemcitabine administration, and at 0, 15, 30, 60, 90, 120, and 240 minutes after the termination of the infusion. Fifty microliters of 1% tetrahydrouridine was immediately added to these samples to prevent ex vivo deamination. Plasma levels of gemcitabine and dFdU were determined using the high-performance liquid chromatography method previously reported.[23] The area under the curve (AUC) and mean residence time from 0 to infinity, peak concentration (C_{max}), clearance (CL/m^2) and distribution volume based on the terminal phase (Vz/m^2) were calculated using WINNonlin (Scientific Consultant, Apex, NC) version 4.01 (Pharsight Corporation, Mountain View,

CA). AUC and C_{max} were corrected for dose, assuming that all patients received 1,000 mg/m^2 of gemcitabine.

CDA Activities in Plasma

Determination of CDA activities was performed using the method by Richards et al[30] with slight modifications (modifications are as follows: gemcitabine was used as a substrate as well as cytidine, internal standards for analysis [3'-dFT for gemcitabine or dFdU for cytidine] were added to the mixtures at the beginning of the reaction, and high-performance liquid chromatography was used for detection of reaction products). CDA activity was expressed by unit, and one unit of enzyme activity was defined as the concentration that produces 0.1 nmol of dFdU or uridine per minute per milliliter of plasma.[30]

Statistical Analysis

Kruskal-Wallis, Mann-Whitney, and Pearson's correlation tests were performed using the JMP software (SAS Institute Inc, Cary, NC). Two ordinally scaled categoric data were subjected to χ^2 analysis for a correlation test. A significance level of .05 was applied to all two-tailed and correlation tests. Multiplicity was adjusted by the false-discovery rate,[31] if necessary.

RESULTS

Genetic Variations and Haplotype Structures of CDA

Twenty-six (14 novel) genetic variations were detected in the 256 Japanese cancer patients enrolled onto this study (Table 2). Three of the novel variations were found in the 5'-untranslated region, one in exon 2, three in the 3'-untranslated region and seven in the introns. Three known SNPs in the coding region of *CDA* were also detected. Among these, the nonsynonymous SNPs, 79A>C (Lys27Gln) and 208G>A (Ala70Thr), exhibited allelic frequencies of 0.207 and 0.037 (Table 2), respectively, and they were comparable to those reported previously.[18] One patient was found to be homozygous for the 208A polymorphism. A novel insertion of an approximately 320-bp Alu element (IVS3-194_-193insAlu) was newly found in intron 3.

The detected variations were used to analyze LD (Fig 1). Four novel variations (IVS3-56G>A, IVS3-36G>A, IVS3-23C>T and

Table 2. Variations of the *CDA* Gene Found

SNP ID				Position				
This Study	NCBI (dbSNP)	JSNP	Location	NT_004610.17	From the Translational Initiation Site or From the Nearest Exon	Nucleotide Change and Flanking Sequences (5' to 3')	Amino Acid Change	Allele Frequency
MPJ6_CDA001	rs532545	IMS-JST008767	5'-Flanking	3739514	−451‡	TGCCTCCTGCCT**C/T**GGGATGCCGCAG		0.199
MPJ6_CDA002	rs603412	IMS-JST008768	5'-Flanking	3739760	−205‡	CACACGTAGGCA**C/G**TGTCTTACACCA		0.266
MPJ6_CDA003	rs12726436		5'-Flanking	3739783	−182‡	CACACCTGCTGA**G/A**TCCAAACCATGG		0.061
MPJ6_CDA004*			Exon 1 (5'-UTR)	3739849	−116‡	CTGAGAGCCTGC**G/A**GTCTGGCTGCAG		0.059
MPJ6_CDA005	rs602950		Exon 1 (5'-UTR)	3739873	−92‡	GGGACACACCCA**A/G**GGGGAGGAGCTG		0.205
MPJ6_CDA006*			Exon 1 (5'-UTR)	3739884	−81‡	AAGGGGAGGAGC**T/C**GCAATCGTGTCT		0.002
MPJ6_CDA007	rs3215400	IMS-JST076939	Exon 1 (5'-UTR)	3739934	−33_−31‡	GCTCCTGTTTCC**C/-**GCTGCTCTGCTG		0.451
MPJ6_CDA008*			Exon 1 (5'-UTR)	3739957	−8‡	TGCCTGCCCGGG**G/A**TACCAACATGGC		0.002
MPJ6_CDA009†	rs2072671	IMS-JST008769	Exon 1	3740043	79‡	CAGGAGGCCAAG**A/C**AGTCAGCCTACT	Lys27Gln	0.207
MPJ6_CDA010	rs12059454		Intron 1	3740155	IVS1+37	CCCAGCCCAGCA**G/A**CCTGGGTGGTGG		0.184
MPJ6_CDA011†			Exon 2	3755816	208‡	GCTGAACGGACC**G/A**CTATCCAGAAGG	Ala70Thr	0.037
MPJ6_CDA012*			Exon 2	3755818	210‡	TGAACGGACCGC**T/C**ATCCAGAAGGCC	Ala70Ala	0.004
MPJ6_CDA013*			Intron 2	3755932	IVS2+58	GCCAACATCTTC**C/T**TTACACATATTA		0.002
MPJ6_CDA014*			Intron 2	3755961_3755962	IVS2+87_+88	TCATTCATTCAT**-/TCAT**CTGACATATGTT		0.135
MPJ6_CDA015*			Intron 2	3756043	IVS2+169	ATAAGGAGATAA**A/G**TAAGAAATGGAG		0.002
MPJ6_CDA016	rs10916825		Intron 2	3756116	IVS2+242	CATACAAGGGCC**A/G**GTATGCCCCTGT		0.289
MPJ6_CDA017	rs818194		Intron 2	3756170	IVS2+296	GTCCTACAAGAT**T/A**TAACAGAAAGGC		0.217
MPJ6_CDA018	rs3738130	IMS-JST083844	Intron 3	3764805	IVS3+71	AGCCACGCCAAG**T/C**TGCAGGCATGGC		0.053
MPJ6_CDA019*			Intron 3	3769093_3769094	IVS3-194_−193	CTGTTCAGTTTC**-/(Alu)**§ACAGCATTCTTT		0.293
MPJ6_CDA020*			Intron 3	3769231	IVS3-56	CAGACCCAGTCC**G/A**TCTCAGCCCCCT		0.293
MPJ6_CDA021*			Intron 3	3769251	IVS3-36	CCCCTCAGCCAC**G/A**CTGTGTCTCTCA		0.293
MPJ6_CDA022*			Intron 3	3769264	IVS3-23	CTGTGTCTCTCA**C/T**GCCAGCTTTGCC		0.293
MPJ6_CDA023†	rs17846527		Exon 4	3769397	435‡	CCTGCAGAAGAC**C/T**CAGTGACAGCCA	Thr145Thr	0.293
MPJ6_CDA024*			Exon 4 (3'-UTR)	3769472	510 (*69)‡	CTCACAGCCCTG**G/T**GGACACCTGCCC		0.002
MPJ6_CDA025*			Exon 4 (3'-UTR)	3769599_3769600	637_638 (*196_197)‡	ACCGCCGCCCCC**-/C**TGCCCCACCTTT		0.293
MPJ6_CDA026*			Exon 4 (3'-UTR)	3769638	676 (*235)‡	GGGCCCTCTTTC**A/G**AAGTCCAGCCTA		0.010

*Novel variations detected in this study.
†Yue et al.[18]
‡A of the translation initiation codon ATG is numbered 1, and the number with * in parentheses indicates the position from the termination codon TGA.
§The sequence of the Alu insertion was as follows: 5' - (T)nGAGACGGAGTCTCGCTGTCGCCCAGGCTGGAGTGCAGTGGCGCAATCTCGGCTCACTGCAGGCTCCG CCCCCTGGGGTTCACGCCATTCTCCTGCCTCAGCCTCCCGAGTAGCTGGGACTACAGGCGCCCGCCACCTCGCCCGGCTAATTTTTTGTATTTTTAGTAGAGACGGGGTT TCACCGTGTTAGCCAGGATGGTCTCGATCTCCTGACCTCGTGATCCGCCCGCCTCGGCCTCCCAAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCCACTG TTCAGTTTC-3' (n = approximately 25).

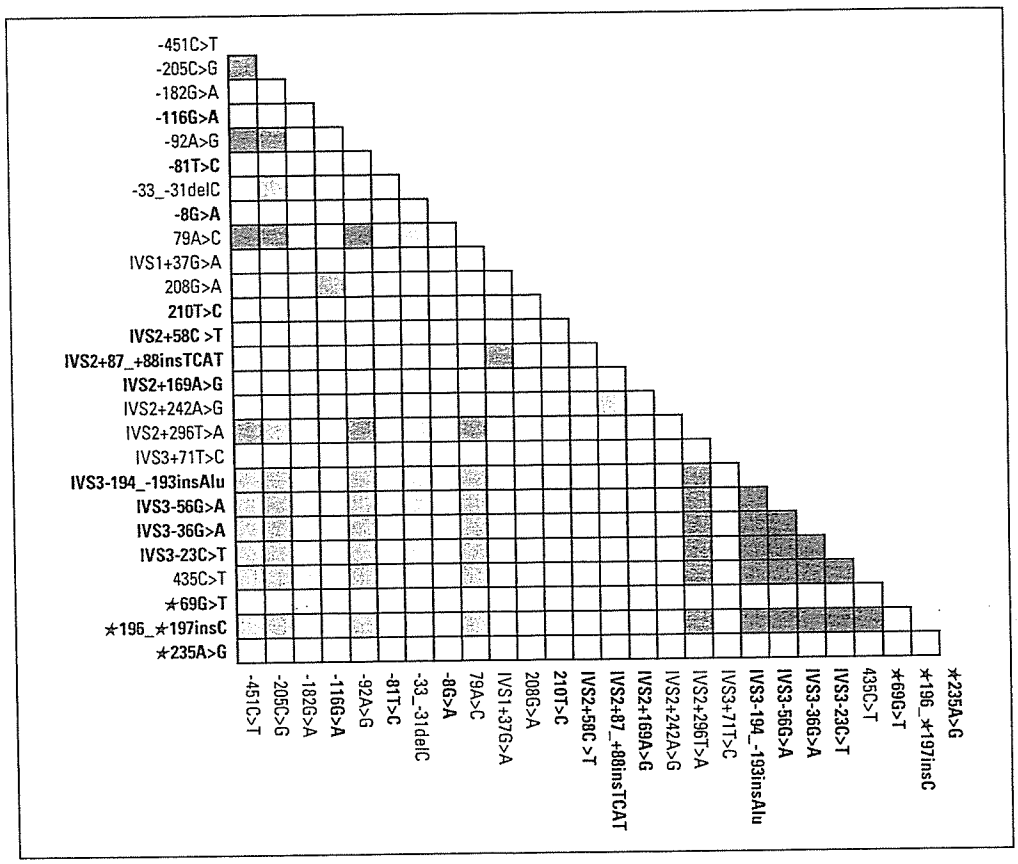

Fig 1. Linkage disequilibrium (LD) among 26 *CDA* variations. Pairwise LD as r^2 (from 0 to 1) is expressed as 10-graded blue color. The density of the blue color increases with higher linkage rates.

*196_*197insC), the Alu element insertion and a known SNP 435C>T (Thr145Thr) showed complete linkage (Fig 1) with a frequency of 0.293. Strong LD ($r^2 \geq 0.93$) was also observed among SNPs −451C>T, −92A>G, and 79A>C. Note that moderate linkages ($r^2 \geq 0.42$) were observed between the two completely and strongly linked groups (Fig 1). Because relatively close linkages were observed throughout the entire *CDA* gene spanning approximately 30 kb, the *CDA* haplotypes were analyzed as one LD block.

The haplotypes determined/inferred in this study are summarized in Table 1. Haplotypes without amino acid changes were defined as the **1* group. These harboring the nonsynonymous SNPs 79A>C and 208G>A were designated **2* and **3*, respectively. The most frequent haplotype was **1a* (frequency, 0.342), followed by **2a* (0.164), **1b* (0.123), and **1c* (0.102).

Effects of Patient Background Factors on Gemcitabine Pharmacokinetics

Characteristics of the 250 patients recruited for the pharmacokinetic study are shown in Table 3. As previously reported, the patient who was homozygous for 208A showed extraordinarily high gemcitabine and low dFdU plasma concentrations.[23] Therefore, this patient was excluded when effects of patient background factors on the pharmacokinetic parameters of gemcitabine were analyzed.

The effects of age and sex on pharmacokinetic parameters are summarized in Table 4. Vz/m^2 was significantly higher in males than in females, even after adjustments for their body surface areas (Mann-Whitney $P = .0031$). The C_{max}, AUC, CL/m^2, and Vz/m^2 of gemcitabine showed significant correlations with age ($P < .0001$ for all parameters). Values of any clinical tests, including creatinine concentration, did not correlate with pharmacokinetic parameters of gemcitabine. Although approximately 30% of patients in this study underwent combined chemotherapy, no clinically significant effects of coadministered drugs on pharmacokinetic parameter values of gemcitabine were detected.

Effects of CDA *Genetic Polymorphisms on Gemcitabine Pharmacokinetics*

Because age and sex were unbiasedly distributed among the patients, with the various genotypes compared in the following analysis (data not shown), the 250 patients were not further stratified.

After careful examination, the data did not identify any **1*, **2*, or **3* subtypes that showed statistically significant differences from each major subtype within the three groups (Table 5; unpublished data). Therefore, each subtype was combined into one group (the **1*, **2*, or **3* group) to investigate the association between pharmacokinetic parameters and genetic groups.

The relationships between the diplotype groups and the pharmacokinetic parameters of gemcitabine are shown in Figure 2 and summarized in Table 6. The data clearly showed a haplotype **3*–dependent decrease in clearance and increases in C_{max} and AUC values (χ^2 trend $P < .0001$ for all parameters). The values of C_{max}, AUC, and CL/m^2 observed in the patient bearing a homozygous 208G>A (**3/*3*) were two-fold, five-fold, and one-fifth of the means of the **1/*1* group, respectively (Table 6). In contrast, the pharmacokinetic parameters of gemcitabine except for mean residence time (data not shown) were not significantly influenced by the haplotype **2*.

Table 3. Characteristics of Patients Recruited to Pharmacokinetic Studies (N = 250)

Characteristic	
Sex	
Male	165
Female	85
Age, years	
Mean	62.6
Range	32-80
SD	9.2
Body surface area, m^2	
Mean	1.57
Range	1.18-1.99
SD	0.17
Weight, kg	
Mean	54.8
Range	34.4-80.3
SD	9.7
Performance status	
0	122
1	118
2	10
Primary tumor	
Pancreas	205
Lung	38
Mesothelium	7
Dose, mg/m^2	
1,000	246
800	4
Regimen	
Gemcitabine alone	180
Gemcitabine-based combination	70
Cisplatin	30
Carboplatin	16
Fluorouracil	14
Vinorelbine ditartrate	10
Previous treatment	
None	134
Surgery	66
Radiation	74
Chemotherapy	65

*Effect of Haplotypes *2 and *3 on Plasma CDA Activity*

Plasma CDA activities were measured in 121 patients of the 250 patients in this study. One patient in the **1/*2* group who showed extremely high plasma CDA activities to both gemcitabine and cytidine (43.04 and 29.04 units, respectively; far higher than the 99% upper confidence limits of plasma CDA activities for the **1/*2* group) was excluded as an outlier from the following statistical analysis, although his pharmacokinetic parameters were quite normal.

Haplotype **2* failed to show any significant effects on the plasma CDA activities toward both gemcitabine and cytidine. On the other hand, activity decreased depending on the number of haplotype **3* (Table 6; Fig 3). The plasma CDA activities in the homozygous **3* (208A) patient were 12% (gemcitabine) and 25% (cytidine) of the median activities for the **1/*1* patients. As shown in Figure 4, a statistically significant correlation between the plasma CDA activity toward gemcitabine and the AUC values of gemcitabine was observed ($r = -0.30$; $P = .0009$). However, the correlations were not remarkable.

*Effect of Haplotype *3 on Toxicities*

Then, associations of haplotype **3* with toxicities were analyzed. Nadir grades of neutrophil counts were compared between the patient groups with and without haplotype **3* under the individual therapeutic regimens. As shown in Table 7, there were no significant differences in incidences of grade 3 or higher neutropenia between the two groups under the gemcitabine monotherapy. However, when gemcitabine was administered with carboplatin, cisplatin, or fluorouracil, grade 3 or higher neutropenia was more frequently observed in the haplotype **3*–bearing group than in the group without haplotype **3*. The increases in incidences were statistically significant. AUC values were also increased in the group with haplotype **3* under concomitant therapeutic regimen as under the monotherapy.

DISCUSSION

The pharmacokinetic parameters summarized in Table 4 showed great similarity to those obtained with adult American patients.[32] The age-dependent decrease in gemcitabine clearance in Japanese patients in this study is in agreement with the description for Gemzar injections (Eli Lilly Japan K.K.), which is based on a population pharmacokinetic study performed outside Japan. The main route of gemcitabine elimination is its metabolism into dFdU, and there was no correlation between plasma creatinine level and gemcitabine clearance. Therefore, the aging effect on gemcitabine clearance is likely to result from a decrease in distribution volume or liver function. It is

Table 4. Effects of Patient Background Factors on Pharmacokinetic Parameters of Gemcitabine

Factor	C_{max} (μg/mL) Median	C_{max} (μg/mL) 1/4-3/4 Quantiles	AUC (hr · μg/mL) Median	AUC (hr · μg/mL) 1/4-3/4 Quantiles	CL/m^2 (L/hr/m^2) Median	CL/m^2 (L/hr/m^2) 1/4-3/4 Quantiles	Vz/m^2 (L/m^2) Median	Vz/m^2 (L/m^2) 1/4-3/4 Quantiles
Sex								
Male	23.1	18.4-26.1	9.9	8.6-11.8	100.3	83.7-115.9	42.4*	35.13-52.0
Female	24.0	19.8-28.8	10.2	9.0-11.5	97.6	86.1-111.2	38.7	32.7-43.5
Mann-Whitney *U* test	NS		NS		NS		$P < .005$	
Age								
Spearman r	0.32		0.39		−0.39		−0.39	
P value	< .0001		< .0001		< .0001		< .0001	

Abbreviations: C_{max}, peak concentration; AUC, area under the curve; CL/m^2, clearance; Vz/m^2, distribution volume based on the terminal phase.
*Significantly different from the value for female (Mann-Whitney *U* test $P = .0031$).

Table 5. Pharmacokinetic Parameters of Gemcitabine in Patients With Various *CDA* Diplotypes

Diplotype	No. of Patients	Median Gemcitabine PK Parameters				
		C_{max} (μg/mL)	AUC (hr · μg/mL)	CL/m² (L/hr/m²)	MRT (hours)	AUC Ratio (dFdU/gemcitabine)
*1a/*1a	30	22.40	10.54	94.24	0.37	8.86
*1a/*1b	17	22.75	10.08	97.91	0.35	9.08
*1b/*1b	6	20.81	9.19	108.60	0.36	9.19
P value*		0.82	0.40	0.59	0.97	0.83
*1a/*1c	23	23.23	10.87	94.31	0.35	8.73
*1c/*1c	1	25.84	16.62	60.16	0.55	8.40
P value*		0.77	0.57	0.94	0.97	0.83
*1a/*1d	7	22.05	9.07	108.30	0.36	9.04
*1d/*1d	1	26.43	9.99	100.10	0.31	7.70
P value*		0.82	0.45	0.90	0.86	0.57
*2a/*2a	8	23.94	9.34	107.20	0.33	9.70
*2a/*2b	4	23.02	9.78	100.13	0.38	8.59
*2a/*2c	2	21.50	9.22	111.63	0.36	10.99
P value†		0.66	0.98	0.76	0.077	0.46

Abbreviations: PK, pharmacokinetics; C_{max}, peak concentration; AUC, area under the curve; CL/m², clearance; MRT, mean residence time; dFdU, 2′,2′-difluorodeoxyuridine.
**P* value of a correlation test among *1a/*1a, *1a/(*1b, *1c, or *1d), and (*1b, *1c, or *1d)/(*1b, *1c, or *1d). Multiplicity is adjusted by false-discovery rate.
†*P* value of a Kruskal-Wallis test among *2a/*2a, *2a/*2b, and *2a/*2c.

also indicated on the label that the elimination half-life of gemcitabine was longer in females than in males in a population pharmacokinetic study using 45 Japanese non–small-cell lung cancer patients. The present study did not reveal any significant sex-based difference in clearance. However, the distribution volume was significantly smaller in females than in males.

Human CDA is involved in the salvaging of pyrimidines,[33,34] and plays a key role in detoxifying gemcitabine. Although the activities of 27Gln or 70Thr variant (the products of 79A>C or 208G>A) toward cytidine and cytarabine were reported to be lower than those of the "prototype" in a yeast expression system,[18] the decreased CDA activity in patients bearing these SNPs has not been reported. Kreis et al[35] reported that the response of leukemic patients to cytarabine correlated with the phenotype of CDA deamination determined based on the ratio of plasma concentrations of a cytarabine metabolite and cytarabine.[35] They reported that 70% of subjects were slow metabolizers. However, the relationship between genetic polymorphisms and phenotypes remained to be clarified.

In our study, the haplotype *2 harboring 79C (27Gln) did not show clear effects on the AUC and CL/m² values. In contrast, the 208A (Thr70, *3) -dependent decreases in gemcitabine clearance and plasma CDA activities were clearly demonstrated in this study. These results suggest that the *CDA* variant loses its in vivo deamination activities toward gemcitabine considerably. Moreover, the decreased plasma CDA activities toward gemcitabine and cytidine ex vivo also strongly suggest that the reduced enzymatic activity was caused by the genetic variation.

In the monotherapy group, the increased AUC in the patient with haplotype *3 did not clearly augment the incidence of toxicities including neutropenia. However, the incidences of grade 3 or higher neutropenia were higher in patients heterozygous for haplotype *3 compared with in the patients without haplotype *3 when they received concomitant chemotherapy with fluorouracil or platinum compounds. As we reported recently, one patient homozygous for haplotype *3 who received both gemcitabine and cisplatin suffered from extremely severe adverse effects including grade 3 anathema.[23] However, he experienced neither of the specific toxicities associated with cisplatin, nephrotoxicity, and neurotoxicity. Abbruzzese et al[36] reported the gemcitabine dose-dependent increase in incidence of thrombocytopenia (one of seven at 525 mg/m²/wk, three of nine at 790 mg/m²/wk, and three of six at 1,000 mg/m²/wk).[36] Therefore, we concluded that extremely high exposure to gemcitabine (AUC five times higher than the average) due to the decreased deamination activity caused the life-threatening severe toxicities in this patient. In contrast, the gemcitabine AUC of the patients with heterozygous haplotype *3 was only slightly (23% to 48%) increased from that of the patients having no haplotype *3 (Table 6). This finding coincides with the lack of life-threatening severe toxicities in the heterozygotes for *3, although the incidences of grade 3 or higher neutropenia in the heterozygotes in combined chemotherapy groups were higher in the group without haplotype *3.

CDA is also involved in the activation of capecitabine to its active form fluorouracil.[37] Therefore, capecitabine activation would be inefficient in patients who are homozygous for 208A. The allele frequency of the 208G>A SNP, a tagging SNP of haplotype *3, was reported to be 0.125 in Africans, while it was not detected in Europeans.[38] The frequency of homozygous carriers of the variant could be higher in Africans than in the Japanese population. However, the frequency of 208G>A in Africans is still controversial, because it was not detected in 60 African Americans in a recent report.[17] Extra attention may be necessary for patients with the allele before treatments with gemcitabine or cytarabine are initiated, especially to *3/*3 patients, although more studies are necessary to confirm the clinical importance of this allele in the treatments using gemcitabine or cytarabine.

A number of studies have investigated the associations between cellular CDA activity and drug responses to cytarabine.[24-27,39] However, correlation between plasma CDA activity and the