therapy are limited to ~10% (20–22), and the majority of pancreatic cancers do not show significant tumor regression. Given that the ultimate goal of gemcitabine therapy for pancreatic cancer is to achieve prolonged survival, it would be desirable to stratify patients according to survival rather than tumor response (9). In the present study, using 2DICAL, we compared the base-line plasma proteome of two extreme populations of patients who had shown distinct clinical courses after identical gemcitabine treatment.

EXPERIMENTAL PROCEDURES

Patients—Samples were collected from a total of 304 patients who had all been included in our previous study (8). All patients had metastatic (stage IVb; n = 285) or locally advanced (stage IVa; n = 19) (23) histologically or cytologically proven pancreatic ductal adenocarcinoma and had received at least two cycles of gemcitabine monotherapy (1,000 mg/m^2 intravenously over 30 min on days 1, 8, and 15 of a 28-day cycle). Two hundred eighty-one patients (92%) received gemcitabine as a first line therapy (supplemental Table S1).

Two hundred sixty-two patients (86%) were treated consecutively at the National Cancer Center (NCC) Hospital (Tokyo, Japan) between September 2002 and June 2007, and 42 patients (14%) were treated consecutively at the NCC Hospital East (Kashiwa, Japan) between September 2002 and July 2004. Survival times were determined as of May 2008. During this period, 248 patients (82%) died, and 56 patients (18%) were censored. Tumor response was evaluated after the first two cycles of gemcitabine using the Response Evaluation Criteria in Solid Tumors guideline.

Sample Preparation—Blood was collected before the first administration of gemcitabine. Plasma or serum was separated by centrifugation at 1,050 × g for 10 min at 4 °C and frozen until analysis as reported previously (8, 24). Macroscopically hemolyzed samples were excluded from the current analysis. Two hundred fifty-two plasma samples (83%) were collected from the NCC Hospital and Hospital East, and 52 serum samples (17%) were collected from the NCC Hospital. Written informed consent was obtained from all patients before blood sampling. The protocol of this retrospective study was reviewed and approved by the institutional ethics committee boards of the NCC (Tokyo, Japan) and the National Institute of Health Sciences (Tokyo, Japan).

LC-MS—Samples were blinded, randomized, and passed through an IgY-12 High Capacity Spin Column (Beckman Coulter, Fullerton, CA) in accordance with the manufacturer's instructions. The flow-through portion was digested with sequencing grade modified trypsin (Promega, Madison, WI) and analyzed in triplicate using a nanoflow high performance LC system (NanoFrontier nLC, Hitachi High Technologies, Tokyo, Japan) connected to an electrospray ionization quadrupole time-of-flight mass spectrometer (Q-Tof Ultima, Waters, Milford, MA). LC-MS run order was also randomized to eliminate any potential bias.

MS peaks were detected, normalized, and quantified using the in-house 2DICAL software package as described previously (16). A serial identification (ID) number was applied to each of the MS peaks detected (1–45,277). The stability of LC-MS was monitored by calculating the correlation coefficient (CC) and coefficient of variance (CV) of every triplicate measurement. The mean CC and CV ± S.D. for all 45,277 peaks observed in the 60 triplicate runs were as high as 0.970 ± 0.022 and as low as 0.056 ± 0.017, respectively.

Protein Identification by MS/MS—Peak lists were generated using the Mass Navigator software package (version 1.2) (Mitsui Knowledge Industry, Tokyo, Japan) and searched against the NCBInr database (downloaded on May 20, 2008) using the Mascot software package (version 2.2.1) (Matrix Science, London, UK). The search parameters used were as follows. A database of human proteins was selected.

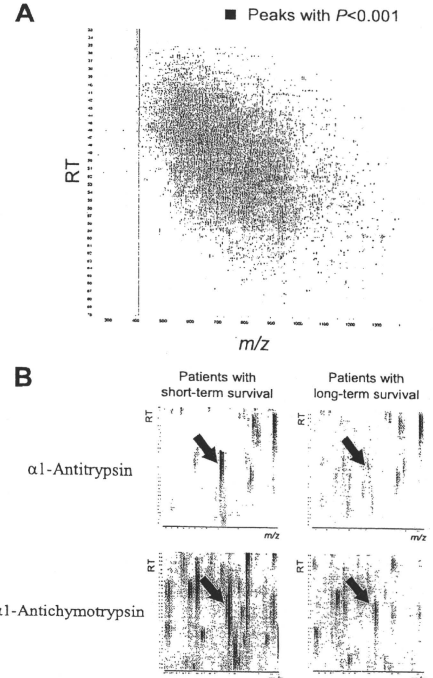

FIG. 1. *A*, two-dimensional display of all (>45,000) the MS peaks. The 637 MS peaks whose mean intensity differed significantly between patients with short term and long term survival ($p < 0.001$, Welch's t test) are highlighted in *red*. *B*, two MS peaks with the smallest p values (*upper*, $p = 2.57 \times 10^{-4}$; *bottom*, $p = 5.03 \times 10^{-4}$) in representative patients with short term (*left*) and long term (*right*) survival. *RT*, retention time.

Trypsin was designated as the enzyme, and up to one missed cleavage was allowed. Mass tolerances for precursor and fragment ions were ±2.0 and ±0.8 Da, respectively. The score threshold was set to $p < 0.05$ based on the size of the database used in the search. If a peptide matched to multiple proteins, the protein name with the highest Mascot score was selected.

Western Blot Analysis—Primary antibodies used were rabbit polyclonal antibody against human α_1-antitrypsin (Dako, Glostrup, Denmark), rabbit polyclonal antibody against human α_1-antichymotrypsin (Dako), and mouse monoclonal antibody against human complement C3b-α (Progen, Heidelberg, Germany). Ten microliters of partitioned sample was separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane. The membrane was then incubated with the primary antibody and subsequently with the relevant horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG as described previously (25, 26). Blots were developed using an ECL detection system (GE Healthcare).

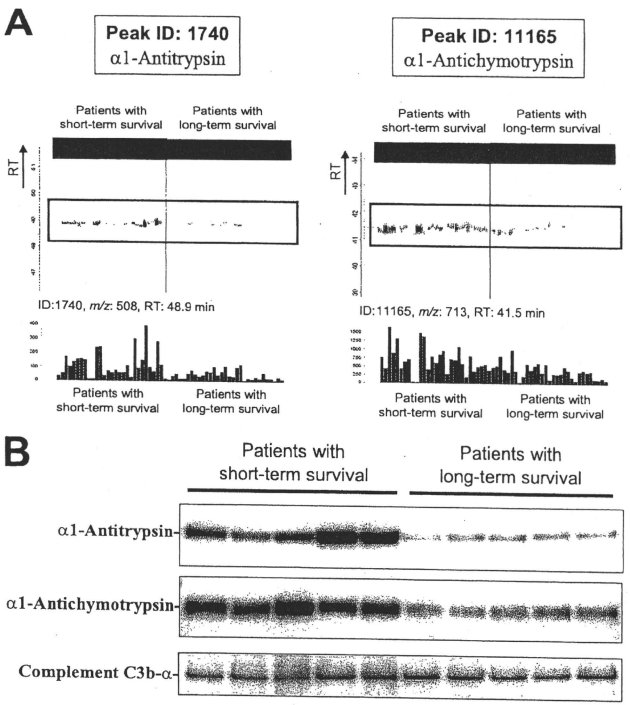

FIG. 2. *A*, representative MS peaks in 60 triplicate LC-MS runs (29 with short term survival (*red*) and 31 with long term survival (*blue*)) aligned along the retention time (*RT*) of LC. *Columns* represent the mean intensity of triplicates (*bottom*). *B*, detection of α_1-antitrypsin, α_1-antichymotrypsin, and complement C3b-α (loading control) by immunoblotting.

Reverse-phase Protein Microarray—Samples were serially diluted 1:500, 1:1,000, 1:2,000, and 1:4,000 using a Biomek 2000 Laboratory Automation Robot (Beckman Coulter) and randomly plotted onto ProteoChip® glass slides (Proteogen, Seoul, Korea) in quadruplicate in a 6,144-spot/slide format using a Protein Microarrayer Robot (Kaken Geneqs Inc., Matsudo, Japan). The spotted slides were incubated overnight with the same primary antibodies as those used in Western blotting. The slides were incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and subsequently with streptavidin-horseradish peroxidase conjugate (GE Healthcare). The peroxidase activity was detected using the Tyramide Signal Amplification (TSA®) Cyanine 5 System (PerkinElmer Life Sciences). The slides were counterstained with Alexa Fluor® 546-labeled goat anti-human IgG (Invitrogen) (spotting control).

The stained slides were scanned on a microarray scanner (InnoScan® 700AL, Innopsys, Carbonne, France). Fluorescence intensity, determined as the mean net value of quadruplicate samples, was determined using the Mapix® software package (Innopsys). All determined intensity values were transformed into logarithmic variables.

The reproducibility of reverse-phase protein microarray assay was revealed by repeating the same experiment. A plasma sample was serially diluted within a range of 1,024–16,384-fold. Each diluted sample was spotted in quadruplicate onto glass slides and blotted with anti-α_1-antitrypsin antibody. In a representative quality control experiment, the CC value was 0.977 between days, and the median CV was 0.026 among quadruplicate samples.

Statistical Analysis—Overall survival time was defined as the period from the date of starting gemcitabine monotherapy until the date of death from any cause or until the date of the last follow-up at which point the data were censored. We used the Kaplan-Meier method to plot overall survival curves. Statistical significance of intergroup differences was assessed with Welch's *t* test, Wilcoxon test, χ^2 test, or log rank test as appropriate. The maximally selected statistics (27) using the fitness of univariate Cox model (log likelihood) was used to determine which level (optimal cutoff point) of each factor best segregated patients in terms of survival.

Multivariate regression analysis was performed using ordinal Cox regression modeling. Factors included in the prediction model were selected with a forward stepwise selection procedure using Akaike's information criterion (AIC), and the result was confirmed using a backward stepwise procedure. The significance of differences between models with and without α_1-antitrypsin was assessed with the likelihood ratio test. The survival prediction model was internally validated by measuring both discrimination and calibration (28). Discrimination was evaluated using the concordance index, which is similar in concept to the area under the receiver operating characteristic curve. Calibration was evaluated with a calibration curve whereby patients are categorized by predicted survival and then

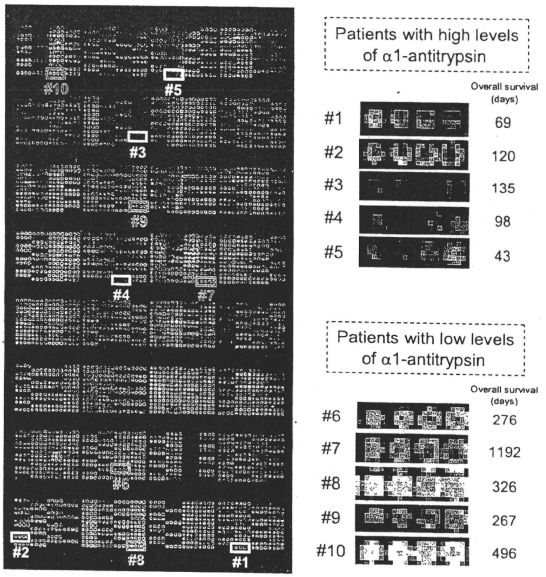

FIG. 3. *Left*, representative reverse-phase protein microarray slide stained with anti-α_1-antitrypsin antibody. *Right*, samples were randomly assigned, and quadruplicate spots of representative patients with high and low levels of α_1-antitrypsin were extracted.

plotted as actual *versus* predicted survival. Both discrimination and calibration were evaluated for the whole study cohort using 200 cycles of bootstrap resampling. Statistical analyses were performed using the open source statistical language R (version 2.7.0) with the optional module Design package.

RESULTS

The median survival estimate for the present study was 236 days (95% CI, 216–254 days), which is comparable to those of previous large scale studies (10, 22). To identify a prognostic factor in patients with advanced pancreatic cancer, we compared the base-line plasma proteome between 29 patients showing short term survival (<100 days) and 31 patients showing long term survival (>400 days) using 2DICAL. There was no significant difference in age, sex, body surface area, prior therapy, clinical stage, or gemcitabine pharmacokinetics (24) (Table I) between the two groups, but the patients with short term survival had significantly poorer base-line conditions such as liver function and Eastern Cooperative Oncology Group (ECOG) performance status than those with long term survival (Table I).

Among a total of 45,277 independent MS peaks detected within the range 250–1,600 *m/z* and within the time range of 20–70 min, we found that the mean intensity of triplicates differed significantly for 637 peaks ($p < 0.001$, Welch's *t* test). Fig. 1*A* is a representative two-dimensional view of all the MS peaks displayed with *m/z* along the *x axis* and the retention time of LC along the *y axis*. The 637 MS peaks whose expression differed significantly between patients with short term and long term survival are highlighted in *red*.

MS peaks that were increased in patients with short term survival with the highest statistical significance ($p = 2.57 \times 10^{-4}$) (Fig. 1*B*) matched the amino acid sequences of the α_1-antitrypsin (*AAT*) gene product (supplemental Fig. S1A). The MS peak with the second highest statistical significance ($p = 5.03 \times 10^{-4}$) was revealed to be derived from the α_1-antichymotrypsin (*AACT*) gene product (supplemental Fig. S1B). We calculated the false discovery rate (FDR) (29) and confirmed the significance of these MS peaks (FDR = 0.0327 for α_1-antitrypsin and FDR = 0.0428 for α_1-antichymotrypsin). Fig. 2*A* shows the distribution of the two peaks (ID 1740 (at 508 *m/z* and 48.9 min; α_1-antitrypsin) and ID 11165 (at 713 *m/z* and 41.5 min; α_1-antichymotrypsin)) in patients with short term (*red*) and long term survival (*blue*). The differential expression and identification of α_1-antitrypsin and α_1-antichymotrypsin were confirmed by denaturing SDS-PAGE and immunoblotting (Fig. 2*B*).

Correlation of α_1-Antitrypsin and α_1-Antichymotrypsin with Overall Survival—The relative levels of α_1-antitrypsin and α_1-antichymotrypsin in plasma or serum samples obtained from 304 patients with advanced pancreatic cancer prior to gemcitabine treatment (including 60 patients used in 2DICAL)

TABLE II

Univariate and multivariate Cox regression analyses of overall survival since the start of gemcitabine therapy (n = 304)

Factors except sex are regarded as continuous variables. A forward stepwise selection based on Akaike's information criterion was used to select parameters for multivariate analysis. *p* values of <0.050 are shown in bold. AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.

	Univariate analysis		Multivariate analysis	
	Hazard ratio[a] (95% CI)	*p*	Hazard ratio[a] (95% CI)	*p*
Age (years)	0.99 (0.98–1.01)	0.380		
Female sex (*vs.* male)	1.07 (0.83–1.38)	0.610		
ECOG performance status	1.49 (1.22–1.80)	**<0.001**	1.36 (1.11–1.67)	**0.003**
Body surface area (m^2)	0.70 (0.33–1.50)	0.360		
Leukocytes	1.08 (1.05–1.11)	**<0.0001**	1.04 (1.00–1.08)	0.066
Platelets	1.07 (0.90–1.28)	0.450		
Hemoglobin (g/dl)	0.93 (0.85–1.01)	0.098		
Albumin (g/dl)	0.61 (0.45–0.82)	**0.001**		
Creatinine (mg/dl)	1.13 (0.60–2.14)	0.700		
AST (IU/liter)	1.01 (1.00–1.01)	**<0.001**		
ALT (IU/liter)	1.00 (1.00–1.01)	**0.033**		
ALP	1.09 (1.06–1.11)	**<0.0001**	1.07 (1.05–1.10)	**<0.0001**
α_1-Antitrypsin[b]	5.92 (3.09–11.37)	**<0.0001**	3.66 (1.89–7.11)	**0.0001**
α_1-Antichymotrypsin[b]	11.60 (2.69–50.01)	**0.001**		
Clinical stage IVa[c] (*vs.* IVb)	1.10 (0.85–1.38)	0.453		

[a] Hazard ratios are per 1,000/mm^3 increase for leukocytes, per 10 × 10^4/mm^3 increase for platelets, and per 100 units/liter increase for ALP. Hazard ratios for other continuous variables are per 1 unit increase for each variable.

[b] Logarithmic variable determined by reverse-phase protein microarray.

[c] According to Ref. 23.

were measured using reverse-phase protein microarrays (Fig. 3). Quadruplicate spots for representative patients with high and low levels of α_1-antitrypsin are shown in Fig. 3. There were no differences between plasma (n = 252) and serum (n = 52) with regard to the levels of α_1-antitrypsin and α_1-antichymotrypsin (plasma *versus* serum (mean ± S.D.): α_1-antitrypsin, 2.10 ± 0.19 *versus* 2.16 ± 0.16, p = 0.06; α_1-antichymotrypsin, 4.44 ± 0.10 *versus* 4.45 ± 0.08, p = 0.67).

Although the levels of α_1-antitrypsin and α_1-antichymotrypsin were not mutually correlated (Pearson's r = 0.274), either level showed a significant correlation with overall survival (Table II). When the most optimal cutoff value was determined by maximally selected analysis, the median survival time of patients with high levels of α_1-antitrypsin (>2.09 arbitrary units) was significantly shorter than that of patients with low levels (≤2.09) (201 days (95% CI, 176–219 days) *versus* 327 days (95% CI, 271–439 days), log rank $p = 2.26 \times 10^{-9}$; Fig. 4*A*). Similarly, the median survival time was significantly shorter in patients with α_1-antichymotrypsin levels of >4.41 (211 days (95% CI, 193 to 235 days)) than in those with levels of ≤4.41 (327 days (95% CI, 255–416 days)) ($p = 2.02 \times 10^{-4}$; Fig. 4*B*). Even when the 60 patients used for 2DICAL were excluded, the differences in survival separated by α_1-antitrypsin and α_1-antichymotrypsin levels were still significant (supplemental Fig. S2, A and B). However, the level of either α_1-antitrypsin or α_1-antichymotrypsin was not associated with tumor response (Spearman's ρ = 0.090 and ρ = 0.017, respectively). The increased level of α_1-antitrypsin in 58 patients who subsequently developed progressive diseases was statistically significant (p = 0.020; supplemental Fig. S3) but quite modest, confirming that it is not a predictive biomarker of tumor response.

Construction and Validation of Model Predicting Overall Survival Time—Univariate Cox regression analysis revealed that ECOG performance status and laboratory values including leukocyte count, albumin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, α_1-antitrypsin, and α_1-antichymotrypsin were correlated with overall survival of the 304 patients ($p < 0.05$; Table II). Because none of the parameters were able to predict survival outcome satisfactorily when used individually (data not shown), we attempted to construct a multivariate predictive model for estimation of overall survival. We searched for parameters using a forward stepwise selection procedure by AIC from all the clinical and laboratory data listed in Table II (available for all 304 cases) and found that a combination of α_1-antitrypsin, alkaline phosphatase, leukocyte count, and ECOG performance status provided the lowest AIC value. We also searched for parameters using a backward elimination algorithm and found that this identified the same combination of factors as that selected by a forward stepwise procedure. The base-line α_1-antitrypsin level was the second most significant contributor to the model (Table II). The prediction model using this combination of parameters was significantly compromised when the level of α_1-antitrypsin was excluded ($\Delta\chi^2 = 14.12$, $df = 1$, $p = 0.0002$, likelihood ratio test).

Based on the results of multivariate Cox regression analysis, we constructed a scoring system (nomogram) in which the values of the four parameters (α_1-antitrypsin, alkaline phosphatase, leukocyte count, and ECOG performance status) were integrated into a single score (total point) to estimate

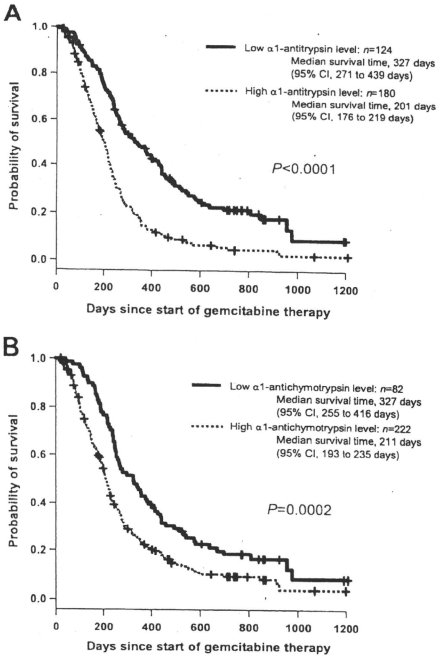

FIG. 4. **Kaplan-Meier plots of overall survival according to α_1-antitrypsin (*A*) and α_1-antichymotrypsin (*B*) levels.**

the survival outcome (Fig. 5*A*). The accuracy of the nomogram for prognostication was internally validated. The bootstrap-corrected concordance index was 0.672, and the calibration curve demonstrated good agreement between the predicted and observed outcomes (Fig. 5*B*). It was possible to estimate high risk patients by calculating the total points using the nomogram. The median survival time was 150 days (95% CI, 123–187 days) for patients with a total point score of >94 (n = 98) and 282 days (95% CI, 255–328 days) for patients with a score of ≤94 (n = 206), and the difference was significant ($p = 2.00 \times 10^{-15}$, log rank test; Fig. 5*C*). Even when the 60 patients used for 2DICAL analyses were excluded from the total points calculation, the difference was still significant ($p = 5.23 \times 10^{-10}$; supplemental Fig. S2C). The median survival time was 171 days (95% CI, 147–205 days) for patients with a score of >92 (n = 83) and 270 days (95% CI, 243–299 days) for patients with a score of ≤92 (n = 161). The cutoff value that optimally segregated patients into subgroups with a poor and good prognosis was determined by using the maximally selected statistics.

DISCUSSION

Currently, no diagnostic tool has been established for stratifying patients with advanced pancreatic cancer according to their likelihood of obtaining a survival benefit from gemcitabine treatment. Because some high risk patients may achieve prolonged survival through modification (or even withdrawal) of therapeutic protocols, a diagnostic method that can accurately identify such patients is necessary. We first compared the plasma proteome of two groups of patients who showed distinct clinical courses after receiving the same gemcitabine protocol (Fig. 1) and found that individuals who showed poor clinical courses had shown high base-line levels of plasma α_1-antitrypsin and α_1-antichymotrypsin (Figs. 1*B* and 2*A*). α_1-Antitrypsin is an abundant plasma protein that usually cannot be measured by MS. However, antibody-based protein depletion (30) allowed us to accentuate the differences in α_1-antitrypsin levels.

The results obtained by 2DICAL were then validated in a 5-fold larger cohort using a different methodology: high density reverse-phase protein microarray (Figs. 3 and 4 and Table II). Reverse-phase protein microarray is an emerging proteomics technology capable of validating new biomarkers because of its overwhelmingly high throughput (31, 32). Furthermore, reverse-phase protein microarrays require significantly smaller amounts of clinical samples for quantification than established clinical tests, such as ELISA. The prognostic significance of α_1-antitrypsin was further supported by multivariate survival analysis with stepwise covariate selection. The level of α_1-antitrypsin was selected as the second most significant factor following alkaline phosphatase (Table II), but α_1-antichymotrypsin was not selected. To derive clinical applicability from the above findings, we constructed a model (nomogram) including α_1-antitrypsin to estimate the survival period of pancreatic cancer patients (Fig. 5*A*), and its significance was internally validated (Fig. 5*B*). One previous study has demonstrated a correlation between an increased serum level of α_1-antitrypsin and short survival in patients treated surgically for pancreatic cancer (33). Although the number of cases examined was small (n = 44), the results support our present findings.

α_1-Antitrypsin and α_1-antichymotrypsin are members of the serine protease inhibitor (serpin) superfamily that plays key roles in the regulation of inflammatory cascades (34, 35). α_1-Antitrypsin and α_1-antichymotrypsin interact mainly with neutrophil elastase and neutrophil cathepsin G, respectively, and inhibit their protease activities (36). A protease-to-protease inhibitor imbalance in patients with genetic α_1-antitrypsin deficiency is reported to confer a higher risk of chronic pancreatitis (37). However, the serum level of α_1-antitrypsin in patients with pancreatic cancer varied significantly from case to case, and its clinical significance has remained unclear. We showed that increased concentrations of α_1-antitrypsin and α_1-antichymotrypsin in plasma/serum correlated with poor

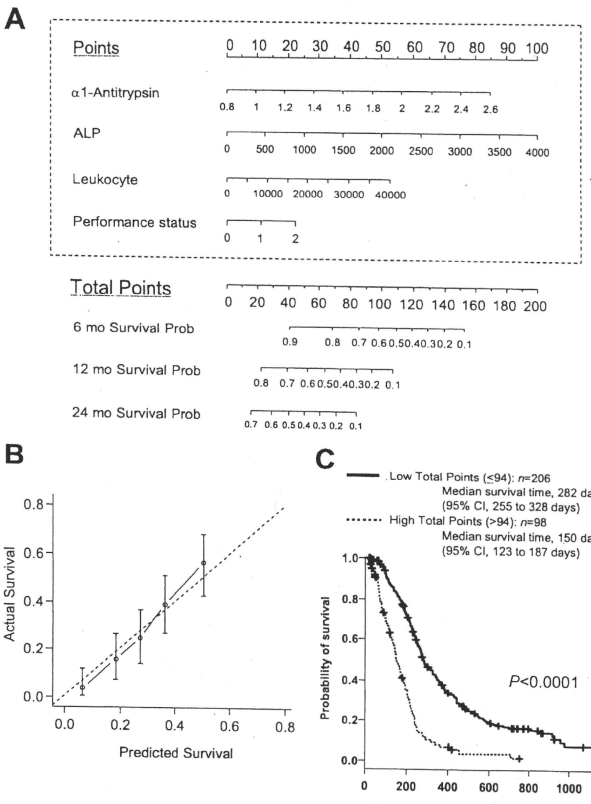

FIG. 5. *A*, nomogram for estimating the probability (*Prob*) of survival 6, 12, and 24 months (*mo*) after gemcitabine treatment. See supplemental Fig. S4 and its legend for details of usage. *B*, calibration curve demonstrating the correlation between predicted and actual survival at 12 months after gemcitabine treatment. *Bars* represent 95% CI. *C*, Kaplan-Meier plots of overall survival according to total points. *ALP*, alkaline phosphatase.

survival, indicating that patients with poor outcomes have lower base-line protease activities than those with favorable outcomes. How such a protease imbalance affects the progression of pancreatic cancer awaits further clarification in future studies.

In conclusion, we identified a prognostic biomarker potentially useful for selecting high risk patients with advanced pancreatic cancer who are unlikely to gain adequate survival benefit from the standard treatment. This may be of great clinical importance, especially when an alternative therapeutic option becomes available for patients with advanced pancreatic cancer in the future. However, the level of α_1-antitrypsin was not significantly correlated with the efficacy of gemcitabine, indicating that it may reflect the natural course of pancreatic cancer irrespective of treatment. Therefore, an independent prospective validation study will be definitely necessary to confirm the universality of the present findings. The absolute concentration of α_1-antitrypsin can be measured by nephelometry, but this measurement requires a larger sample volume than reverse-phase microarrays and for this reason could not be performed in this study. While bearing all these limitations in mind, the present findings may not only help to stratify patients with pancreatic cancer but also provide novel insights into the molecular mechanisms behind the malignant progression of this neoplasm, possibly leading to the development of novel therapeutic strategies.

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[S] This article contains supplemental Figs. S1–S4 and Table S1.

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A Multi-Institution Phase I/II Trial of Triweekly Regimen with S-1 Plus Cisplatin in Patients with Advanced Non-small Cell Lung Cancer

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Introduction: To determine the dose-limiting toxicity and recommended dose (RD) of cisplatin (CDDP) combined with S-1 (tegafur, 5-chloro-2, 4-dihydroxypyridine, and potassium oxonate) for patients with non-small cell lung cancer and to evaluate efficacy and toxicity of this regimen at RD.

Methods: Patients with stages III and IV non-small cell lung cancer received 3-week cycles of treatment, each consisting of oral administration of S-1 at 80 mg/m^2 in 2 divided doses per day for 14 consecutive days, intravenous administration of CDDP (60 mg/m^2, 70 mg/m^2, or 80 mg/m^2) on the first day, and no medication during the subsequent 7 days. The primary objective of phase I study was to estimate the maximum tolerable dose and the RD, and the primary end point of phase II study was response.

Results: RD of CDDP in the analysis of 18 eligible patients was 60 mg/m^2. Evaluation of efficacy and toxicity at RD in 55 eligible patients showed that partial response was observed in 18 patients (32.7%, 95% confidence interval: 20.7–46.7%). The median survival time was 18.1 months, and the time to disease progression was 3.8 months. Grade 3 or severer adverse events were observed in 27 patients (49.1%).

Conclusions: CDDP combined with S-1 showed a satisfactory overall survival time and acceptable toxicity profile. However, the response as the primary end point did not reach the predetermined threshold level.

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Lung cancer, with its high mortality rate, is the most common cause of death from malignant tumors. Among the various types of lung cancer, non-small cell lung cancer (NSCLC) accounts for more than 80% of all patients with lung cancer. Platinum-based two-drug combinations with third-generation agents such as docetaxel,[1] paclitaxel,[2] gemcitabine,[3] and vinorelbine[4] are standard first-line treatment for metastatic NSCLC. Cisplatin (CDDP) plus pemetrexed or carboplatin, paclitaxel plus bevacizumab is an option for nonsquamous NSCLC.[5,6] Platinum-based chemotherapy has also been applied to combined modality treatment with thoracic radiotherapy or surgery in stage II, IIIA, or IIIB NSCLC.[7–9] Although chemotherapy plays an important role in the management of patients with NSCLC, the benefits of chemotherapy are modest and standard platinum-based regimens have significant toxicities; thus, more effective and less toxic regimens are needed. Although an important goal of such development is to raise the survival rate, it is also crucial to minimize adverse events, cost, and improve quality of life.

S-1 (Taiho Pharmaceutical Co., Ltd., Tokyo, Japan) is an oral antineoplastic drug consisting of tegafur, which is a prodrug of 5-fluorouracil (5-FU), and 2 modulators, 5-chloro-2,4-dihydroxypyridine and potassium oxonate.[10] Tegafur has advantages of high bioavailability and small individual differences in absorption. This substance is gradually converted to 5-FU in liver. On the other hand, 5-chloro-2,4-dihydroxypyridine antagonizes dihydropyrimidine dehydrogenase and suppresses the metabolism of 5-FU in liver to help maintain the blood level of 5-FU[11] and prevents neurotoxicity by suppressing the generation of metabolite F-β-alanine. Potassium oxonate prevents gastrointestinal toxicity through inhibition of orotate phosphoribosyl transferase and suppresses the phosphorylation of 5-FU in the digestive tract.[12–14]

Ichinose et al.[15] conducted a phase II study on the combination of S-1 and CDDP chemotherapy in patients with

advanced NSCLC, using the schedule defined by a phase I study in patients with advanced gastric cancer (3-week administration of S-1 with CDDP at 60 mg/m^2 on day 8). Because the approved dose of CDDP for NSCLC in Japan is 70 to 90 mg/m^2 and many regimens use day 1 administration of CDDP in a 3-week schedule, the Health, Labor and Welfare Ministry of Japan requested to evaluate the day 1 administration of CDDP and S-1 in a 3-week schedule. For this reason, we conducted the phase I/II study with 2-week administration of S-1 combined with CDDP on day 1.

The purpose of the study is to define maximum tolerated dose (MTD) and recommended dose (RD) of CDDP in the phase I study and evaluated response rate, survival, and adverse events in the phase II study.

PATIENTS AND METHODS

Patient Eligibility

Patients satisfying the following criteria were eligible: (1) clinical stage of IIIB (no indications of radical radiotherapy) or IV with a diagnosis of NSCLC confirmed by histology or cytology; (2) presence of a measurable lesion; (3) age 20 to 74 years at the time of enrollment; (4) Eastern Cooperative Oncology Group performance status of 0 to 1; and (5) expected survival time of 3 months or more. Other eligibility criteria included white blood cells (4 to 12 $\times$ 10^3/μl), platelets ($\geq$100 $\times$ 10^3/μl), hemoglobin ($\geq$9.0 g/dl), total bilirubin ($\leq$1.5 times laboratory reference value), aspartate aminotransferase and alanine aminotransferase ($\leq$100 U/liter), alkaline phosphatase ($\leq$2 times normal laboratory reference value), creatinine clearance ($\geq$60 ml/min), and oxygen partial pressure ($\geq$60 mmHg). Exclusion criteria included (1) patients with a history of severe drug sensitivity (not specified); (2) patients taking other anticancer medication; (3) patients with active infection; (4) patients with significant comorbid medical conditions, including, but not limited to, heart failure, renal failure, hepatic failure, hemorrhagic peptic ulcer, mechanical or paralytic ileus, or poorly controlled diabetes; (5) patients with pleural effusion, ascites, or pericardial fluid requiring drainage; (6) patients with symptomatic brain metastasis; (7) patients with difficulty in controlling bowel movements; (8) patients with prior malignancies within the past 5 years of nontreatment or disease-free interval, with the exception of carcinoma in situ; (9) pregnant, nursing, or potentially pregnant women; and (10) patients considered inappropriate by the principal or subinvestigator. The patients meeting enrollment criteria were registered after obtaining their written informed consent. This protocol was reviewed and approved by institutional review boards at all participating institutes.

Treatment Schedule

CDDP was administered on the first day with 14 consecutive day administration of S-1 and no medication on the subsequent 7 days (21 days in total). S-1 was prescribed at a dose of 80 mg/d if body surface area was less than 1.25 m^2, 100 mg/d if body surface area was 1.25–1.5 m^2, or 120 mg/d if body surface area was 1.5 m^2 or more, divided into 2 doses/d. The dose of S-1 was reduced by one level (20 mg/d) in patients with BSA $\geq$1.25 m^2 if there were grade 4 leukocytopenia, neutropenia, or platelet counts below 10,000/mm^3 or grade 3 or more nonhematological toxicity including diarrhea, stomatitis, or rash. If the dose reduction was required in patients who received 80 mg/d (patients with BSA $<$1.25 m^2 or who had dose reduction) it was reduced to a minimum of 50 mg/d.

Analysis for MTD and RD of CDDP (Phase I Trial)

RD Analysis

Doses of CDDP in the estimation of MTD were set in increments of 10 mg/m^2 starting from a dose of 70 mg/m^2 (level 1). RD was defined as the dose that was one level lower than MTD. If MTD was defined as more than 80 mg/m^2 (level 2), no further dose increase was made, and RD was set at 80 mg/m^2.

MTD Analysis

MTD was estimated based on the analysis of dose-limiting toxicity (DLT) as follows: (1) If DLT occurrence was 0/3, CDDP dose was increased to 80 mg/m^2; (2) If DLT occurrence ranged from 1/3 to 2/3, 3 patients were added, and 70 mg/m^2 of CDDP was repeated; (3) If DLT occurrence was 3/3, this level was judged as MTD; (4) If DLT occurrence after adding 3 patients ranged from 1/6 to 2/6, CDDP dose was increased to 80 mg/m^2; and (5) If DLT occurrence was 3/6 or more, this level was judged as MTD. The same procedures were repeated at the CDDP dose of 80 mg/m^2, except the dose was not increased further. If MTD was not determined at 80 mg/m^2, it was estimated to be more than 80 mg/m^2. The level was evaluated at least 1 week after the completion of protocol treatment.

Definition of DLT

DLT was defined by the following adverse events: (1) persistence of grade 4 neutropenia for 4 days or more; (2) neutropenic fever of 38°C or more; (3) grade 3 or 4 for thrombocytopenia; (4) grade 3 or more severe nonhematologic toxicity other than nausea, vomiting, anorexia, and hyponatremia; and (5) any adverse event requiring reduction of total S-1 dose below 75% of the planned dose per cycle.

Assessment of Response

Antitumor response was evaluated by computed tomography scan and magnetic resonance imaging at 4-week intervals after the beginning of administration in comparison with the baseline lesions taken within 2 weeks before enrollment using "Response Evaluation Criteria in Solid Tumors (RECIST) Guidelines."

Safety Assessment

Adverse events were identified according to "National Cancer Institute Common Toxicity Criteria (NCI-CTC), Version 2.0."

Sample Size Determination and Statistical Analysis

Assuming the response would follow a binomial distribution, enrollment of 54 patients was planned, so that an expected response of 50% would be significant in a test at the one-tailed significance level of $\alpha/2 = 2.5\%$ when the thresh-

old response is 30% and the power of the test ($1 - \beta$) is 80%. The survival curve for eligible patients was estimated using Kaplan-Meier method,[16] and median survival time (MST), 1-year, and 2-year survival proportions were calculated.

RESULTS

Eligible Patients and Doses

From seven institutions in Japan, 18 patients were enrolled in the phase I and 55 eligible patients in the phase II study. The latter included six patients accrued in phase I at RD. Table 1 shows characteristics of the total 67 patients. Patients were enrolled between July 2004 and August 2005.

Recommended Dose

MTD and RD of CDDP were estimated with six patients in the 70 mg/m^2 dose group (level 1) and six patients in the 80 mg/m^2 dose group (level 2), respectively. Severe adverse events that should be regarded as DLT, such as shock, disseminated intravascular coagulation, and bloody stool, occurred in three patients (50.0%, three of six) during second and later cycles in level 1. Thus, we considered that level 1 corresponded to MTD and performed an additional evaluation at the CDDP dose of 60 mg/m^2 assigned as level 0 in six patients. In the 60 mg/m^2 group (level 0), one patient developed pneumonia during the second cycle, but no other events corresponding to DLT were observed. Therefore, we estimated that MTD of CDDP in this schedule was 70 mg/m^2 and RD was level 0.

Compliance

We regarded as completion of a cycle if CDDP was administered on the first day, and the patient took 28 doses of S-1. The number of patients completing 1, 2, 3, and 4 or more cycles was 50, 36, 34, and 25, respectively. The reasons of noncompletion were adverse events in 11 patients, disease progression in 6 patients, and refusal and inadvertent skipping in 3 patients. In this trial, it was possible to administer S-1 without dose reduction 90.9% of patients (50 of 55).

TABLE 1. Patient Characteristics

	Phase I		Phase II
CDDP (mg/m^2)	70	80	60
No. of patients	6	6	55
Gender			
Male	6	3	32
Female	0	3	23
Age, yr			
Median	57	54	59
Range	49–69	37–66	36–74
ECOG performance status			
0	2	2	20
1	4	4	35
Clinical stage			
IIIB	3	1	14
IV	3	5	41
Histology			
Adenocarcinoma	6	5	43
Squamous cell carcinoma	0	0	7
Others	0	1	5

CDDP, cisplatin; ECOG, Eastern Cooperative Oncology Group.

TABLE 2. Response by Patient Characteristics

Characteristics	No. of Patients	Response					Response (%)
		CR	PR	SD	PD	NE	
All	55	0	18	18	16	3	32.7[a]
Gender							
Male	32	0	11	10	10	1	34.4
Female	23	0	7	8	6	2	30.4
Stage							
IIIB	14	0	5	4	4	1	35.7
IV	41	0	13	14	12	2	31.7
Histology							
Adenocarcinoma	43	0	14	12	14	3	32.6
Squamous cell carcinoma	7	0	2	4	1	0	28.6
Others	5	0	2	2	1	0	40.0

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluated.
[a] 95% confidence interval: 20.7–46.7%.

Response and Survival

Table 2 shows the antitumor response (RECIST) in the 55 patients in phase II part of the study, as determined by the central review. Response was 32.7% (18 of 55), and 95% confidence interval (CI) was 20.7 to 46.7%.

In the 18 responding patients, the median time to 30% reduction in tumor size was 38 days (range, 19–60 days). The reduction occurred during the first cycle in three patients (16.7%), second cycle in seven patients (38.9%), and third cycle in eight patients (44.4%). Median time to confirmation of partial response was 75.5 days (range, 51–95 days). This occurred during the third cycle in 10 patients (55.6%), fourth cycle in 6 patients (33.3%), and fifth cycle in 2 patients (11.1%). The median response duration was 104 days (range, 57–176 days).

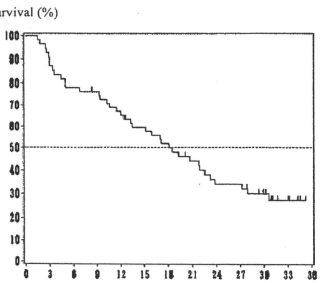
At the time of analysis, there were 32 cases of progression and 38 death events. MST of the 55 patients was 18.1 months (95% CI: 13.3–23.2 months), 1-year survival proportion was 65.2%, and 2-year survival proportion was 34%. The median follow-up at the time of analysis was 31.0 months (range, 29.3–33.0 months). The median time to disease progression was 3.8 months (95% CI: 3.1–5.7 months). Kaplan-Meier curves are shown in Figures 1 and 2.

Adverse Events

Major adverse events observed in this trial were myelosuppression, gastrointestinal toxicity, abnormal liver, or renal function (Table 3). No treatment-related deaths were observed in this trial.

DISCUSSION

This study was conducted to determine the optimal dose of CDDP on day1 in combination with 14-day admin-



Number of patients analyzed: 55

Number of deaths: 38

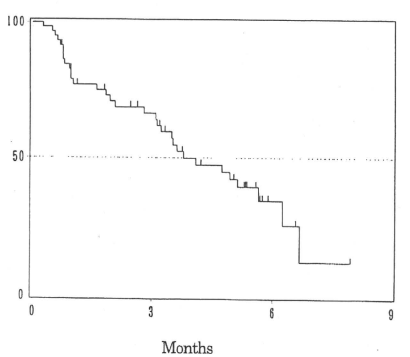
Median survival time: 18.1 months (95% CI:13.3–23.2 months)

1-year survival proportion: 65.2%

2-year survival proportion: 34.0%

Time (months)	0	3	6	9	12	15	18	21	24	27	30	33
At risk	55	50	43	41	36	31	27	22	17	17	12	5

FIGURE 1. Overall survival.



Number of patients analyzed: 55

Number of deaths: 32

Median survival time: 3.8months (95% CI:3.1–5.7 months)

Time (months)	0	1	2	3	4	5	6	7	8
At risk	55	44	35	31	20	16	4	1	1

FIGURE 2. Time to progression.

TABLE 3. Hematologic and Nonhematologic Toxicities (All Cycles)[a]

	Phase I					Phase II (Including 60 mg/m² in Phase I)									
CDDP (mg/m²)	70					80					60				
No. of Patients	6					6					55				
	Grade					Grade					Grade				
Toxicity	**1**	**2**	**3**	**4**	**≧3**	**1**	**2**	**3**	**4**	**≧3**	**1**	**2**	**3**	**4**	**≧3**
Leukopenia	1	1	2	1	3	1	1	2	—	2	16	11	3	—	3
Neutropenia	1	1	1	2	3	1	2	—	1	1	12	9	9	3	12
Anemia	3	1	1	1	2	3	—	2	—	2	20	18	5	2	7
Thrombocytopenia	3	1	1	—	1	1	—	1	—	1	21	7	3	—	3
Aspartate aminotransferase	3	—	—	—	—	3	—	—	—	—	13	1	3	1	2
Alanine aminotransferase	2	1	—	—	—	2	—	—	—	—	17	2	1	1	2
Creatinine	2	1	—	—	—	1	—	—	—	—	14	2	1	—	1
Anorexia	1	1	2	—	2	3	1	1	—	1	28	12	10	1	11
Vomiting	1	—	—	—	—	—	3	—	—	—	16	10	3	—	3
Diarrhea	3	—	1	—	1	3	—	1	—	1	19	5	4	—	4
Stomatitis	2	—	1	—	1	—	2	—	—	—	16	6	1	—	1
Pigmentation	2	1	—	—	—	3	—	—	—	—	16	—	—	—	—
Shock	—	—	1	—	1	—	—	—	—	—	—	—	—	—	—
DIC	—	—	1	—	1	—	—	—	—	—	—	—	—	—	—
Bloody stool	—	—	—	—	—	—	1	—	1	—	—	—	—	—	—

[a] This table shows no. of patients.
DIC, disseminated intravascular coagulation.

istration of S-1 as 3-week cycle and to evaluate efficacy and safety of the regimen at RD in patients with advanced NSCLC. In phase I part of the study, MTD and RD of CDDP were defined as 70 mg/m² and 60 mg/m², respectively. RD of CDDP on day 1 was identical to day 8 administration of CDDP used in the NSCLC phase II study by Ichinose et al.,[15] which was based on RD of CDDP in combination chemotherapy for gastric cancer reported by Koizumi et al.[17]

The previous phase II study of 3-week administration of S-1 combined with 60 mg/m² of CDDP on day 8 showed objective response of 47%, MST of 11.2 months, and 1-year and 2-year survival proportions of 45% and 17%, respectively. Although we must be careful to historically compare, objective response was numerically higher in the previous study with CDDP on given day 8 than this study with CDDP on given day 1. Phase II part of this study demonstrated that response in 55 patients was 32.7% (18 of 55, 95% CI: 20.7–46.7%) and did not reach the predetermined threshold level.

Although objective response did not meet the threshold level, the results of survival were encouraging with a MST of 18.1 months, a 1-year survival proportion of 65.2%, and a 2-year survival proportion of 34%. The relatively good survival results might in part be due to second-line treatment with third-generation antineoplastic agents and/or molecular-targeted agents: 74.5% of patients received second-line chemotherapy including endothelial growth factor receptor tyrosine kinase inhibitors as poststudy treatment.

Preclinical studies showed the strongest antitumor effect was produced by the treatment with tegafur-uracil administered both before and after CDDP.[18] In addition, Satouchi et al. conducted a randomized phase II study of two different schedules of S-1 and gemcitabine in patients with advanced NSCLC. S-1 was administered daily from day 1 to 14, and gemcitabine was given on days 1 and 8 or days 8 and 15. Objective response, median time to progression, and MST were favored for gemcitabine on days 8 and 15 schedule.[19] Optimum sequence of S-1 in combination with other agents has not been determined; however, S-1 and CDDP or gemcitabine trials suggest that day 8 administration of other agents is more effective than day 1 administration in combination with S-1.

In conclusion, the RD of CDDP on day 1 was identical to that of day 8 administration of CDDP in combination with S-1 in patients with advanced NSCLC. We have chosen day 8 administration schedule of CDDP and S-1 from days 1 to 21 as experimental arm in the phase III trial currently underway.

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Randomized Phase 2 Dose-finding Study of Weekly Administration of Darbepoetin Alfa in Anemic Patients with Lung or Ovarian Cancer Receiving Multicycle Platinum-containing Chemotherapy

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Objective: This is the first clinical trial for Japanese to evaluate the dose–response and determine the clinically effective dose of darbepoetin alfa by weekly subcutaneously administration in anemic patients with lung cancer or ovarian cancer receiving chemotherapy.

Methods: Eligible patients were required to have anemia (hemoglobin level of ≤11.0 g/dl). Patients were randomized in a 1:1:1 ratio to receive darbepoetin alfa (1.0, 2.25 or 4.5 μg/kg) subcutaneously once a week for up to 12 weeks. The study drug was withheld from patients who had a hemoglobin level >15.0 g/dl (for men) or 14.0 g/dl (for women), and reinstated at 50% of the previous weekly dose when the hemoglobin level decreased to ≤13.0 g/dl. Quality-of-life assessments were conducted using the Japanese version of the Functional Assessment of Cancer Therapy-anemia (FACT-an) questionnaire.

Results: Hemoglobin response rate was 31.6%, 55.6% and 70.3% in 1.0, 2.25 and 4.5 μg/kg groups, respectively. The dosages of 2.25 and 4.5 μg/kg thus met the clinically effective dose criterion of at least 50% of patients achieving a hemoglobin response. The FACT-fatigue subscale had a high internal consistency with Cronbach's α score. Although no improvement in FACT-fatigue subscale score from baseline to the end of the treatment phase was confirmed for any dose group, there was a correlation between FACT-fatigue subscale score and hemoglobin concentration. Darbepoetin alfa appears to be well tolerated in this setting and no dose-dependent adverse events were observed.

Conclusions: Darbepoetin alfa alleviated anemia caused by platinum-based chemotherapy, and the dosage of 2.25 μg/kg was the lowest dose that met the clinically effective dose criteria when administered once weekly.

Key words: chemotherapy-induced anemia – erythropoietin – lung cancer – ovarian cancer – quality of life

INTRODUCTION

Anemia is a frequent complication in cancer patients receiving multicycle chemotherapy. Anemia is associated with a plethora of symptoms, including fatigue and dyspnea. Fatigue is the most frequently reported symptom in patients with cancer and has been found to have severe detrimental effects on their lives (1). The etiology of anemia is multifactorial (2–4). In particular, direct effects on the renal tubules by platinum-based compounds lead to a decrease in the production of erythropoietin (EPO), which is responsible for terminal differentiation, proliferation and survival of red

blood cell (RBC) precursors (5). If a patient with cancer develops severe or symptomatic anemia, RBC transfusions may be required, with their attendant risks. Acute transfusion reactions can occur, and although the blood supply is now safer with respect to infection than before, the risk of transmission of infectious agents still exists (6,7). In addition, there are some concerns that frequent RBC transfusions with allogeneic blood may adversely affect the immune system of patients with cancer, thereby increasing the tendency to develop infections and hastening the time to relapse or shortening survival (8).

Erythropoiesis-stimulating agents (ESAs), such as recombinant human EPO (rHuEPO) or darbepoetin alfa (DA), have provided another treatment option for anemic patients with cancer receiving chemotherapy and have been shown to reduce the need for transfusions in this setting (9,10). Previous studies have indicated that ESAs increase hemoglobin (Hb) concentration, relieve the symptoms of anemia, improve quality of life (QOL) and reduce transfusion requirements in patients with solid tumors (11) or lymphoproliferative malignancies (12–14).

DA is a unique EPO protein with higher sialic acid content, longer terminal half-life and higher biological activity than rHuEPO (15), allowing less frequent administration with a similar efficacy and safety profile (16–18). Previous studies of DA have demonstrated that it is effective for the treatment of anemia across a wide range of tumor types, with a similar dose–response curve observed in nonmyeloid malignancies (19). Furthermore, in foreign countries, a Phase 3, randomized, double-blind, placebo-controlled study conducted on patients with lung cancer receiving chemotherapy confirmed that a DA starting dose of 2.25 μg/kg administered once weekly (QW) significantly reduced the percentage of patients who required an RBC transfusion and increased Hb concentrations compared with a placebo (10).

In Europe and the USA, ESAs have been widely used since the 1990s for the treatment of chemotherapy-induced anemia. However, they have not been approved yet in Japan. In this prospective study, we first planned a Phase 2 dose-finding study of QW dosing of DA in patients with lung or ovarian cancer who were expected to receive cyclic platinum-containing chemotherapy once every 3 or 4 weeks.

PATIENTS AND METHODS

Study Population

The protocol was approved by the institutional review boards of each of the 31 participating centers, and all patients gave written informed consent before any study-related procedures were carried out.

For entry into the study, patients were required to have been diagnosed with lung or ovarian cancer and expected to receive cyclic platinum-containing chemotherapy once every 3 or 4 weeks for at least two courses after enrollment. Eligible patients were 20–74 years of age and were required to have anemia (Hb level of $\leq$11.0 g/dl). Patients were required to have an Eastern Cooperative Oncology Group (ECOG) performance status of 0–2, and adequate hepatic and renal functions.

Patients were excluded if they were iron deficient; had primary or metastatic malignancy of the central nervous system; had a thrombotic tendency; had received more than three RBC transfusions within 4 weeks or any RBC transfusions within 2 weeks of randomization; were pregnant, breastfeeding or not using adequate birth control measures; or had a history of seizure disorders, active cardiac disease, uncontrolled hypertension, active infection or inflammation or a primary hematologic disorder as the cause of their present anemia.

Study Design and Treatment Schedule

This study was a Phase 2, multicenter, randomized, open-label, sequential dose-finding study (Fig. 1). DA (Kyowa Hakko Kirin Co., Ltd, Japan) was supplied in vials as a clear, colorless, sterile protein solution containing 500 μg/ml of the drug.

After registration, patients were randomized in a 1:1:1 ratio to receive DA (1.0, 2.25 or 4.5 μg/kg) subcutaneously once a week for up to 12 weeks, with a 2-week follow-up period after the last dose of DA. Randomization was performed using a central computerized system and was stratified to balance the treatment groups with respect to tumor type (lung cancer, ovarian cancer), Hb level ($<$9.0, 9.0 $\leq$ Hb level $<$ 10.0 and $\geq$10.0 g/dl) and treatment site. The patients received the first dose of DA on the first day of a chemotherapy cycle.

The study drug was withheld from patients who had an Hb level of $>$15 g/dl (for men) or 14 g/dl (for women), and reinstated at 50% of the previous weekly dose once the Hb concentration decreased to $\leq$13.0 g/dl. Patients with a serum ferritin concentration of $<$10 ng/ml or a serum transferrin saturation of $<$15% received iron therapy to prevent iron deficiency.

RBC transfusion policies were left to the discretion of the investigators, although RBC transfusions were recommended for patients with an Hb level of $\leq$8.0 g/dl or symptoms of anemia, regardless of the patient's Hb level.

Study Endpoints

The primary objective of this study was to determine the clinically effective dose (CED) of DA. The criteria for CED are shown in Table 1.

Efficacy was assessed using Hb endpoints and the incidence of RBC transfusions. The primary measure of efficacy was the percentage of patients achieving an Hb response, defined as an increase in Hb of $\geq$2.0 g/dl from the baseline

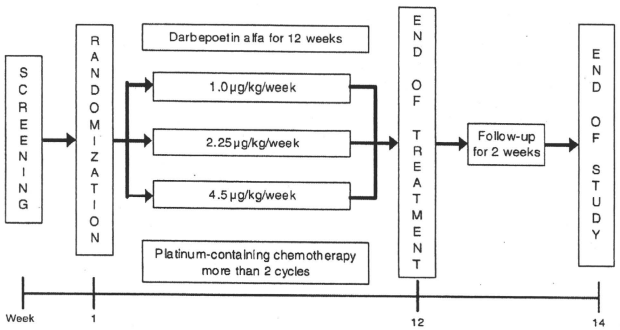


Figure 1. Study design and treatment schema. Darbepoetin alfa was administered once every week.

Table 1. Criteria for clinically effective dose

Efficacy
≥50% of patients achieve an Hb response
≤20% of patients in the safety analysis set experience a dose-limiting toxicity [treatment-related adverse events (>Grade 3 and SAE)]
Safety
≤20% of patients whose Hb concentration is >15.0 g/dl for men or >14.0 g/dl for women

Hb response: ≥2.0 g/dl increase over baseline in the absence of any red blood cell transfusions in the preceding 28 days; Hb, hemoglobin; SAE, serious adverse event.

in the absence of any RBC transfusions during the previous 28 days. The secondary efficacy endpoints were the change in Hb concentration from baseline during the treatment and the incidence of RBC transfusions.

QOL assessments were conducted at baseline, during 7–11 weeks, at the beginning of a chemotherapy course and at the end of a treatment phase after the initiation of DA administration. The Japanese version of the Functional Assessment of Cancer Therapy-anemia (FACT-an) questionnaire was used, which is composed of the FACT-general, a 20-item FACT-anemia subscale and 13 items of which make up the FACT-fatigue subscale.

The safety of DA was evaluated by monitoring adverse events, Hb level, changes in laboratory values and vital signs, and antibody formation resulting from DA administration.

Statistical Analysis

The efficacy analyses were conducted using a per-protocol set that included all patients who received seven or more doses of the study drug and at least two courses of platinum-containing chemotherapy, without major protocol deviations. The proportion of patients exhibiting an Hb response was estimated by subtracting the Kaplan–Meier estimate of the survivor function during week 1 until the end of treatment phase in the absence of an RBC transfusion during the previous 28 days with 95% confidence intervals (CIs), because of the anticipated withdrawal rate. The same analysis for patients in the FAS and analysis using a crude proportion were also performed as part of the sensitivity analysis. For secondary analysis, the percentage of patients exhibiting an Hb correction and patients who received at least one RBC transfusion were also estimated using the Kaplan–Meier method. Cronbach's α coefficient was calculated to assess the reliability of the FACT-an scales. Summary statistics by Hb levels were used to assess the validity of FACT-an scales.

Safety analyses were conducted on all patients who received at least one dose of the study drug. Adverse events were summarized by primary system organ class and by preferred term.

Baseline demographic and clinical characteristics were summarized by the summary statistics.

This study was determined to require a sample size of 120 patients (~40 patients in each dose cohort accounting for patients with drop-out). With 30 patients evaluated in each dose cohort, the proportion of Hb response could be estimated within a standard error of 0.09 if the true proportion is almost 50%.

RESULTS

Patient Demographics and Disposition

Of the 145 patients screened, 132 were enrolled into the study and randomized. Four patients withdrew from the study before receiving the first dose of the study drug. One

hundred and twenty-eight patients (42 patients in the 1.0 μg/kg group and 43 patients in each of the 2.25 and 4.5 μg/kg groups) received at least one dose of the study drug. Twenty-two patients (12 patients received less than seven doses of the study drug, 9 patients received less than two courses of platinum-containing chemotherapy and 1 patient did not have laboratory data after administration) were excluded from the efficacy evaluation due to protocol deviations. One hundred and six patients (33 patients in the 1.0 μg/kg group, 36 patients in the 2.25 μg/kg group and 37 patients in the 4.5 μg/kg group) were included for all efficacy endpoints. Demographic characteristics were similar among the groups, except for age (Table 2).

Efficacy

The proportion of patients that exhibited an Hb response is shown in Fig. 2. The Kaplan–Meier percentages of patients exhibiting an Hb response were 31.6% (95% CI = 14.3–48.9%), 55.6% (95% CI = 35.9–75.2%) and 70.3% (95% CI = 28.0–100.0%) in the 1.0, 2.25 and 4.5 μg/kg groups, respectively. Although there was no reduction in the median time to an Hb response at 4.5 μg/kg compared with 2.25 μg/kg (10 weeks for the 2.25 μg/kg group and 13 weeks for the 4.5 μg/kg group), the dosages of 2.25 and 4.5 μg/kg met the CED criterion that at least 50% of patients exhibited an Hb response.

The mean change in Hb level associated with administration of the various doses of DA was examined (Fig. 3). Although, in this study, there was no difference in the mean change in Hb concentration between the 2.25 and 4.5 μg/kg groups, a trend toward greater increases in Hb level with higher doses of DA was observed: the increase was 0.71 g/dl in the 1.0 μg/kg cohort compared with 1.71 g/dl in the 2.25 μg/kg and 1.72 g/dl in the 4.5 μg/kg cohorts at the end of the treatment phase.

Table 2. Patient characteristics at baseline (per-protocol set population)

	Darbepoetin alfa			Total (n = 106)
	1.0 μg/kg, n = 33	2.25 μg/kg, n = 36	4.5 μg/kg, n = 37	
Sex (n/%)				
Male	12 (36.4)	14 (38.9)	13 (35.1)	39 (36.8)
Female	21 (63.6)	22 (61.1)	24 (64.9)	67 (63.2)
Age (years)				
Mean (SD)	61.2 (9.9)	56.2 (10.2)	56.1 (12.8)	57.7 (11.2)
Body weight (kg)				
Mean (SD)	53.29 (9.68)	55.59 (9.64)	53.86 (9.36)	54.27 (9.51)
Primary disease (n/%)				
Lung	16 (48.5)	17 (47.2)	20 (54.1)	53 (50.0)
NSCLC	13 (39.4)	13 (36.1)	16 (43.2)	42 (39.6)
SCLC	3 (9.1)	4 (11.1)	4 (10.8)	11 (10.4)
Ovarian	17 (51.5)	19 (52.8)	17 (45.9)	53 (50.0)
ECOG PS (n/%)				
0	17 (51.5)	22 (61.1)	16 (43.2)	55 (51.9)
1	16 (48.5)	14 (38.9)	21 (56.8)	51 (48.1)
2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
>3/unknown	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Hb (g/dl)				
Mean (SD)	9.81 (1.27)	10.29 (0.98)	10.03 (1.07)	10.05 (1.11)
Hb < 9.0 (n/%)	7 (21.2)	4 (11.1)	6 (16.2)	17 (16.0)
9.0 ≤ Hb < 10.0 (n/%)	14 (42.4)	9 (25.0)	13 (35.1)	36 (34.0)
Hb ≥ 10.0 (n/%)	12 (36.4)	23 (63.9)	18 (48.6)	53 (50.0)
Endo-EPO (mIU/ml)				
Mean (SD)	98.56 (81.91)	57.15 (40.08)	66.41 (60.66)	73.27 (64.41)

Per-protocol set population: all patients who received seven or more doses of study drug and at least two courses of platinum-containing chemotherapy, without considerable protocol deviations; SD, standard deviation; NSCLC, non-small cell lung cancer; ECOG, Eastern Cooperative Oncology Group: PS, performance status; EPO, erythropoietin.

The Kaplan–Meier percentage of patients who received at least one RBC transfusion from week 5 to the end of the treatment phase was lower in the 2.25 μg/kg group [5.8% (95% CI = 0.0–13.7%)] than in the other groups [13.4% (95% CI = 1.1–25.8%) for 1.0 μg/kg group and 15.4% (95% CI = 0.7–30.1%) for 4.5 μg/kg group], although there was no significant difference.

Of the 128 patients, FACT-fatigue subscale score data were available for 127 (41 patients in the 1.0 μg/kg group and 43 patients in each of the 2.25 and 4.5 μg/kg groups). The Japanese version of the FACT-fatigue subscale score had a high internal consistency with Cronbach's α score, which was 0.908 at baseline and 0.932 at the end of the treatment phase. In this study, although no improvement in FACT-fatigue subscale score from baseline to the end of the treatment phase was observed for any dose group, FACT-fatigue subscale score was correlated with Hb concentration at the end of the treatment phase (Fig. 4). In addition, subscale score was also correlated with ECOG performance status score.

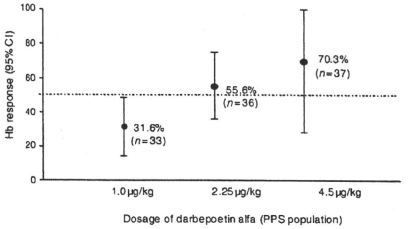
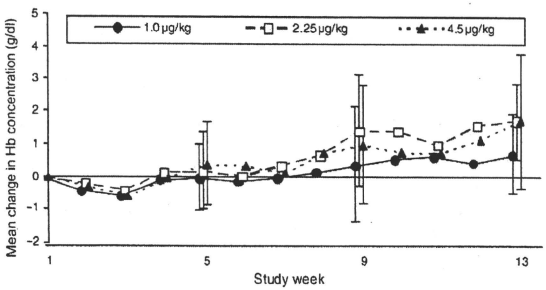


Figure 2. Kaplan–Meier rates of hemoglobin (Hb) response by treatment group [per-protocol set (PPS) population].

Safety

The incidence of adverse events that were considered by the investigators to be related to the study drug was similar among the cohorts: 15 patients (35.7%) in the 1.0 μg/kg group, 15 patients (34.9%) in the 2.25 μg/kg group and 15 patients (34.9%) in the 4.5 μg/kg group. The most frequently reported event was headache [one patient (2.4%) in the 1.0 μg/kg group, two patients (4.7%) in the 2.25 μg/kg group and three patients (7.0%) in the 4.5 μg/kg group]. Other treatment-related adverse events seen in two or more patients were sporadic in each dose cohort (Table 3). The treatment-related adverse events of Grade 3 or greater were angina, sudden hearing, adrenal hemorrhage, nausea, fatigue, increased blood pressure, increased blood uric acid, hypernatremia and prostate induration and each of them was observed in one patient. The incidences of serious adverse events and adverse events of Grade 3 or greater that were considered by the investigators to be related to the study drug were also similar in each dose cohort: three patients in each dose cohort (7.1% in the 1.0 μg/kg group, 7.0% in the 2.25 μg/kg group and 7.0% in the 4.5 μg/kg group). The incidence of adverse events regardless of relationship was at a level expected in a population of cancer patients receiving chemotherapy and occurred at a similar frequency within each dose cohort. The incidences of serious adverse events and adverse events of Grade 3 or greater were similar in each dose cohort.

The percentage of patients who exceeded the Hb thresholds (14.0 g/dl for women and 15.0 g/dl for men) was under 20%

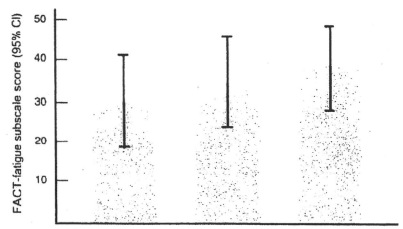


	Week 5	Week 9	Week 13
1.0 μg/kg (*n*=33)	−0.02±1.05 (*n*=31)	0.37±1.74 (*n*=27)	0.71±1.24 (*n*=20)
2.25 μg/kg (*n*=36)	0.19±1.19 (*n*=36)	1.41±1.70 (*n*=27)	1.71±1.13 (*n*=17)
4.5 μg/kg (*n*=37)	0.40±1.30 (*n*=37)	0.98±1.82 (*n*=24)	1.72±2.16 (*n*=15)

Figure 3. Mean change in Hb concentration from baseline to the end of the treatment phase in PPS population (mean ± SD).

in each cohort [one patient (2.4%) in the 1.0 μg/kg group, four patients (9.3%) in the 2.25 μg/kg group and six patients (14.0%) in the 4.5 μg/kg group].

Five patients (3.9%) [two patients (4.8%) in the 1.0 μg/kg group, two patients (4.7%) in the 2.25 μg/kg group and one patient (2.3%) in the 4.5 μg/kg group] died during the study, but none of the deaths were considered by the investigators to be related to the study drug. One venous thromboembolism, a renal vein thrombosis (Grade 1), was observed in one patient with ovarian cancer in 1.0 μg/kg group (2.4%). No anti-DA antibodies were detected in this population of patients receiving DA.



	Hb< 10	10≤ Hb< 12	Hb≥ 12
Number of patients	36	49	37
Mean ± SD	30.1±11.2	34.5±10.6	38.4±10.5
Median (range)	31.0 (5-51)	36.0 (2-52)	39.0 (7-52)

Figure 4. Correlation between FACT-fatigue subscale score and Hb concentration at the end of the treatment phase.

DISCUSSION

In this study, the proportion of patients who exhibited a ≥2.0 g/dl increase in Hb level from baseline was investigated. Dosages of both 2.25 and 4.5 μg/kg met the CED criterion, although there was no reduction in the median time to Hb response at 4.5 μg/kg group compared with 2.25 μg/kg group (10 weeks for the 2.25 μg/kg group and 13 weeks for the 4.5 μg/kg group). Meanwhile, in a study in the US study, there was an obvious dose-dependent increase in the percentage of patients exhibiting an Hb response at 4.5 μg/kg group compared with 2.25 μg/kg group (18). In this study, the median numbers of doses administered were 12, 10 and 9 in the 1.0, 2.25 and 4.5 μg/kg groups, respectively. The median number of doses in the 4.5 μg/kg group was smaller than that in the other groups irrespective of safety. There was no dose-dependent difference in the number of subjects not completing the study. This discrepancy in dose-dependency between the US study and this study may be related to the fact that the treatment duration in

Table 3. Adverse events related to study drug reported for two or more patients receiving darbepoetin alfa (safety analysis population)

Event (PT)	Darbepoetin alfa			Total (n = 128)
	1.0 μg/kg, n = 42	2.25 μg/kg, n = 43	4.5 μg/kg, n = 43	
Headache	1 (2.4)	2 (4.7)	3 (7.0)	6 (4.7)
Rush	2 (4.8)	2 (4.7)	1 (2.3)	5 (3.9)
Liver dysfunction	3 (7.1)	—	1 (2.3)	4 (3.1)
Back pain	1 (2.4)	1 (2.3)	1 (2.3)	3 (2.3)
Increased brood pressure	2 (4.8)	1 (2.3)	—	3 (2.3)
Urinary occult blood positive	—	1 (2.3)	2 (4.7)	3 (2.3)
Epigastric pain	—	—	2 (4.7)	2 (1.6)
Increased bilirubin	—	—	2 (4.7)	2 (1.6)
Constipation	—	2 (4.7)	—	2 (1.6)
Dizziness	1 (2.4)	—	1 (2.3)	2 (1.6)
Hypertension	—	1 (2.3)	1 (2.3)	2 (1.6)
Nausea	—	—	2 (4.7)	2 (1.6)
Peripheral edema	—	2 (4.7)	—	2 (1.6)
Mclalgia	2 (4.8)	—	—	2 (1.6)
Palpitation	1 (2.4)	—	1 (2.3)	2 (1.6)
Fever	—	—	2 (4.7)	2 (1.6)
Positive urine protein	—	1 (2.3)	1 (2.3)	2 (1.6)

Values are expressed as n (%). PT, preferred term.

the 4.5 μg/kg group of this study was shorter than that for other groups. The incidence of RBC transfusions was assessed throughout the study. The period from week 5 to the end of the treatment phase in patients receiving at least one RBC transfusion was analyzed (14). The percentage of patients who received at least one RBC transfusion was lower in the 2.25 μg/kg group than in the other groups from week 5 to the end of the treatment phase, although there was no significant difference. It has been reported that once-weekly DA treatment reduced the percentage of patients receiving RBC transfusions (18). The enrollment of more subjects is considered necessary to assess the reduction in transfusion rate, because this study was designed to assess the percentage Hb response as the primary endpoint. Further large-scale studies focusing on RBC transfusion are needed in Japan.

ESAs have been shown to improve health-related QOL in several studies (20–22). A FACT-an questionnaire was used widely to evaluate cancer patients with anemia, but there are few Japanese reports of studies conducted using FACT-an. Therefore, in this study, the feasibility, reliability and validity of the FACT-an questionnaire were assessed. The collection rate of questionnaires was nearly 100%. FACT-fatigue showed a higher internal consistency (Cronbach's α score range = 0.908 and 0.932 before and after treatment) than other subscales. This internal consistency was consistent with previously reported results and other subscales as well (23). Investigation of the correlation between QOL score and Hb level with FACT-fatigue and FACT-an showed a trend of higher QOL score with increasing Hb level as well as a validation study of FACT (24). These results indicated that the use of the FACT-an questionnaire was a feasible, reliable and valid method of assessing anemia and fatigue in Japanese cancer patients.

In a US study, QOL score increased with increasing Hb concentration (18). In this study, no correlation between FACT-fatigue score and Hb concentration was found. Reasons may include that the QOL baseline score for Japanese patients is slightly higher than for others. A meta-analysis indicated that the baseline of FACT-fatigue is about 26, but in this study, the baseline is 36, which reflects less fatigue (25). A high baseline score may affect the efficacy's resistance to the change in QOL score. FACT-fatigue uses the minimum important difference (MID). MID is the 'smallest difference in score in the domain of interest that patients perceive as important, either beneficial or harmful, and that would lead a clinician to consider a change in the patient's management'. Because FACT-fatigue MID is already known as 3–4, characteristics may have been different between this study and those described in existing reports (26). This baseline difference in Japanese patients may cause difficulty for interpretation.

The results from this study suggest that DA is safe when administered to patients with anemia who are undergoing chemotherapy. The adverse event profile was dominated by findings, e.g. neutropenia, nausea, and vomiting, that are predictable in a population of patients with advanced malignancy receiving multicycle chemotherapy. No unexpected trends were noted in the incidence or severity of adverse events. Although the correlation between the rate of Hb concentration increase and adverse events was investigated, no relationship was apparent. Specifically, the incidence of hypertension and thrombotic events was reported to be associated with a rapid Hb concentration increase in patients with renal failure undergoing dialysis. In this study, the incidence of these complications in all patients was not associated with a rapid increase in Hb concentration. ESA-associated pure red cell aplasia cases have been reported, but almost all cases were observed among hemodialysis patients who received several months of one type of subcutaneously administered rHuEPO (Eprex; Johnson & Johonson, New Brunswick, NJ) (27). No evidence of antibodies to DA was detected for any patient in this study.

Several reports suggested that ESAs had a potential to increase the risk of mortality and/or disease control (28–35) and the negative safety signals were incorporated into the product labels in a boxed warning. It should also be noted that the recently published meta-analyses have indicated a negative impact of ESA use on mortality in cancer patients but the increases on mortality or disease progression were not detected in the patients with chemotherapy-induced anemia (36–39). Several non-clinical studies also have indicated that ESAs do not promote the tumor growth and improve chemotherapeutic outcome in cancer-bearing animals (40–42). Therefore, Aapro and Spivak (43) suggested that the benefit of ESAs outweighs their risks when used for labeled indication and guidelines. The impact of ESAs on mortality and/or disease progression could not be assessed since a long-term follow-up surveillance was not planned in this study. Therefore, further research is needed to clarify the increased risk of them in Japanese patients with chemotherapy-induced anemia.

In conclusion, DA was effective and well tolerated for the treatment of anemia in patients with lung or ovarian cancer receiving platinum-containing chemotherapy and dosages of DA 2.25 μg/kg/QW were the lowest dose that met the CED criteria. Therefore, dosage of DA 2.25 μg/kg/QW was determined as a recommended dose for randomized, placebo-controlled, Phase 3 trial in Japan.

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Conflict of interest statement

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