

Table 7. Comparison of Adverse Reaction Incidence and Pharmacokinetic Parameters of Gemcitabine Between Two Patient Groups With and Without Haplotype *3

| Chemotherapy | Genotype | Incidence of Neutropenia (nadir)* | | | | | | AUC† (hr·µg/mL) |
|-------------------|------------------------------|-----------------------------------|-----------------------|--------------|--------------|-----------------------|--------------|--------------------|
| | | ≥ Grade 3 | | | ≥ Grade 4 | | | |
| | | No. of Cases | Total No. of Patients | Probability | No. of Cases | Total No. of Patients | Probability | |
| Monotherapy | <i>non</i> *3/ <i>non</i> *3 | 66 | 167 | 0.40 | 8 | 67 | 0.05 | 9.91 |
| | <i>non</i> *3/*3 | 6 | 10 | 0.60 | 1 | 10 | 0.10 | 13.13 |
| | <i>P</i> | | | 0.205 | | | 0.514 | 0.0017 |
| With fluorouracil | <i>non</i> *3/ <i>non</i> *3 | 3 | 12 | 0.25 | 2 | 12 | 0.17 | 8.11 |
| | <i>non</i> *3/*3 | 2 | 2 | 1.00 | 1 | 2 | 0.50 | 11.98 |
| | <i>P</i> | | | 0.029 | | | 0.327 | 0.055 |
| With carboplatin | <i>non</i> *3/ <i>non</i> *3 | 9 | 13 | 0.69 | 1 | 13 | 0.08 | 9.87 |
| | <i>non</i> *3/*3 | 3 | 3 | 1.00 | 2 | 3 | 0.67 | 12.48 |
| | <i>P</i> | | | 0.163 | | | 0.033 | 0.031 |
| With cisplatin | <i>non</i> *3/ <i>non</i> *3 | 8 | 28 | 0.29 | 2 | 28 | 0.07 | 9.53 |
| | <i>non</i> *3/*3 | 1 | 1 | 1.00 | 0 | 1 | 0.00 | 11.71 |
| | *3/*3 | 1 | 1 | 1.00 | 1 | 1 | 1.00 | 52.86 |
| | <i>P</i> ‡ | | | 0.030 | | | 0.128 | 0.061 |

Note. No analyses were performed in patients who received gemcitabine with vinorelbine, because only one patient bore the haplotype *3. Boldfacing indicates a statistically significant difference ($P < .05$).

* χ^2 -test.

†Kruskal-Wallis test.

‡A P value for comparison between *non**3/*non**3 and (*non**3/*3 + *3/*3).

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Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan

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Background: To compare the efficacy and toxicity of three platinum-based combination regimens against cisplatin plus irinotecan (IP) in patients with untreated advanced non-small-cell lung cancer (NSCLC) by a non-inferiority design.

Patients and methods: A total of 602 patients were randomly assigned to one of four regimens: cisplatin 80 mg/m² on day 1 plus irinotecan 60 mg/m² on days 1, 8, 15 every 4 weeks (IP); carboplatin AUC 6.0 min × mg/mL (area under the concentration–time curve) on day 1 plus paclitaxel 200 mg/m² on day 1 every 3 weeks (TC); cisplatin 80 mg/m² on day 1 plus gemcitabine 1000 mg/m² on days 1, 8 every 3 weeks (GP); and cisplatin 80 mg/m² on day 1 plus vinorelbine 25 mg/m² on days 1, 8 every 3 weeks (NP).

Results: The response rate, median survival time, and 1-year survival rate were 31.0%, 13.9 months, 59.2%, respectively, in IP; 32.4%, 12.3 months, 51.0% in TC; 30.1%, 14.0 months, 59.6% in GP; and 33.1%, 11.4 months, 48.3% in NP. No statistically significant differences were found in response rate or overall survival, but the non-inferiority of none of the experimental regimens could be confirmed. All the four regimens were well tolerated.

Conclusion: The four regimens have similar efficacy and different toxicity profiles, and they can be used to treat advanced NSCLC patients.

Key words: carboplatin, cisplatin, gemcitabine, irinotecan, non-small-cell lung cancer, paclitaxel, randomized phase III study, vinorelbine

Introduction

Nearly 60 000 patients in Japan died of lung cancer in 2004, and the mortality rate is still increasing [1]. Even old-generation cisplatin-based chemotherapy provides a survival benefit and symptom relief in patients with inoperable non-small-cell lung cancer (NSCLC) [2]. Several anticancer agents including irinotecan, paclitaxel, docetaxel, gemcitabine, and vinorelbine, were developed in the 1990s and most of them have mechanisms of action that differ from those of the old-generation agents [3–7]. The combinations of platinum and these new agents developed in the 1990s are more useful against advanced NSCLC than old-generation combination

chemotherapy, and doublets of platinum and new-generation anticancer agents are considered standard chemotherapy regimens for advanced NSCLC, although no consistent standard regimens have yet been established [8–17].

Two phase III studies comparing cisplatin plus irinotecan (IP) with cisplatin plus vindesine for advanced NSCLC have been conducted in Japan [18, 19]. Fukuoka et al. [20] reported the results of a combined analysis of the 358 eligible stage IV patients in these studies. They carried out a multivariate analysis using the Cox regression model with adjustment for well-known prognostic factors, and the Cox regression analysis demonstrated that treatment with IP was one of significant independent favorable factor. Based on their data, we selected IP for the reference arm in our study.

The Ministry of Health, Labour and Welfare of Japan approved the prescription of paclitaxel, gemcitabine, and

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vinorelbine for NSCLC in 1999 and requested a phase III study to confirm the efficacy and safety of these agents. The Japanese investigators and the pharmaceutical companies decided to conduct a four-arm randomized phase III study for NSCLC, the so-called FACS, Four-Arm Cooperative Study. The purpose of the study was to compare the efficacy and toxicity of three platinum-based combination regimens, carboplatin plus paclitaxel (TC), cisplatin plus gemcitabine (GP), cisplatin plus vinorelbine (NP), with IP as the reference arm.

patients and methods

patient selection

Patients with histologically and/or cytologically documented NSCLC were eligible for participation in the study. Each patient had to meet the following criteria: clinical stage IV or IIIB (including only patients with no indications for curative radiotherapy, such as malignant pleural effusion, pleural dissemination, malignant pericardiac effusion, or metastatic lesion in the same lobe), at least one target lesion >2 cm, no prior chemotherapy, no prior surgery and/or radiotherapy for the primary site, age 20–74 years, Eastern Cooperative Oncology Group performance status (PS) of 0 or 1, adequate hematological, hepatic and renal functions, partial pressure of arterial oxygen (paO₂) ≥60 torr, expected survival >3 months, able to undergo first course treatment in an inpatient setting, and written informed consent. The study was approved by the Institutional Review Board at each hospital. Written informed consent was obtained from every patient.

treatment schedule

All patients were randomly assigned to one of the four treatment groups by the central registration office by means of the minimization method. Stage, PS, gender, lactate dehydrogenase (LDH) and albumin values, and institution were used as adjustment variables. The first group received the reference treatment, 80 mg/m² of cisplatin on day 1 and 60 mg/m² of irinotecan on days 1, 8, and 15, and the cycle was repeated every 4 weeks. The second group received 200 mg/m² of paclitaxel (Bristol-Myers K.K., Tokyo, Japan) over a 3-h period followed by carboplatin at a dose calculated to produce an area under the concentration–time curve of 6.0 min × mg/mL on day 1 and the cycle was repeated every 3 weeks. The third group received 80 mg/m² of cisplatin on day 1 and 1000 mg/m² of gemcitabine (Eli Lilly Japan K.K., Kobe, Japan) on days 1, 8 and the cycle was repeated every 3 weeks. The fourth group received 80 mg/m² of cisplatin on day 1 and 25 mg/m² of vinorelbine (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) on days 1, 8 and the cycle was repeated every 3 weeks. Each treatment was repeated for three or more cycles unless the patient met the criteria for progressive disease or experienced unacceptable toxicity.

response and toxicity evaluation

Response was evaluated according to the Response Evaluation Criteria in Solid Tumors, and tumor markers were excluded from the criteria [21]. Objective tumor response in all responding patients was evaluated by an external review committee with no information on the treatment group. Toxicity grading criteria in National Cancer Institute Common Toxicity Criteria Ver 2.0 were used to evaluate toxicity.

quality of life assessment

Quality of life (QoL) was evaluated by means of the Functional Assessment of Cancer Therapy—Lung (FACT-L) Japanese version and the QoL Questionnaire for Cancer Patients Treated with Anticancer Drugs (QoL-ACD), before treatment, immediately before the second cycles of chemotherapy, and 3 and 6 months after the start of treatment [22–24].

statistical analysis and monitoring

The primary end point of this study was overall survival (OS), and the secondary end points were response rate, response duration, time to progressive disease (TTP), time to treatment failure (TTF), adverse event, and QoL. The 1-year survival rate of the control group in this study was estimated to be 43% based on the data in published papers, and the 1-year survival rate in the other treatment group was expected to be 50%. The lower equivalence limit for 1-year survival rate was set as '–10%'. The criterion for the non-inferiority of each treatment was a lower limit of the two-sided 95% confidence interval (CI) of the 1-year survival rate of treatment minus that of control larger than the lower equivalence limit. Because the non-inferiority of each treatment versus the control was to be evaluated independently, a separate null hypothesis was stated for each treatment, and for that reason no multiple comparison adjustment was included in the study. Based on the above conditions and binomial distribution, 135 patients were needed per arm for a one-sided Type I error of 2.5% and 80.0% power. In view of the possibility of variance inflation due to censoring, the sample size was set at 600 (150 per arm).

Central registration with randomization, monitoring, data collection, and the statistical analyses were independently carried out by a contract research organization (EPS Co., Ltd, Tokyo, Japan).

results

patient characteristics

From October 2000 to June 2002, a total of 602 patients were registered by 44 hospitals in Japan. All patients had been followed up for >2 years, and 447 patients had died as of June 2004. Of the 602 patients registered, 151 were allocated to the reference treatment, IP, and 150, 151, and 150 patients were allocated to TC, GP, and NP, respectively. Since 10 patients did not receive chemotherapy and 11 patients were subsequently found to be ineligible, 592 patients were assessable for toxicity and 581 patients were assessable for efficacy. Four patients did not receive chemotherapy due to electrolytic disorder, fever, symptomatic brain metastases, and rapid tumor progression in IP, two patients due to refusal and pneumonia in TC, four patients due to lower WBC counts (two patients), rapid tumor progression, and nephritic syndrome in NP. Two patients were ineligible due to wrong stage in IP, two patients were wrong stage and one patient had double cancer in TC, two patients were wrong diagnosis, one patient had massive pleural effusion, one patient received prior chemotherapy in GP, one patient had no target lesions in NP. Age, gender, PS, stage, and LDH and albumin values were well balanced in each arm (Table 1). Fewer patients with adenocarcinoma and more patients with squamous cell carcinoma were, however, entered in three experimental arms than in IP.

objective tumor response and response duration

Objective tumor response is shown in Table 2. Forty-five partial responses occurred in the 145 assessable patients in the reference arm, IP, for an objective response rate of 31.0% with a median response duration of 4.8 months. The response rate and median response duration were 32.4% and 4.0 months in TC, 30.1% and 3.5 months in GP, and 33.1% and 3.4 months in NP. The response rates in TC, GP, and NP were not statistically different from the rate in IP according to the results of the χ^2 test.

Table 1. Patient characteristics and treatment delivery

| | Cisplatin + irinotecan | Carboplatin + paclitaxel | Cisplatin + gemcitabine | Cisplatin + vinorelbine |
|-------------------------|---------------------------|-----------------------------|----------------------------|----------------------------|
| Assessable patients | 145 | 145 | 146 | 145 |
| Gender (male/female) | 97/48 | 99/46 | 101/45 | 101/44 |
| Age, median (range) | 62 (30–74) | 63 (33–74) | 61 (34–74) | 61 (28–74) |
| PS (0/1) | 44/101 | 44/101 | 45/101 | 45/100 |
| Histology | | | | |
| Adenocarcinoma | 121 | 104 | 108 | 109 |
| Squamous cell carcinoma | 16 | 31 | 29 | 29 |
| Others | 8 | 10 | 9 | 7 |
| Stage (IIIB/IV) | 31/114 | 28/117 | 30/116 | 26/119 |
| No. of cycles | | | | |
| Mean \pm SD | 3.0 \pm 1.3 | 3.5 \pm 1.5 | 3.2 \pm 1.2 | 3.1 \pm 1.3 |
| Median | 3 | 3 | 3 | 3 |
| Range | 1–7 | 1–10 | 1–7 | 1–8 |

PS, performance status; SD, standard deviation.

Table 2. Survival, TTP, TTTF, response rate, and response duration

| | N | Median survival, months | 1-year survival (%) | Difference in 1-year survival from IP | 2-year survival (%) | TTP (median), months | TTTF (median), months | Response rate (%) | Response duration (median), months |
|-----------------------------|-----|-------------------------------|---------------------------|---|---------------------------|------------------------------|------------------------------|-------------------------------|---|
| Cisplatin + irinotecan | 145 | 13.9 | 59.2 | – | 26.5 | 4.7 | 3.3 | 31.0 | 4.8 (n = 45) |
| Carboplatin + paclitaxel | 145 | 12.3 | 51.0 | –8.2% (95% CI –19.6% to 3.3%) | 25.5 | 4.5 (P = 0.355) ^a | 3.2 (P = 0.282) ^a | 32.4 (P = 0.801) ^b | 4.0 (n = 47) |
| Cisplatin + gemcitabine | 146 | 14.0 | 59.6 | 0.4% (95% CI –10.9% to 11.7%) | 31.5 | 4.0 (P = 0.170) ^a | 3.2 (P = 0.567) ^a | 30.1 (P = 0.868) ^b | 3.5 (n = 44) |
| Cisplatin + vinorelbine | 145 | 11.4 | 48.3 | –10.9% (95% CI –22.3% to 0.5%) | 21.4 | 4.1 (P = 0.133) ^a | 3.0 (P = 0.091) ^a | 33.1 (P = 0.706) ^b | 3.4 (n = 48) |

^aCompared with IP by the generalized Wilcoxon test.

^bCompared with IP by the χ^2 test.

CI, confidence interval; IP, cisplatin plus irinotecan; TTP, time to progressive disease; TTTF, time to treatment failure.

OS, TTP disease, and TTTF

OS and TTP are shown in Figure 1. Median survival time (MST), the 1-year, and 2-year survival rate in IP were 13.9 months, 59.2%, and 26.5%, respectively. The MSTs, 1-year, and 2-year survival rates were, respectively, 12.3 months, 51.0%, and 25.5% in TC; 14.0 months, 59.6%, and 31.5% in GP; and 11.4 months, 48.3%, and 21.4% in NP. The lower limits of the 95% CI of the difference in 1-year survival rate between IP and TC (–19.6%), GP (–10.9%), and NP (–22.3%) were below –10%, which was considered the lower equivalence limit (Table 2). Thus, the results did not show non-inferiority in three experimental regimens compared with reference treatment. Median TTP and median TTTF were 4.7 and 3.3 months, respectively in IP. Median TTP and TTTF were, respectively, 4.5 and 3.2 months in TC, 4.0 and 3.2 months in GP, and 4.1 and 3.0 months in NP. There were no statistical differences in either TTP or TTTF in TC, GP, or NP, compared with IP according to the results of the generalized Wilcoxon test (Table 2).

hematologic and non-hematologic toxicity

In IP, 47.6% and 83.7% of patients developed grade 3 or worse leukopenia and neutropenia, respectively (Table 3). The incidences of grade 3 or worse leukopenia (33.1%, $P = 0.010$) and neutropenia (62.9%, $P < 0.001$) were significantly lower in GP than in IP. The incidence of grade 3 or worse leukopenia (67.1%, $P < 0.001$) was significantly higher in NP than in IP. Grade 3 or worse thrombocytopenia developed in 5.4% of the patients in IP, and the incidence was significantly higher in GP (35.1%, $P < 0.001$). The incidence of febrile neutropenia in IP was 14.3%, and was significantly lower in GP (2.0%, $P < 0.001$).

Grade 2 or worse nausea, vomiting, anorexia, and fatigue occurred in 60.5%, 51.0%, 65.3%, and 38.8%, respectively, of the patients in IP. The incidences of grade 2 or worse nausea (TC: 25.0%, $P < 0.001$, NP: 47.3%, $P = 0.022$), vomiting (TC: 22.3%, $P < 0.001$, NP: 36.3%, $P = 0.011$), and anorexia (TC: 32.4%, $P < 0.001$, NP: 49.3%, $P = 0.005$) were significantly lower in TC and NP than in IP. Grade 2 or worse diarrhea was

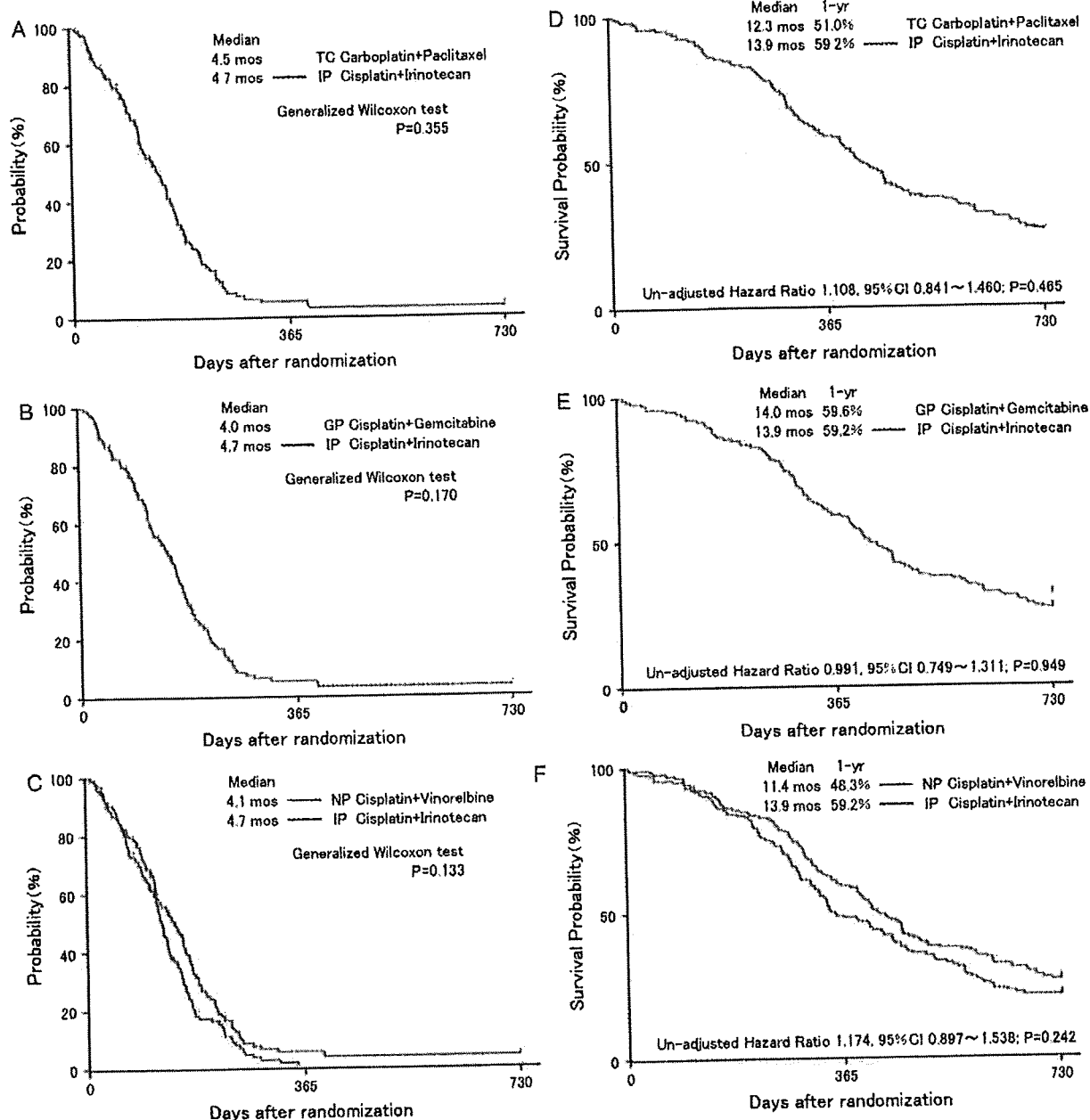


Figure 1. Overall survival (OS) and time to progressive (TTP) disease. TTP and OS in the carboplatin plus paclitaxel (TC) (A, D), cisplatin plus gemcitabine (GP) (B, E), and cisplatin plus vinorelbine (NP) (C, F) were not statistically significantly different from the values in the cisplatin plus irinotecan.

significantly less frequent in TC (6.8%), GP (8.6%), and NP (11.6%) than in IP (48.3%, $P < 0.001$). The incidences of grade 2 or worse sensory neuropathy (16.9%, $P < 0.001$), arthralgia (21.6%, $P < 0.001$), and myalgia (17.6%, $P < 0.001$) were significantly higher in TC than in IP. Grade 2 alopecia occurred in 30.6% of the patients in IP, and its incidence was significantly higher in TC (44.6%, $P = 0.013$) and significantly lower in GP (15.2%, $P = 0.001$) and NP (8.9%, $P < 0.001$). Grade 2 injection site reactions were more frequent in NP (26.7%) than in IP (4.8%, $P < 0.001$).

A total of five patients died of treatment-related toxicity: three in IP (cerebral hemorrhage, interstitial pneumonia, acute circulatory failure/disseminated intravascular coagulation: 2.0%), one in TC (acute renal failure: 0.7%), and one in NP (pulmonary embolism: 0.7%).

second-line treatment

Data on second-line treatment, but not third-line or later treatment, was available in this study, and they showed that

Table 3. Toxicity

| | IP (n = 147) | | | TC (n = 148) | | | GP (n = 151) | | | NP (n = 146) | | |
|-------------------------|--------------|----|----|-----------------|-----------------|----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| | Grade (%) | | | Grade (%) | | | Grade (%) | | | Grade (%) | | |
| | 2 | 3 | 4 | 2 | 3 | 4 | 2 | 3 | 4 | 2 | 3 | 4 |
| Leukocytes | 42 | 43 | 5 | 39 | 42 | 3 | 40 | 31 ^a | 2 ^a | 25 | 51 ^b | 16 ^b |
| Neutrophils | 11 | 39 | 45 | 5 | 19 | 69 | 21 | 40 | 23 ^a | 5 | 16 | 72 |
| Hemoglobin | 42 | 24 | 7 | 42 | 13 ^a | 2 ^a | 44 | 22 | 5 | 43 | 25 | 5 |
| Platelets | 6 | 5 | 1 | 9 | 11 | 0 | 22 | 35 ^b | 0 ^b | 3 | 1 ^a | 0 ^a |
| Febrile neutropenia | — | 14 | 0 | — | 18 | 0 | — | 2 ^a | 0 ^d | — | 18 | 0 |
| Nausea | 32 | 29 | — | 14 ^c | 11 ^c | — | 35 | 23 | — | 33 ^c | 14 ^c | — |
| Vomiting | 38 | 13 | 0 | 17 ^c | 5 ^c | 0 ^c | 34 | 14 | 0 | 29 ^c | 7 ^c | 0 ^c |
| Anorexia | 30 | 33 | 2 | 15 ^c | 17 ^c | 1 ^c | 31 | 26 | 1 | 29 ^c | 20 ^c | 1 ^c |
| Fatigue | 27 | 12 | 1 | 26 | 2 | 1 | 17 ^c | 3 ^c | 0 ^c | 23 ^c | 3 ^c | 0 ^c |
| Diarrhea | 33 | 15 | 1 | 4 ^c | 3 ^c | 0 ^c | 7 ^c | 2 ^c | 0 ^c | 8 ^c | 4 ^c | 0 ^c |
| Constipation | 27 | 7 | 0 | 30 | 8 | 0 | 33 | 9 | 0 | 40 ^d | 14 ^d | 0 ^d |
| Neuropathy, motor | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Neuropathy, sensory | 1 | 0 | 0 | 14 ^d | 3 ^d | 0 ^d | 0 | 0 | 0 | 0 | 0 | 0 |
| Alopecia | 31 | — | — | 45 ^d | — | — | 15 ^c | — | — | 9 ^c | — | — |
| Arthralgia | 2 | 0 | 0 | 20 ^d | 2 ^d | 0 ^d | 0 | 0 | 0 | 1 | 0 | 0 |
| Myalgia | 1 | 0 | 0 | 16 ^d | 2 ^d | 0 ^d | 0 | 0 | 0 | 1 | 1 | 0 |
| Injection site reaction | 5 | 0 | — | 5 | 0 | — | 5 | 0 | — | 27 ^d | 0 ^d | — |
| Pneumonitis | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Creatinine | 8 | 1 | 0 | 2 ^c | 0 ^c | 0 ^c | 7 | 0 | 0 | 8 | 1 | 0 |
| AST | 7 | 1 | 1 | 5 | 1 | 0 | 6 | 3 | 0 | 1 | 3 | 0 |
| Fever | 2 | 0 | 0 | 5 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| Treatment-related death | 3 (2.0%) | | | 1 (0.7%) | | | 0 | | | 1 (0.7%) | | |

^aIncidence of grade 3 or 4 toxicity significantly ($P < 0.05$) lower than that with IP.

^bIncidence of grade 3 or 4 toxicity significantly ($P < 0.05$) higher than that with IP.

^cIncidence of grade 2 or worse toxicity is significantly ($P < 0.05$) lower than that with IP.

^dIncidence of grade 2 or worse toxicity significantly ($P < 0.05$) higher than that with IP.

GP, cisplatin plus gemcitabine; IP, cisplatin plus irinotecan; NP, cisplatin plus vinorelbine; TC, carboplatin plus paclitaxel.

AST, aspartate aminotransferase; —, no category in the criteria.

60%–74% of the patients received chemotherapy and 6%–9% received thoracic irradiation as second-line treatment (Table 4). The percentages of patients in each treatment group who received second-line chemotherapy were not significantly different ($P = 0.081$).

quality of life

The details of the QoL analysis will be reported elsewhere. No statistically significant difference in global QoL was observed among the four treatment groups based on either the FACT-L Japanese version or the QoL-ACD. Only the physical domain evaluated by QoL-ACD was significantly better in TC, GP, and NP than in IP.

discussion

Many randomized phase III studies have compared platinum-plus-new-agent doublets in NSCLC, but, this is the first to evaluate the efficacy of an irinotecan-containing regimen in comparison with other platinum-plus-new-agent doublets in NSCLC [14–17]. Although non-platinum-containing chemotherapy regimens are used as alternatives, doublets of platinum and a new-generation anticancer agent, such as TC, GP, and NP, are considered standard chemotherapy regimens for advanced NSCLC worldwide [13–17, 25]. Although the non-

inferiority of none of the three experimental regimens could be confirmed in this study, no statistically significant differences in response rate, OS, TTP, or TTTF were observed between the reference regimen and the experimental regimens. All four platinum-based doublets have similar efficacy against advanced NSCLC but different toxicity profiles. Nevertheless, IP was still regarded as the reference regimen in this study because the non-inferiority of none of the three experimental regimens could be confirmed.

OS in this study was relatively longer than previously reported. The estimated 1-year survival rate in the reference arm was 43%, but the actual 1-year survival rate was 59.2%, much higher than expected. The MSTs reported for patients treated with TC, GP, and NP in recent phase III studies have ranged from 8 to 10 months, and in the present study they were 12.3, 14.0, and 11.4 months, respectively [14–17]. One reason for the good OS in this study was the difference in patient selection criteria, for example exclusion of PS2 patients. Ethnic differences in pharmacogenomics have also been indicated as a possible reason for the good OS in this study [26]. The OS in IP in this study, however, was better than in previous Japanese studies [18, 19]. TTP in this study ranged from 4.0 to 4.7 months, and was similar to the TTP of 3.1–5.5 months reported in the literature [15, 16]. OS not TTP was longer in this study

Table 4. Second-line treatment

| | Cisplatin + irinotecan | Carboplatin + paclitaxel | Cisplatin + gemcitabine | Cisplatin + vinorelbine | |
|----------------------|------------------------|--------------------------|-------------------------|-------------------------|-----------|
| Number of patients | 145 | 145 | 146 | 145 | P = 0.081 |
| Chemotherapy | 107 (74%) | 87 (60%) | 101 (69%) | 95 (66%) | |
| Docetaxel | 39 | 25 | 50 | 51 | |
| Gefitinib | 11 | 9 | 18 | 12 | |
| Paclitaxel | 15 | 14 | 7 | 11 | |
| Gemcitabine | 24 | 28 | 17 | 28 | |
| Vinorelbine | 9 | 12 | 2 | 9 | |
| Irinotecan | 15 | 4 | 3 | 3 | |
| Thoracic irradiation | 8 | 10 | 13 | 10 | |

than previously reported, and higher 2-year survival rates, 21.4%–31.5%, were observed in the minimum 2-year follow-up in this study. Second-line or later treatments may affect survival, because docetaxel has been established as standard second-line chemotherapy for advanced NSCLC [27, 28]. Gefitinib is also effective as second-line or later chemotherapy for advanced NSCLC, especially in Asian patients, never smokers and patients with adenocarcinoma [29–32].

The toxicity profile of each treatment differed and the toxicity of all four regimens was well tolerated. Overall QoL was similar in the four platinum-based doublets. Only physical domain QoL evaluated by the QoL-ACD was statistically better in TC, GP, and NP than in IP. This finding is presumably attributable to the fact that diarrhea is a statistically less frequent adverse effect of TC, GP, and NP than of IP.

In conclusion, all four platinum-based doublets had similar efficacy for advanced NSCLC but different toxicity profiles. All the four regimens can be used to treat advanced NSCLC patients in clinical practice.

appendix

Institutions of the FACS Cooperative Group: National Hospital Organization (NHO) Hokkaido Cancer Center, Tohoku University Hospital, Yamagata Prefectural Central Hospital, Niigata Cancer Center Hospital, Tochigi Cancer Center, NHO Nishigunma National Hospital, Saitama Cancer Center, National Cancer Center Hospital East, Chiba University Hospital, National Cancer Center Hospital, Tokyo Medical University Hospital, Japanese Foundation for Cancer Research, Kanagawa Cancer Center, Yokohama Municipal Citizen's Hospital, Kanagawa Cardiovascular and Respiratory Center, Aichi Cancer Center Hospital, Prefectural Aichi Hospital, Nagoya City University Hospital, NHO Nagoya Medical Center, Nagoya University Hospital, Gifu Municipal Hospital, NHO Kyoto Medical Center, Osaka City General Hospital, Osaka City University Hospital, Osaka Medical Center for Cancer and Cardiovascular Diseases, NHO Toneyama Hospital, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Kinki University School of Medicine, Rinku General Medical Center Izumisano Municipal Hospital, Kobe Central General Hospital, The Hospital of Hyogo College of Medicine, Hyogo Medical Center for Adults, Tokushima University Hospital, Kagawa Prefectural Central Hospital, NHO Shikoku Cancer Center Hospital, Hiroshima University Medical Hospital, NHO

Kyushu Cancer Center Hospital, Kyushu University Hospital, National Nagasaki Medical Center, Nagasaki Municipal Hospital, Nagasaki University Hospital of Medicine and Dentistry, Kumamoto Chuo Hospital, Kumamoto Regional Medical Center, NTT West Osaka Hospital.

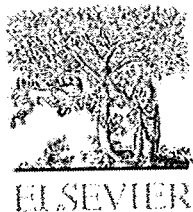
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Randomized trial of drip infusion versus bolus injection of vinorelbine for the control of local venous toxicity

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KEYWORDS

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Chemotherapy;
Toxicity;
Phlebitis;
Randomized trial

Summary Vinorelbine is a moderate vesicant that is well known to cause local venous toxicity such as drug induced-phlebitis. We conducted a prospective randomized trial to determine whether a 1-min bolus injection (1 min bolus) of vinorelbine reduced the incidence of local venous toxicity compared with a 6-min drip infusion (6 min infusion). Non-small cell lung cancer patients who were to receive chemotherapy containing vinorelbine were randomly assigned to receive either 6 min infusion or 1 min bolus of the drug. All infusions were administered through a peripheral vein. Local venous toxicity was evaluated at each infusion up to two cycles. Eighty-three patients were randomized into the study and 81 of them assessable for analysis. One hundred thirty-eight infusions to 40 patients in 6 min infusion and 135 infusions to 41 patients in 1 min bolus were delivered. Vinorelbine induced-local venous toxicity was observed in 33% of patients in 6 min infusion and 24% in 1 min bolus. There was no statistically significant difference between the two arms ($P=0.41$). The incidence of local venous toxicity per infusions was 16% (22 of 138 infusions) in 6 min infusion and 11% (15 of 135 infusions) in 1 min bolus ($P=0.47$). No severe local venous toxicity was seen in either arm. In this study, the administration of 1 min bolus of vinorelbine did not significantly reduce the incidence of local venous toxicity compared with 6 min infusion. Further studies for the control of local venous toxicity of vinorelbine are warranted.

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1. Introduction

Vinorelbine is a second-generation semi-synthetic vinca alkaloid whose antitumor activity is related to its ability to depolymerize microtubules and disrupt the mitotic spindle apparatus [1]. Vinorelbine has been shown to have clearly higher activity and lower neurotoxicity than the other vinca

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alkaloids, and is currently one of the most active agents for the treatment of non-small cell lung cancer (NSCLC) or other solid tumors [2–4].

Vinorelbine is most commonly administered through a peripheral vein as drip infusion over a period of between 6 and 10 min [5]. However, vinorelbine is a moderate vesicant that is well documented to cause local venous toxicity such as drug induced-phlebitis and venous irritation, and its incidence of approximately 30% has been reported in patients who received vinorelbine via a 6–10 min drip infusion [6,7]. Although local venous toxicity is not life threatening, it can result in discomfort or pain and can be a disincentive of chemotherapy to the patients. Therefore local venous toxicity should be managed effectively to decrease patient discomfort.

Recently, a retrospective study on drug induced-phlebitis with bolus injection of vinorelbine has been reported. In the analysis of 39 patients who received the administration of bolus injection of vinorelbine, drug induced-phlebitis occurred in only 1 of 39 patients (2.6%). The results suggested that the administration of bolus injection of vinorelbine might decrease the incidence of drug induced-phlebitis when compared common drip infusion [8]. Furthermore, shortening the infusion time of vinorelbine has also been reported to reduce the incidence of drug induced-phlebitis [9], although a randomized trial evaluating the bolus injection of vinorelbine has not been performed.

We conducted a prospective randomized trial to determine whether a 1-min bolus injection (1 min bolus) of vinorelbine reduced the incidence of local venous toxicity compared with a 6-min drip infusion (6 min infusion). In addition, we assessed the incidence of acute lower back pain, which has been reported to occur in shorter time infusions of vinorelbine [10] as other toxicity.

2. Patients and methods

2.1. Patient eligibility

Patients who had histological or cytological evidence of cancer, and planned to receive vinorelbine-containing chemotherapy as peripheral infusion, were eligible for this study. The patients were required to be 20 years of age or older and have an Eastern Cooperative Oncology Group performance status (PS) of 0–2. Patients were excluded if they had previous treatment with vinorelbine, medical condition that required regular use of steroids, or were pregnant or nursing. All patients provided written informed consent before randomization for this study, and the study was approved by the institutional review board at the National Cancer Center.

2.2. Study design

This study was a randomized trial comparing 1 min bolus of vinorelbine with 6 min infusion for the control of local venous toxicity. The study was performed in the National Cancer Center Hospital East. Patients were randomly assigned to receive either 6 min infusion or 1 min bolus by a minimization method. Before randomization, patients were stratified by chemotherapy regimens (stra-

tum I: vinorelbine plus cisplatin, stratum II: vinorelbine plus gemcitabine, stratum III: vinorelbine alone) and body mass index (BMI) (stratum I: normal (BMI < 24), stratum II: high (BMI 24 or more)). We reported previously that high BMI was associated with a significant increased risk of vinorelbine irritation [6].

2.3. Treatment plan

Patients received either 6 min infusion or 1 min bolus of vinorelbine. Vinorelbine was diluted in 50 ml (6 min infusion) or 20 ml (1 min bolus) normal saline, respectively. All infusions were administered through a peripheral vein and followed by flushing the vein with approximately 200 ml of fluid. The administration of other drugs for the prevention of local venous toxicity was not allowed. Vinorelbine-containing chemotherapy regimens consisted of vinorelbine 20–25 mg/m² on days 1 and 8 plus cisplatin 80 mg/m² on day 1 every 3 weeks, vinorelbine 20–25 mg/m² plus gemcitabine 1000 mg/m² on days 1 and 8 every 3 weeks, or vinorelbine 20–25 mg/m² alone on days 1, 8 and 15 every 4 weeks.

2.4. Outcome assessment

The primary endpoint of this study was the incidence of local venous toxicity per patient. Local venous toxicity was evaluated at each infusion up to two cycles and graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 2.0 for injection site reaction by attending physician: grade 0, none; grade 1, pain, itching or erythema; grade 2, pain or swelling, with inflammation or phlebitis; and grade 3, ulceration or necrosis that is severe or prolonged or requires surgery. After the administration of vinorelbine, patients self-recorded in personal dairies symptoms of pain, itching, swelling, blister, or ulceration at injection. The patient's dairies were also used for support of diagnosis of local venous toxicity. Local venous toxicity was categorized as positive or negative, with positive defined as experience of grade 1 or more local venous toxicity at least once during treatment. The secondary endpoint of this study was the incidence of local venous toxicity per infusions and other toxicity. The incidence of acute lower back pain, which was reported to occur in shorter time infusion of vinorelbine, and hematological toxicity were mainly assessed as the other toxicity, and graded according to NCI-CTC version 2.0.

2.5. Statistical analysis

The purpose of this study was to determine whether 1 min bolus of vinorelbine reduced the incidence of local venous toxicity compared with 6 min infusion. The calculation of sample size was based on the estimated incidence of local venous toxicity per patient in the two treatment groups. On the basis of previous reports [6,8], an incidence of local venous toxicity per patients of 30% in 6 min infusion and of 5% in 1 min bolus was assumed. To demonstrate this hypothesis with an alpha of 5% and a power of 80% in a two-sided test, thirty-five patients from each group were required. A total of 80 patients were projected to be accrued. All comparisons between proportions were performed by the Chi-square test

or Fisher's exact test, as appropriate. Multivariate analysis was performed by logistic regression procedure to determine the relationship between the incidence of local venous toxicity and the clinical variables. *P* values < 0.05 were considered significant. The reported *P* values were based on two-sided tests. Statistical analysis software (StatView-J Ver.5.0, Macintosh) was used for the analyses.

3. Results

3.1. Patient characteristics

Between October 2002 and April 2003, 83 patients were enrolled and randomly assigned into the study. Baseline patient characteristics according to treatment group are shown in Table 1. The two treatment groups were well balanced in regards to age, PS, chemotherapy regimens, and BMI. All patients had advanced NSCLC and no prior chemotherapy. Two patients were not assessable for analysis because they refused to receive chemotherapy after randomization.

Treatment delivery is shown in Table 2. One hundred and thirty-eight infusions to 40 patients in 6 min infusion and 135 infusions to 41 patients in 1 min bolus were delivered. There was no significant difference between the two arms for treatment delivery of vinorelbine.

3.2. The incidence of local venous toxicity

The incidence of local venous toxicity was 33% (95% confidence interval (CI), 18.6–49.1%) in 6 min infusion (13 of the 40 patients) and 24% (95% CI, 12.4–40.3%) in 1 min bolus (10 of the 41 patients) (Fig. 1a). There was no statistically

Table 2 Treatment delivery

| | 6 min drip infusion | 1 min bolus injection |
|-----------------------|---------------------|-----------------------|
| Evaluable patients | 40 | 41 |
| Vinorelbine infusions | | |
| 1 | 1 | 3 |
| 2 | 9 | 8 |
| 3 | 1 | 4 |
| 4 | 29 | 26 |
| Total infusions | 138 | 135 |
| Vinorelbine (mg)/body | | |
| Median (range) | 39 (30–48) | 40 (27–48) |

significant difference between the two arms (*P*=0.41; relative risk, 0.67; 95% CI, 0.25–1.77). In 6 min infusion, grade 1 local venous toxicity was observed in 12 patients, grade 2 in 1 patient; in 1 min bolus, grade 1 local venous toxicity was observed in 8 patients, grade 2 in 2 patients. No severe local venous toxicity was seen with both arms. The incidence of local venous toxicity per infusions was 16% in 6 min infusion (22 of 138 infusions) and 11% in 1 min bolus (15 of 135 infusions) (*P*=0.47) (Fig. 1b).

The incidence of local venous toxicity according to chemotherapy regimens were 29% (18/60) in the vinorelbine plus cisplatin group, 22% (2/9) in the vinorelbine plus gemcitabine group, and 25% (1/4) in the vinorelbine alone group, respectively. The incidence of local venous toxicity in the normal BMI group was 30% compared with 24% in the high BMI group (*P*=0.77). There was no statistically significant difference among the stratified factors. We used multivariate logistic regression analysis to determine the relationship

Table 1 Baseline patients characteristics

| Characteristic | 6 min drip infusion (n=41) | | 1 min bolus injection (n=42) | | <i>P</i> |
|-------------------------|----------------------------|------------------|------------------------------|------------------|----------|
| | No. | % | No. | % | |
| Age (years) | | | | | |
| Median | 65 | | 65 | | 0.37 |
| Range | 42–76 | | 49–78 | | |
| Sex | | | | | |
| Male | 29 | 71 | 36 | 86 | 0.10 |
| Female | 12 | 29 | 6 | 14 | |
| ECOG performance status | | | | | |
| 0/1 | 7/29 | 88 | 11/28 | 93 | 0.48 |
| 2 | 5 | 12 | 3 | 7 | |
| Chemotherapy regimen | | | | | |
| Vinorelbine/cisplatin | 35 | 85 | 35 | 83 | 0.95 |
| Vinorelbine/gemcitabine | 4 | 10 | 5 | 12 | |
| Vinorelbine alone | 2 | 5 | 2 | 5 | |
| Body mass index | | | | | |
| Median (range) | | 21.7 (13.5–34.2) | | 21.2 (14.7–29.9) | 0.79 |
| Normal ≤ 24 | 31 | 76 | 31 | 74 | |
| High > 24 | 10 | 24 | 11 | 26 | |

ECOG, Eastern Cooperative Oncology Group.

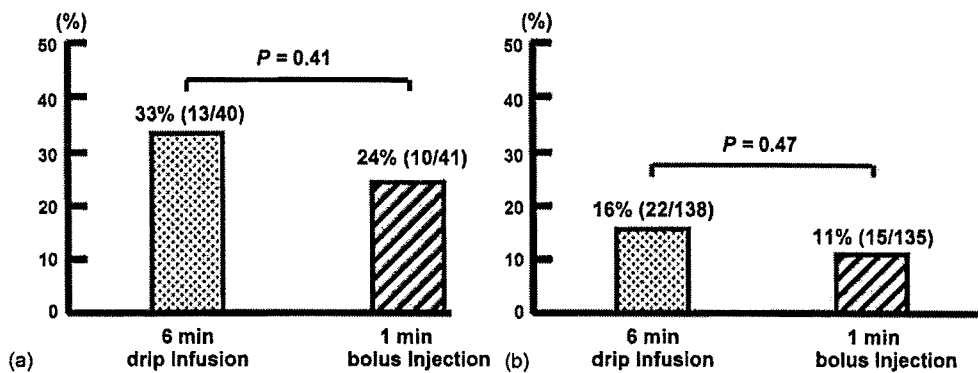


Fig. 1 The incidence of local venous toxicity: (a) per patient, (b) per infusions.

between local venous toxicity and the clinical variables (sex, age, chemotherapeutic regimen, BMI, the dose of VNR, and treatment arm). No significant correlations between the incidence of local venous toxicity and the clinical variables were found.

According to the patient's self-recorded diary, 43% (17/40) of patients in 6 min infusion had at least one symptom at injection site and 34% (14/41) of patients in 1 min bolus ($P=0.43$).

3.3. Other toxicity

Acute lower back pain (>grade 1) was observed in 8% of 6 min infusion, and in 7% of 1 min bolus. There was no statistically significant difference between the two arms ($P>0.99$). Grade 3/4 neutropenia and thrombocytopenia occurred with similar frequency in both arms.

4. Discussion

Local venous toxicity such as drug induced-phlebitis is one of the discomforting toxicities for patients in cancer chemotherapy. Vinorelbine is generally well tolerated and can be administered safely in an outpatient setting; however, it is a moderate vesicant with the potential to cause local venous toxicity. In our study, the incidence of local venous toxicity with the 6-min drip infusion of vinorelbine, which was used as control arm, was 33%, a similar frequency as found in past reports [6,7].

This is the first randomized study that evaluated the incidence of local venous toxicity with the bolus injection of vinorelbine. In this study, the administration of 1 min bolus of vinorelbine did not significantly reduce the incidence of local venous toxicity compared with 6 min infusion. The 24% rate of local venous toxicity with 1 min bolus of vinorelbine, which was observed in our study, was higher than anticipated in the study hypothesis. We speculate that our study hypothesis overestimated the incidence of local venous toxicity with 1 min bolus of vinorelbine because the previous reference reports were not prospective randomized studies [7,8]. Indeed, our study indicated that the administration of 1 min bolus of vinorelbine resulted in a non-statistically significant 27% reduction in rate of local venous toxicity compared with the 33% rate of 6 min infusion. We think that our

study might have no under power to detect a clinically significant difference between the two treatment groups. In our study, an overall incidence of local venous toxicity was 28% although no severe local venous toxicity was seen. If a patient with only poor peripheral venous access receives the administration of vinorelbine, the use of implantable central venous access device should be considered. Moreover, the administration of 1 min bolus of vinorelbine has not been associated with an increased risk of acute lower back pain, which was previously reported to occur in shorter time infusions of vinorelbine [10]. Hematologic toxicity such as neutropenia and thrombocytopenia were also equivalent in both arms. In addition, we examined the clinical risk factors related to local venous toxicity of vinorelbine, but unfortunately there was no significant clinical risk factor in this study.

Two other randomized studies have been performed for the control of local venous toxicity of vinorelbine. Lazano et al. [9] compared the use of heparin-containing solution as anti-thrombotic effect [11] with 10-min infusion of vinorelbine. In their study, a population of 23 patients was randomized to arm A, in which vinorelbine plus 5000 U of heparin was diluted in 500 ml of normal saline and infused over 2 h, or arm B, in which vinorelbine was diluted in 50 ml of normal saline and infused over 10 min. Arm A with heparin was found to be inferior to arm B in terms of pain control at the injection site. Fasce et al evaluated the influence of infusion time of vinorelbine on local venous toxicity in a randomized cross-over trial [10]. Forty-eight patients with solid tumors were randomized to 6-min infusion or 20-min infusion of vinorelbine. Local venous toxicity was recorded in 23 patients (48%) in the 6-min infusion group, and in 26 patients (56%) in the 20-min infusion group, respectively. On the basis of their results, we used the administration of 6 min infusion of vinorelbine as the control arm in this study. The use of defibrotide [12,13] as another anti-thrombotic drug, or cimetidine [14], which was reported to inhibit histamine actions in endothelial cells by vinorelbine [15], have been investigated in an attempt to reduce the incidence of local venous toxicity of vinorelbine. However, there have been no randomized controlled trials to verify the benefit of these methods, and thus a randomized controlled study is needed to draw definitive conclusions about their efficacy.

In conclusion, our findings indicated that the incidence of local venous toxicity with 1 min bolus of vinorelbine was

higher than previously reported. In our study, the administration of 1 min bolus of vinorelbine did not significantly reduce the incidence of local venous toxicity compared with 6 min infusion. Further studies for the control of local venous toxicity of vinorelbine are warranted.

Acknowledgement

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AZD2171 Shows Potent Antitumor Activity Against Gastric Cancer Over-Expressing Fibroblast Growth Factor Receptor 2/Keratinocyte Growth Factor Receptor

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Abstract Purpose: AZD2171 is an oral, highly potent, and selective vascular endothelial growth factor signaling inhibitor that inhibits all vascular endothelial growth factor receptor tyrosine kinases. The purpose of this study was to investigate the activity of AZD2171 in gastric cancer. **Experimental Design:** We examined the antitumor effect of AZD2171 on the eight gastric cancer cell lines *in vitro* and *in vivo*. **Results:** AZD2171 directly inhibited the growth of two gastric cancer cell lines (KATO-III and OCUM2M), with an IC₅₀ of 0.15 and 0.37 $\mu\text{mol/L}$, respectively, more potently than the epidermal growth factor receptor tyrosine kinase inhibitor gefitinib. Reverse transcription-PCR experiments and immunoblotting revealed that sensitive cell lines dominantly expressed COOH terminus-truncated fibroblast growth factor receptor 2 (FGFR2) splicing variants that were constitutively phosphorylated and spontaneously dimerized. AZD2171 completely inhibited the phosphorylation of FGFR2 and downstream signaling proteins (FRS2, AKT, and mitogen-activated protein kinase) in sensitive cell lines at a 10-fold lower concentration (0.1 $\mu\text{mol/L}$) than in the other cell lines. An *in vitro* kinase assay showed that AZD2171 inhibited kinase activity of immunoprecipitated FGFR2 with submicromolar K_i values ($\sim 0.05 \mu\text{mol/L}$). Finally, we assessed the antitumor activity of AZD2171 in human gastric tumor xenograft models in mice. Oral administration of AZD2171 (1.5 or 6 mg/kg/d) significantly and dose-dependently inhibited tumor growth in mice bearing KATO-III and OCUM2M tumor xenografts. **Conclusions:** AZD2171 exerted potent antitumor activity against gastric cancer xenografts over-expressing FGFR2. The results of these preclinical studies indicate that AZD2171 may provide clinical benefit in patients with certain types of gastric cancer.

Various anticancer therapies for gastric cancer have been investigated over the past two decades. Despite intensive studies, the prognosis for patients with unresectable advanced or recurrent gastric cancer remains poor (1, 2), and new therapeutic modalities are needed.

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Fibroblast growth factors (FGF) and their signaling receptors have been found to be associated with multiple biological activities, including proliferation, differentiation, motility, and transforming activities (3–5). The *K-sam* gene was first identified as an amplified gene in human gastric cancer cell line KATO-III (6, 7), and its product was later found to be identical to the bacteria-expressed kinase, or keratinocyte growth factor receptor (KGFR), and FGF receptor 2 (FGFR2). FGFR2/KGFR/K-sam is preferentially amplified in poorly differentiated types of gastric cancers with a malignant phenotype, and its protein expression was detected by immunohistochemical staining from 20 of 38 cases of the undifferentiated type of advanced stomach cancer (8, 9). Thus, FGFR2 signaling may be as a promising molecular target for gastric cancer.

AZD2171 is a potent, ATP-competitive small molecule that inhibits all vascular endothelial growth factor receptors [VEGFR-1, VEGFR-2 (also known as KDR), and VEGFR-3]. *In vitro* studies have shown that recombinant VEGFR-2 tyrosine kinase activity was potently inhibited by AZD2171 (IC₅₀ <1 nmol/L; ref. 10). AZD2171 also showed potent activity versus VEGFR-1 and VEGFR-3 (IC₅₀, 5 and ≤ 3 nmol/L, respectively). VEGF-stimulated proliferation and VEGFR-2 phosphorylation of human umbilical vascular endothelial cells

was inhibited by AZD2171 (IC₅₀, 0.4 and 0.5 nmol/L, respectively). In *in vivo* studies, inhibition of VEGFR-2 signaling by AZD2171 reduced microvessel density and dose-dependently inhibited the growth of various human tumor xenografts (colon, lung, prostate, breast, and ovary; ref. 10). These data are consistent with potent inhibition of VEGF signaling, angiogenesis, neovascular survival, and tumor growth. On the other hand, because it was known that AZD2171 also possesses additional activity against FGFR1 (IC₅₀, 26 nmol/L; ref. 10), we hypothesized that AZD2171 may exhibit the additional anticancer activity against FGFR-overexpressing gastric cancer cells.

Our previous studies showed significant activities of the dual VEGFR-2 and epidermal growth factor receptor inhibitor ZD6474 against poorly differentiated gastric cancer (11) and non-small-cell lung cancer with epidermal growth factor receptor mutations (12, 13), both *in vitro* and *in vivo*. Based on these findings, we proceeded to investigate the anticancer activity of AZD2171 in preclinical models (gastric cell lines and xenografts).

Materials and Methods

Anticancer agents. AZD2171 and gefitinib (Iressa) were provided by AstraZeneca. AZD2171 and gefitinib were dissolved in DMSO for the *in vitro* experiments, and AZD2171 was suspended in 1% (w/v) aqueous polysorbate 80 and administered in a dose of 0.1 mL/10 g per body weight in the *in vivo* experiments.

Cell culture. Human gastric cancer cell lines 44As3, 58As1, OKAJIMA, OCUM2M, KATO-III, MKN-1, MKN-28, and MKN-74 were maintained in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and penicillin-streptomycin.

Established highly tumorigenic cell line. Signet ring cell gastric carcinoma cell line KATO-III was gift from Dr. M. Sekiguchi (University of Tokyo, Tokyo, Japan). All of the presented *in vitro* experiments were done using the KATOIII cell line. We conducted a preliminary experiment to compare the cellular characteristics of TU-KATO-III cells and KATOIII cells, and the results revealed that a high expression level of FGFR2 and high sensitivity to AZD2171 were still maintained in the TU-KATO-III cells (data not shown). KATO-III did not show tumorigenicity following repeated implantation of the cultured cells into BALB/c nude mice. Following s.c. inoculation into nonobese diabetic/severe combined immunodeficient mice, 80% to 100% of the KATO-III cells caused the formation of tumor. Following this result, we cultured the cancer cells isolated from the tumor of mice that developed 2 to 3 months following the implantation of KATO-III cells and attempted s.c. injection into nude mice, in turn, of the incubated cells. This sequence of manipulations was repeated for seven cycles in an attempt to reliably isolate cell lines that would have higher potential to undergo tumor formation over short periods of time. In this way, we obtained a cell line (TU-kato-III) from KATO-III cells that possessed a high tumorigenic potential.

In vitro growth inhibition assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to evaluate the growth-inhibitory effect of AZD2171. Cell suspensions (180 µL) were seeded into each well of 96-well microculture plate and incubated in 10% fetal bovine serum medium for 24 h. The cells were exposed to AZD2171 or gefitinib at concentrations ranging from 4 nmol/L to 80 µmol/L and cultured at 37°C in a humidified atmosphere for 72 h. After the culture period, 20 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent was added, and the plates were incubated for 4 h. After centrifugation, the culture medium was

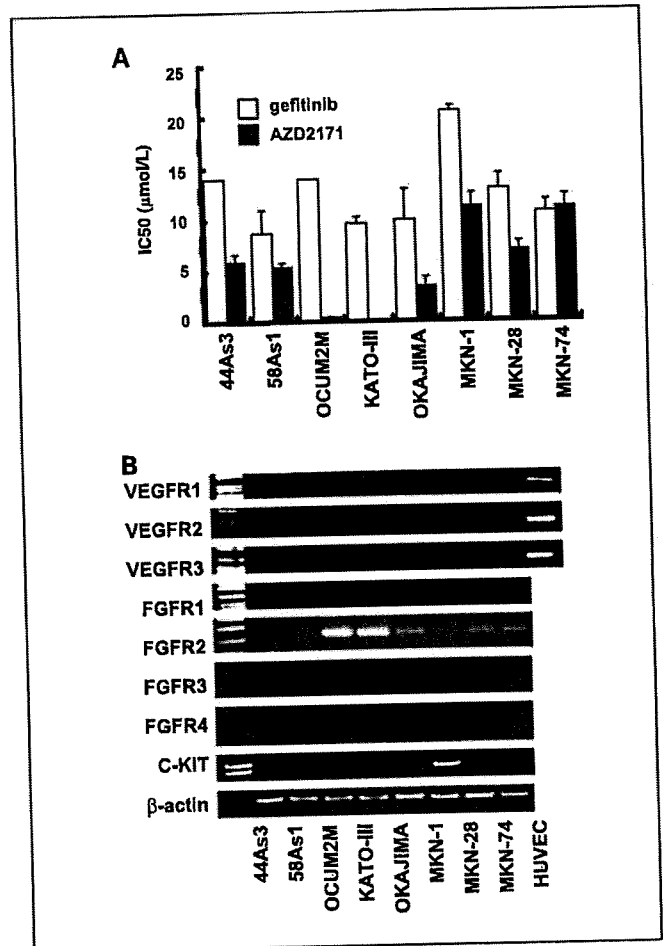


Fig. 1. *A*, *in vitro* growth-inhibitory effect of AZD2171 and gefitinib on eight gastric cancer cell lines. AZD2171 had a growth-inhibitory effect on KATO-III cells and OCUM2M cells (IC₅₀, 0.15 and 0.37 µmol/L, respectively). Columns, mean IC₅₀ of each compound from three independent experiments; bars, SD. □, IC₅₀ of gefitinib; ■, IC₅₀ of AZD2171. *B*, the mRNA expression levels of VEGFRs, FGFRs, and c-KIT in gastric cancer cell lines were determined by reverse transcription-PCR. Human umbilical vascular endothelial cells were used as the positive control for the VEGFRs. No mRNA expression of VEGFRs or c-KIT was detected by reverse transcription-PCR in both sensitive cell lines, but FGFR2 was strongly detected; however, little faint or none was detected in the other cell lines.

discarded, and wells were filled with DMSO. The absorbance of the cultures at 562 nm/L was measured using Delta-soft on a Macintosh computer (Apple) interfaced to a Bio-Tek Microplate Reader EL-340 (BioMatellics). This experiment was done in triplicate.

Reverse-transcription PCR. Using a GeneAmp RNA-PCR kit (Applied Biosystems), 5 µg of total RNA from each cultured cell line was converted to cDNA. The PCR amplification procedure consisted of 28 to 35 cycles (95°C for 45 s, 62°C for 45 s, and 72°C for 60 s) followed by incubation at 72°C for 7 min, and the bands were visualized by ethidium bromide staining. The following primers were used for the PCR: human-specific β-actin, forward 5-GGAAATCGTGGGTGACATT-3 and reverse 5-CATCTGCTGGAAGGTGACAG-3; VEGFR-1, forward 5-TAGCGTCACCAGCAGCGAAAGC-3 and reverse 5-CCITTCITTTGGTCTCTGTGC-3; VEGFR-2, forward 5-CAGACGGACAGTGGTATGTTTC-3 and reverse 5-ACCTGCTGGTGGAAAGAACAAC-3; VEGFR-3, forward 5-AGCCATTCATCAACAAGCCT-3 and reverse 5-GGCAACAGCTGGATGTCATA-3; c-KIT, forward 5-GCCCAATA-GATTGGTATT-3 and reverse 5-AGCATCTTTACAGCGACAGTC-3; FGFR1, forward 5-GGAGGATCGAGCTCACTCGTGG-3 and reverse

5-CGGAGAAGTAGGTGGTGTAC-3; FGFR2, forward 5-CAGTAGACTGTAGACAGTAA-3 and reverse 5-CCGGTAGGCCGATCGCTCACA-3; FGFR3, forward 5-GGTCAAGGATGCCACAGGGCTG-3 and reverse 5-AGCAGCTTCTTGTCCATCCGCT-3; and FGFR4, forward 5-CCGCCTAGAGATTGCCAGCTTC-3 and reverse 5-AGGCCTGTCCATCCTAAGCCA-3.

Real-time reverse transcription-PCR. Real-time reverse transcription-PCR amplification was done by using a Premix Ex Taq and Smart Cycler system (Takara Bio, Inc.) according to the manufacturer's instructions. The following primers were used: FGFR2 (IIIb), forward 5-GATAAATAGTCCAATGCAGAAGTGCT-3 and reverse 5-TGCCCTA-TATAATTGGAGACCTTACA-3 (7); FGFR2 (COOH-terminal), forward 5-GAATACTGGACCTCAGCCAA-3 and reverse 5-AACACTGCCGTT-TATGTGTGG-3; and human-specific β -actin, forward 5-GGAAATC-GTGCCTGACATT-3 and reverse 5-CATCTGCTGGAAGGTGGACAG-3. The experiment was independently done in triplicate using β -actin as a reference to normalize the data.

Western blotting. Cells were cultured overnight in 10% serum-containing medium or serum-starved medium and exposed to 0.1 to 10 μ mol/L of AZD2171 for 3 h before addition of KGF (100 ng/mL) for 15 min. Immunoblotting was done as described previously (14). In brief, after lysing the cells in radioimmunoprecipitation buffer, the lysate was electrophoresed through 10% (w/v) polyacrylamide gels. The proteins were transferred to polyvinylidene difluoride membranes and reacted with the following antibodies: anti-FGFR2 (H-80) and anti-FGFR2 (C-17) antibody (Santa Cruz Biotechnology, Inc.); anti-

phosphotyrosine antibody PY20 (BD Transduction Laboratories); anti-phosphorylated FGFR (Tyr653/654), anti-mitogen-activated protein kinase, anti-phosphorylated mitogen-activated protein kinase antibody, anti-AKT, anti-phosphorylated AKT, and anti-rabbit horseradish peroxidase-conjugated antibody (Cell Signaling Technology); and anti- β -actin antibody (Sigma). Visualization was achieved with an enhanced chemiluminescent detection reagent (Amersham Bioscience).

FGFR2 kinase assay. FGFR2/KGFR kinase activity was quantified by using a Universal Tyrosine Kinase Assay kit (Takara) according to manufacturer's instructions. FGFR2/KGFR proteins were collected from the KATO-III, OCUM2M, and OKAJIMA cell lysates by overnight immunoprecipitation with an anti-FGFR2 antibody. The FGFR2/KGFR immune complexes were washed thrice with radioimmunoprecipitation assay buffer and diluted kinase reaction buffer. Immobilized tyrosine kinase substrate (poly[Glu-Tyr]) was incubated for 30 min at 37°C with each sample in the presence of kinase-reacting solution and ATP. Samples were washed four times, blocked with blocking solution, and incubated with anti-phosphotyrosine antibody (PY20) conjugated to horseradish peroxidase. The absorbance of the phosphorylated substrate was measured at 450 nm.

Chemical cross-link analysis. The chemical cross-link analysis was carried out as described previously (15). In brief, KATO-III cells and OKAJIMA cells were cultured under serum-starved conditions for 24 h, and after stimulation with KGF (100 ng/mL) for 15 min, they were collected and washed with PBS and incubated for 30 min in PBS

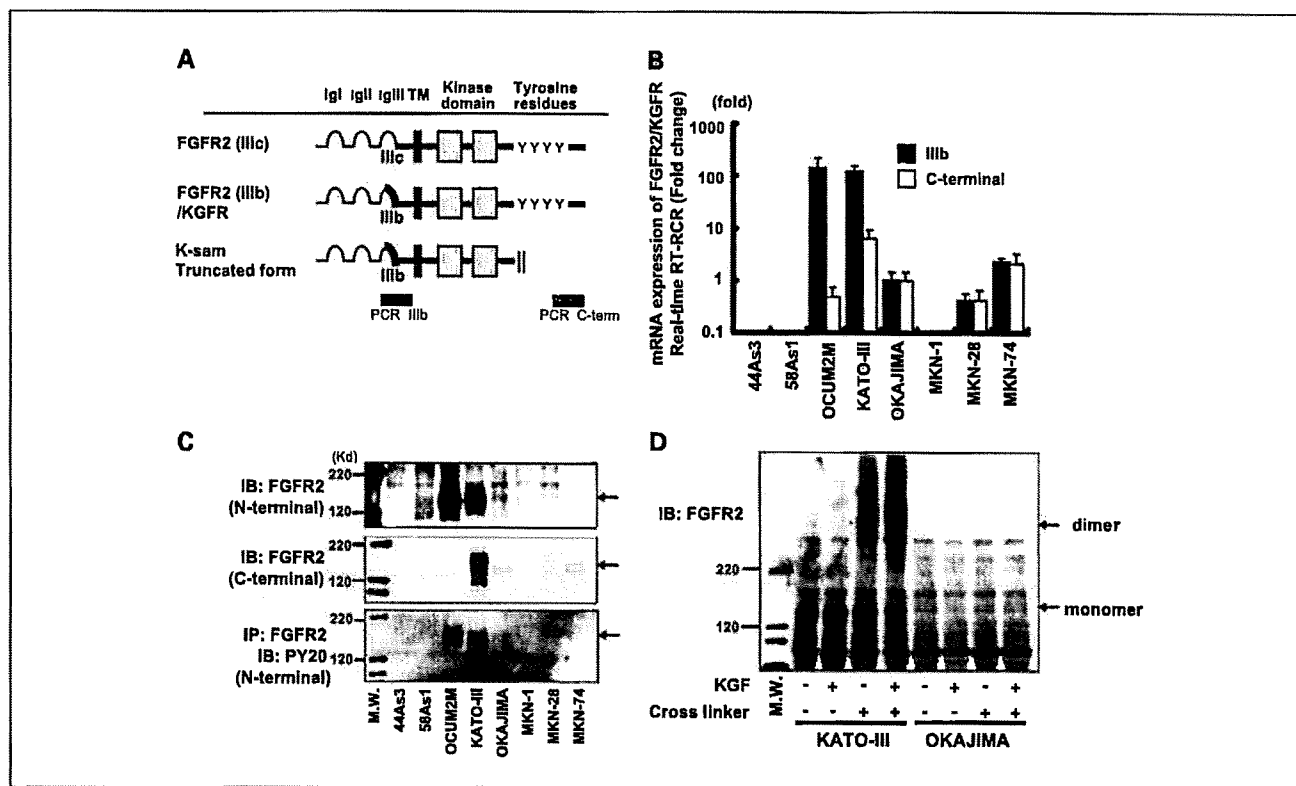


Fig. 2. A, schematic representation of FGFR2 and regions amplified by PCR. B, mRNA expression levels of FGFR2 were quantified by detecting the extracellular domain or COOH-terminal region by real-time reverse transcription-PCR. Expression in the cells is shown as a ratio to expression in OKAJIMA cells. FGFR2 was overexpressed in KATO-III cells and OCUM2M cells by about 100-fold compared with the other cell lines. The majority of the FGFR2 in the sensitive cell lines KATO-III and OCUM2M had no COOH-terminal region. C, protein expression levels of FGFR2 were determined by Western blotting with antibodies to the NH₂ or COOH termini. Both AZD2171-sensitive cell lines overexpressed FGFR2, and the phosphorylation levels were markedly higher. D, chemical cross-linking analysis. Cells were cultured under serum-starved conditions for 24 h and then stimulated with KGF (100 ng/mL) for 15 min. After collecting and washing them with PBS, they were incubated for 30 min in PBS containing cross-linker substrate. The reaction was terminated by adding 250 mmol/L glycine for 5 min. In spite of the serum-starved conditions, high levels of expression of the dimerized form were observed in KATO-III cells in the absence of ligand stimulation. This phenomenon was not observed in the control undifferentiated OKAJIMA cell line. Ligand stimulation resulted in a mild increase in the dimerized form in KATO-III cells. Arrows indicate monomer or dimer formation.

containing 1.5 mmol/L of the non-permeable cross-linker bis-(sulfo-succinimidyl) substrate (Pierce). The reaction was terminated by adding 250 mmol/L glycine for 5 min, and the cells were analyzed by immunoblotting with FGFR2 antibody (Sigma).

FGFR2/KGFR gene silencing with small interfering RNA. Pre-designed small interfering RNA (siRNA) targeting FGFR2 was purchased from Ambion. KATO-III cells were plated on a 96-well plate and incubated in serum-containing medium for 24 h. The cells were then transfected with the FGFR2 targeting siRNA or non-silencing siRNA using RNAiFect Transfection Reagent (Qiagen) according to the

manufacturer's protocol and incubated another 72 h. Cell growth was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. For immunoblotting, 2×10^5 cells per well were plated on a six-well plate for 24 h and transfected with siRNA under the same conditions.

In vivo experiments. Tumorigenic TU-kato-III cells were derived from the gastric cancer cell line KATO-III. Four-week-old female BALB/c nude mice were purchased from CLEA Japan, Inc. and maintained under specific-pathogen-free conditions; 5×10^6 TU-kato-III cells or OCUM2M cells were s.c. injected into both flanks of each mouse. When the tumors had reached a volume of 0.1-0.3 cm³, the mice were randomized into three groups (three per group) and given AZD2171, 1.5 or 6.0 mg/kg/d, or vehicle once daily by oral gavage for 3 weeks. Tumor volume was calculated using the formula: (length \times width) \times $\sqrt{(\text{length} \times \text{width}) \times (\pi/6)}$, where length is the longest diameter across the tumor, and width is the corresponding perpendicular. All mice were sacrificed on day 21, and the tumors were collected. The protocol of the experiment was approved by the Committee for Ethics in Animal Experimentation and conducted in accordance with the Guidelines for Animal Experiments of National Cancer Center.

Results

AZD2171 showed growth-inhibitory activity in vitro. To evaluate the growth-inhibitory activity of AZD2171 *in vitro*, we did 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays on eight gastric cancer cell lines. The epidermal growth factor receptor-specific tyrosine kinase inhibitor gefitinib was used as a reference. The IC₅₀ of gefitinib for all cell lines was between 7 and 20 $\mu\text{mol/L}$. AZD2171 inhibited the growth of KATO-III cells and OCUM2M cells (IC₅₀, 0.15 and 0.37 $\mu\text{mol/L}$, respectively) more potently than the other cell lines (Fig. 1A).

Expression levels of tyrosine kinase receptors. To elucidate the mechanism of action of AZD2171 in the two sensitive cell lines, we measured mRNA expression levels of VEGFRs, FGFRs, and c-KIT, whose kinase activity have been reported to be inhibited by AZD2171 (10). No mRNA expression of VEGFRs or c-KIT was detected by reverse transcription-PCR in either sensitive cell lines. FGFR2 transcripts, however, were strongly expressed in both sensitive cell lines but not strongly in the other cell lines (Fig. 1B). Since we previously found that FGFR2/KGFR/K-sam with a deletion of COOH-terminal exons was amplified in both sensitive cell lines (9), we speculated that amplified FGFR2/KGFR might be associated with sensitivity to AZD2171.

Sensitive cells expressed constitutively active and spontaneously dimerized FGFR2/KGFR. We quantified mRNA expression levels of FGFR2 by real-time reverse transcription-PCR with primers that detect the extracellular domain (IIIb region, see Fig. 2A) and COOH-terminal region. The results show that KATO-III cells and OCUM2M cells expressed FGFR2 100-fold higher than the other cells tested. The COOH-terminal region of FGFR2 was deleted in the KATO-III cells and OCUM2M cells (Fig. 2B). Overexpression and markedly increased phosphorylation of FGFR2 was observed in the AZD2171-sensitive cell lines (Fig. 2C).

Immunoblotting with antibodies for the COOH and NH₂ termini revealed that almost all the FGFR2 expressed by OCUM2M cells, and about half of FGFR2 expressed by KATO-III cells, were truncated (Fig. 2C). Although the KATO-III cells expressed wild-type receptor to some extent, the

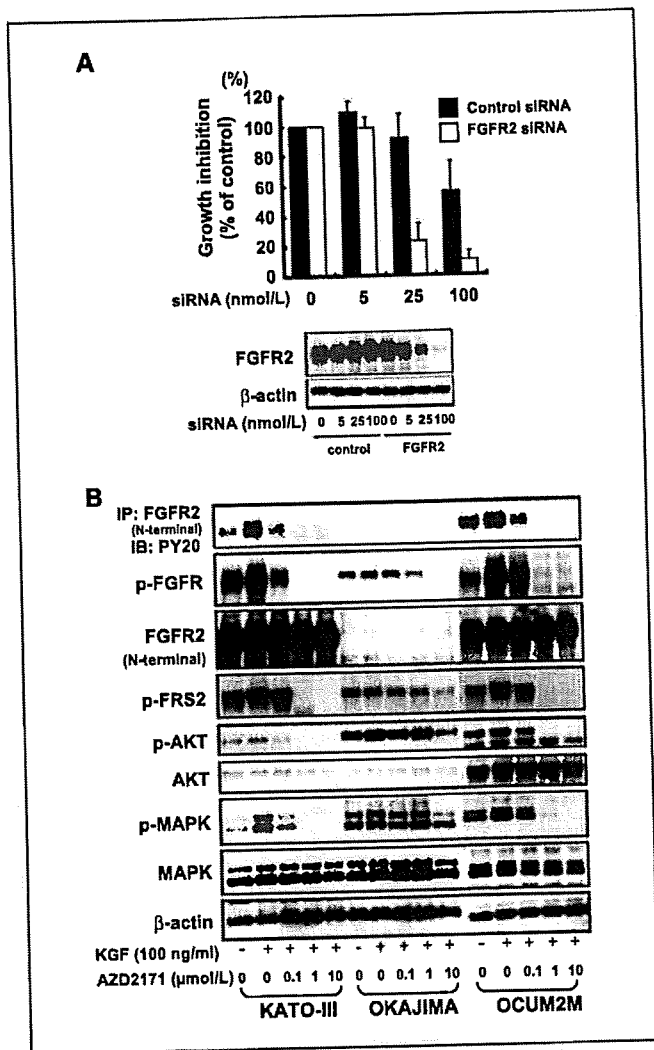


Fig. 3. A. FGFR2 targeting siRNA and cellular growth-inhibitory effect. KATO-III cells were plated on a 96-well plate and incubated in serum-containing medium for 24 h. After incubation, the cells were transfected with FGFR2-targeting or non-silencing siRNA and incubated for another 72 h. Cell growth was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. For immunoblotting, 2×10^5 cells per well were plated on a six-well plate and treated similarly. Marked inhibition of cell growth ($\sim 80\%$) was observed by FGFR2 targeting siRNA compared with control siRNA (*top*). Reduction of FGFR2 protein expression in KATO-III cells was confirmed by immunoblotting (*bottom*). Columns, % control absorbance in three independent experiments; bars, SD. **B.** Western blotting for downstream molecules of FGFR2 signaling. Cells were cultured overnight under serum-starved conditions and exposed to 0.1 to 10 $\mu\text{mol/L}$ AZD2171 for 3 h before adding 100 ng/mL KGF for 15 min. AZD2171 completely inhibited KGF-induced phosphorylation of FGFR2 at 1 $\mu\text{mol/L}$ in the sensitive cell lines, compared with 10 $\mu\text{mol/L}$ in the control cell line OKAJIMA. Similar results were observed for FRS-2, AKT, and mitogen-activated protein kinase (MAPK).

Table 1. *In vitro* kinase assay of AZD2171 against FGFR2

| Cell line | K_m | K_i ($\mu\text{mol/L}$) |
|-----------|----------------|-----------------------------|
| KATO-III | 8.3 ± 3.3 | 0.067 ± 0.017 |
| OCUM2M | 7.1 ± 1.4 | 0.072 ± 0.022 |
| OKAJIMA | 11.0 ± 5.0 | 0.049 ± 0.041 |

COOH-terminal truncated type was dominantly expressed in AZD2171-sensitive cell lines.

A chemical cross-linking analysis was done to evaluate the dimerization of FGFR2. High dimerization of FGFR2 was observed in the KATO-III cells even in the absence of ligand stimulation (Fig. 2D), but no such phenomenon was observed in the control undifferentiated OKAJIMA cell line. Ligand stimulation increased the level of the dimerized-form in KATO-III cells. Taken together, these findings show that the sensitive cell lines expressed high levels of FGFR2 that was highly phosphorylated and spontaneously dimerized without ligand stimulation, suggesting that FGFR2 signaling is constitutively activated in these cells. This evidence is consistent with the widely recognized findings that cancer cells sensitive to other tyrosine kinase inhibitors, such as gefitinib and imatinib, overexpress the highly phosphorylated target receptor with an increased level of dimerization in a ligand-independent manner (12, 16, 17).

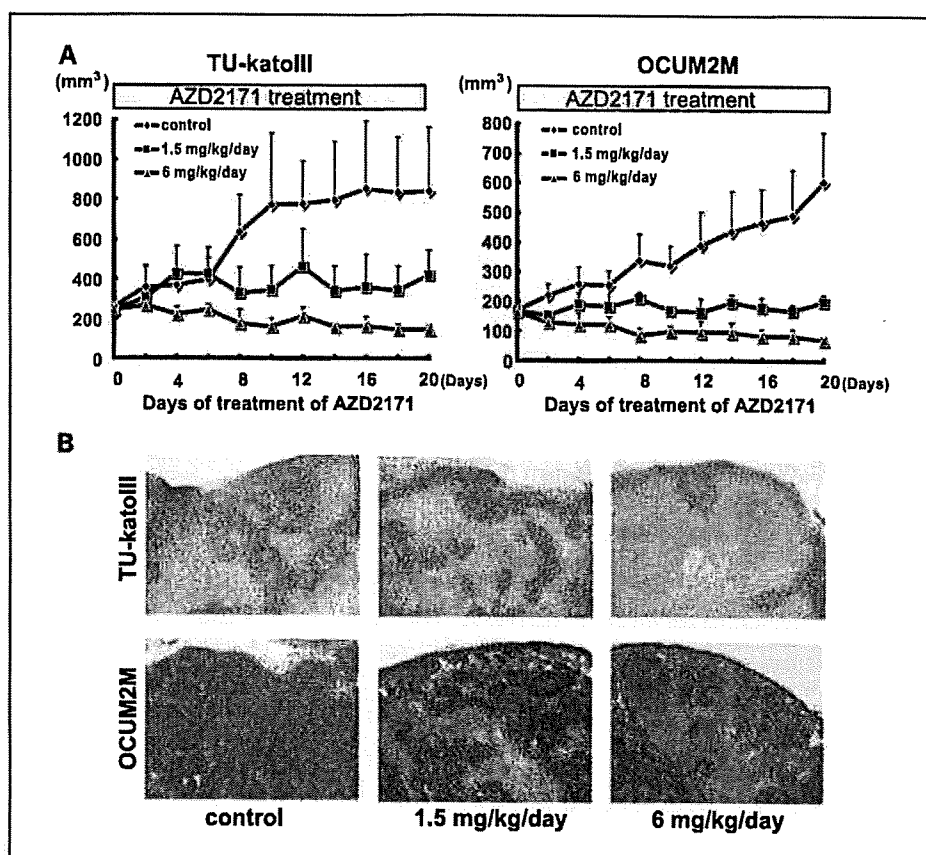
FGFR2 targeting siRNA showed a potent growth-inhibitory effect on KATO-III cells. To investigate the dependency of cell

growth through activated FGFR2 signaling in the AZD2171-sensitive KATO-III cell line, we evaluated the growth-inhibitory effect of siRNA targeted to FGFR2 in KATO-III cells. Targeted siRNA (5-100 nmol/L) decreased FGFR2 and inhibited cell growth (>80%) in a dose-dependent manner (Fig. 3A). The results show that most of the growth of KATO-III cells is dependent on activated FGFR2 signaling, suggesting that the FGFR signaling dependency may be responsible for the higher growth-inhibitory effect of AZD2171 on KATO-III cells.

AZD2171 inhibited FGFR2 signaling. Next, we examined the effect of AZD2171 on FGFR2 downstream phosphorylation signals (i.e., FRS-2, AKT, and mitogen-activated protein kinase). AZD2171 completely inhibited KGF-induced phosphorylation of FGFR2, FRS-2, AKT, and mitogen-activated protein kinase at 1 $\mu\text{mol/L}$ in KATO-III cells, compared with 10 $\mu\text{mol/L}$ in OKAJIMA cells. These results clearly show that AZD2171 possesses inhibitory activity against FGFR2 in cell-based studies and significantly inhibits the phosphorylation of FGFR2 at 1 $\mu\text{mol/L}$ in sensitive cells.

FGFR2 kinase inhibition of AZD2171. To quantify the inhibitory activity of AZD2171 on FGFR2 kinase under cell-free conditions, we calculated the K_i values for immunoprecipitated FGFR2 derived from KATO-III, OCUM2M, and OKAJIMA cells. The K_i values of AZD2171 for FGFR2 in each of these cell lines were 0.067 ± 0.017 , 0.072 ± 0.022 , and 0.049 ± 0.041 $\mu\text{mol/L}$, respectively (Table 1). In contrast, the K_i value of AZD2171 for recombinant VEGFR-2 was 0.0009 $\mu\text{mol/L}$ (data not shown) and was consistent with previous reports (10). At the cellular level, phosphorylation of

Fig. 4. *A.* *In vivo* growth-inhibitory effect of AZD2171 in a tumor xenograft model. After s.c. injecting 5×10^6 TU-kato-III or OCUM2M cells into both flanks of the mice, AZD2171 (1.5 or 6.0 mg/kg/d), or vehicle, was administered orally once daily for 3 wks. A marked tumor growth-inhibitory effect was observed at the low dose (1.5 mg/kg/d) of AZD2171 in both the TU-kato-III tumors and OCUM2M tumors, and the high dose (6.0 mg/kg/d) of AZD2171 completely inhibited the growth of both cell lines. *B.* representative H&E staining of tumor tissue from mice treated with AZD2171. Broad dose-dependent necrosis was observed. Original magnification, $\times 40$.



FGFR2 was inhibited at 10-fold lower concentrations of AZD2171 in the sensitive cell lines (Fig. 3B), but there were no marked differences between the kinase-inhibitory effects among the proteins derived from the cell lines in this cell-free assay. This discrepancy is discussed in the Discussion.

In vivo antitumor activity of AZD2171 against FGFR2-overexpressing gastric cancer. To elucidate the *in vivo* antitumor activity of AZD2171 in mice bearing gastric cancer tumor xenografts, we used the newly established tumorigenic subline TU-kato-III (derived from KATO-III) and OCCUM2M. We attempted to perform control experiments using OKAJIMA cells *in vivo* as suggested by the reviewer. Unfortunately, however, the cell lines grew slowly in the mice, and we could not precisely evaluate the antitumor activity of AZD2171 in the model. However, the results of preliminarily experiments showed that AZD2171 seemed to be less effective against OKAJIMA cells than against KatoIII and OCUM2M cell *in vivo*. Mice implanted the TU-kato-III and OCUM2M tumors were given a low or high dose of AZD2171 (i.e., 1.5 or 6.0 mg/kg/d), or vehicle, orally for 3 weeks. AZD2171 (1.5 mg/kg/d) significantly inhibited tumor growth in the mice bearing TU-kato-III and OCUM2M tumors, and the higher dose (6.0 mg/kg/d) completely inhibited the growth of both tumor models (Fig. 4A). H&E staining showed broad dose-dependent necrosis of core tumor tissue in mice treated with AZD2171 (Fig. 4B). Thus, AZD2171 showed marked antitumor activity *in vivo* against both human gastric tumor xenografts.

Discussion

Recent studies have shown that FGFRs and their ligands are promising therapeutic target molecules for various malignant diseases, such as prostate cancer (18), breast cancer (5, 19), endometrial carcinoma (20), synovial sarcomas (21), thyroid carcinoma (22, 23), and hematopoietic malignancies (24–27). These findings are based on the biological properties of malignant cells expressing activated FGFR, like FGFR fusion tyrosine kinase, involved in chromosomal translocations, gene amplification of FGFRs, or overexpression of FGFs (5, 18–27). In the case of gastric cancer, the results of immunohistochemical analysis of clinical samples revealed that 20 of 38 cases of advanced undifferentiated type of gastric cancer were FGFR2/K-sam positive, whereas none of the 11 cases with the differentiated or intestinal type of cancer showed positive staining for K-sam (8). The results suggest that FGFR2/K-sam overexpression is associated with the undifferentiated type of stomach

cancers. The results of fluorescence *in situ* hybridization analysis of the gastric cancer specimens showed gene amplification of FGFR2/K-sam in 2.9% (28). The clinical implication of FGFR2 overexpression/amplification in gastric cancers remains to be fully clarified, and further investigation is needed.

AZD2171 has the most potent kinase-inhibitory activity against VEGFR-2 ($IC_{50} < 1$ nmol/L); it also possesses additional activity against VEGFR-1, VEGFR-3, and c-Kit (IC_{50} , 5, ≤ 3 , and 2 nmol/L, respectively; ref. 10). AZD2171 showed antiangiogenic activity and broad antitumor activity consistent with potent inhibition of VEGF-induced angiogenesis. We showed kinase-inhibitory activity of AZD2171 against FGFR2 in the present study. When cancer cells are dependent on FGFR2 signaling, AZD2171 can be expected to give additional therapeutic benefit in addition to its antiangiogenic effects.

A cell-based Western blotting analysis showed that phosphorylation of FGFR2 in KATO-III cells and OCUM2M cells was inhibited by AZD2171 at 10-fold lower dose than in OKAJIMA cells (Fig. 3B). However, there was no significant difference in the K_i values of AZD2171 between the FGFR2 derived from KATO-III, OCUM2M, and OKAJIMA in an *in vitro* kinase assay. This may be attributable to the different conditions between the cell-based and cell-free assays. For example, undefined intrinsic intracellular factors may influence kinase activity: (a) differences in baseline intracellular FGFR2 phosphatase activity in each cell line, (b) differences in intracellular concentration of ([transporters, such as ATP-binding cassette transporters, may be involved in this phenomenon refs. 29, 30), and (c) undefined intrinsic inhibitory factors that bind the compounds directly may also be involved (e.g., Brehmer D, et al. have identified various gefitinib binding proteins by affinity chromatography; ref. 31).

In conclusion, AZD2171, a potent inhibitor of all VEGFRs (VEGFR-1, VEGFR-2, and VEGFR-3), was found to have antitumor effect against gastric cancer xenografts in line with previous findings in colon, lung, prostate, breast, and ovarian tumor xenografts (10). The results of this study suggest that activation of the FGFR2 pathway may be a promising target for gastric cancer therapy. AZD2171 may provide a clinical benefit to gastric cancer patients.

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