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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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**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 in 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.


## 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 (stratum 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/m$^2$ on days 1 and 8 plus cisplatin 80 mg/m$^2$ on day 1 every 3 weeks, vinorelbine 20–25 mg/m$^2$ plus gemcitabine 1000 mg/m$^2$ on days 1 and 8 every 3 weeks, or vinorelbine 20–25 mg/m$^2$ 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 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).

**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) |

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 ($n = 42$) | | $P$ |
|---|---|---|---|---|---|
| | 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.

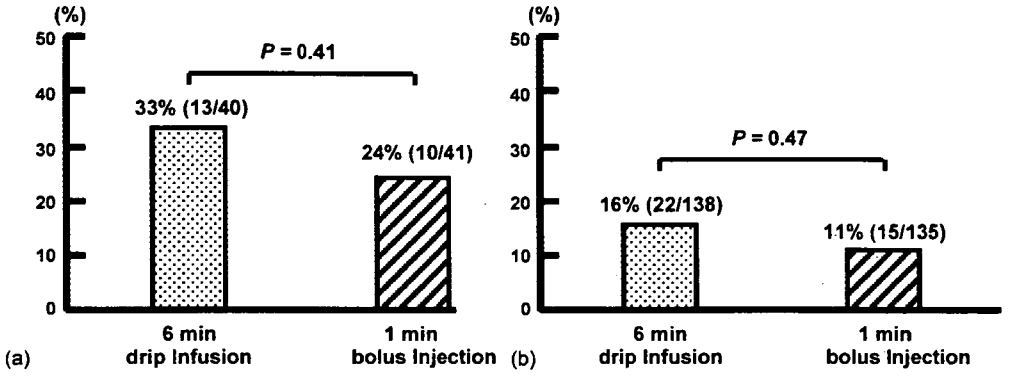


Fig. 1 The incidence of local venous toxicity: (a) per patient, (b) per infusions.

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.

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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 $IC_{50}$ of 0.15 and 0.37 μmol/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 μmol/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 (~0.05 μmol/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.

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 ($IC_{50}$ <1 nmol/L; ref. 10). AZD2171 also showed potent activity versus VEGFR-1 and VEGFR-3 ($IC_{50}$, 5 and ≤3 nmol/L, respectively). VEGF-stimulated proliferation and VEGFR-2 phosphorylation of human umbilical vascular endothelial cells

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was inhibited by AZD2171 ($IC_{50}$, 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 ($IC_{50}$, 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 discarded, and wells were filled with DMSO. The absorbance of the cultures at 562 nmol/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.

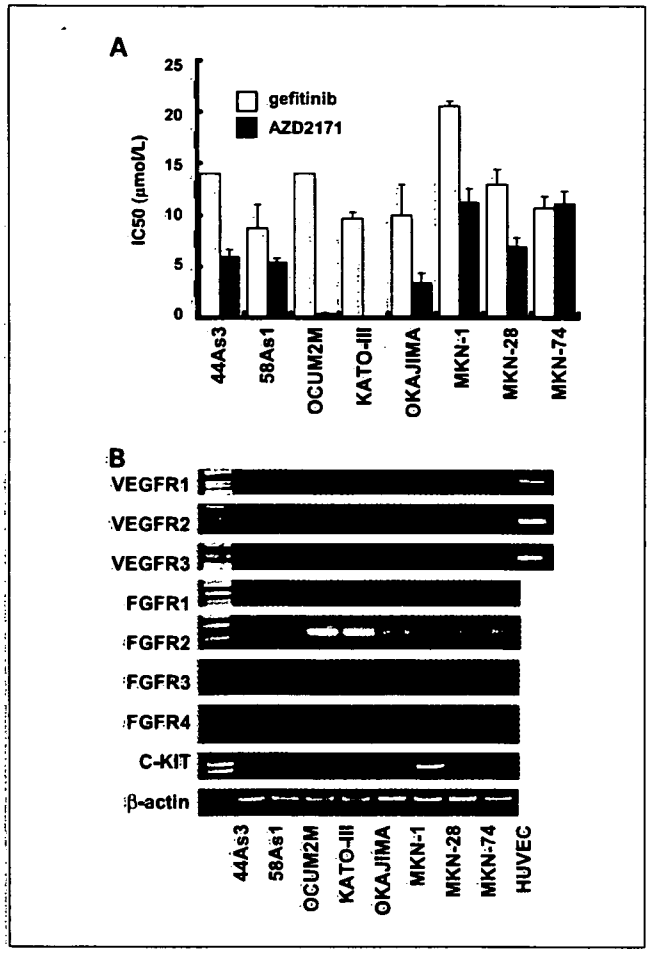


**Fig. 1.** *A, in vitro* growth-inhibitory effect of AZD2171 and gefitinib on eight gastric cancer cell lines. AZD2171 had a growth-inhibitory effect on KATO-III cells and OCUM2M cells ($IC_{50}$, 0.15 and 0.37 μmol/L, respectively). Columns, mean $IC_{50}$ of each compound from three independent experiments; bars, SD. □, $IC_{50}$ of gefitinib; ■, $IC_{50}$ 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.

*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-GGAAATCGTGCGTGACATT-3 and reverse 5-CATCTGCTGGAAGGTGGACAG-3; VEGFR-1, forward 5-TAGCGTCACCAGCAGCGAAAGC-3 and reverse 5-CCTTTCTTTTGGGTCTCTGTGC-3; VEGFR-2, forward 5-CAGACGGACAGTGGTATGGTTC-3 and reverse 5-ACCTGCTGGTGGAAAGAACAAC-3; VEGFR-3, forward 5-AGCCATTCATCAACAAGCCT-3 and reverse 5-GGCAACAGCTGGATGTCATA-3; c-KIT, forward 5-GCCCACAATAGATTGGTATTT-3 and reverse 5-AGCATCTTTACAGCGACAGTC-3; FGFR1, forward 5-GGAGGATCGAGCTCACTCGTGG-3 and reverse

5-CGGAGAAGTAGGTGGTGTCAC-3; FGFR2, forward 5-CAGTAGGACTGTAGACAGTGAA-3 and reverse 5-CCGGTGAGGCGATCGCTCCACA-3; FGFR3, forward 5-GGTCAAGGATGGCACAGGGCTG-3 and reverse 5-AGCAGCTTCTTGTCCATCCGCT-3; and FGFR4, forward 5-CCGCCTAGAGATTGCCAGCTTC-3 and reverse 5-AGGCCTGTCCATCCTTAAGCCA-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-GATAAATAGTTCCAATGCAGAAGTGCT-3 and reverse 5-TGCCCTATATAATTGGAGACCTTACA-3 (7); FGFR2 (COOH-terminal), forward 5-GAATACTTGGACCTCAGCCAA-3 and reverse 5-AACACTGCCGTTTATGTGTGG-3; and human-specific β-actin, forward 5-GGAAATCGTGCGTGACATT-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 μmol/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

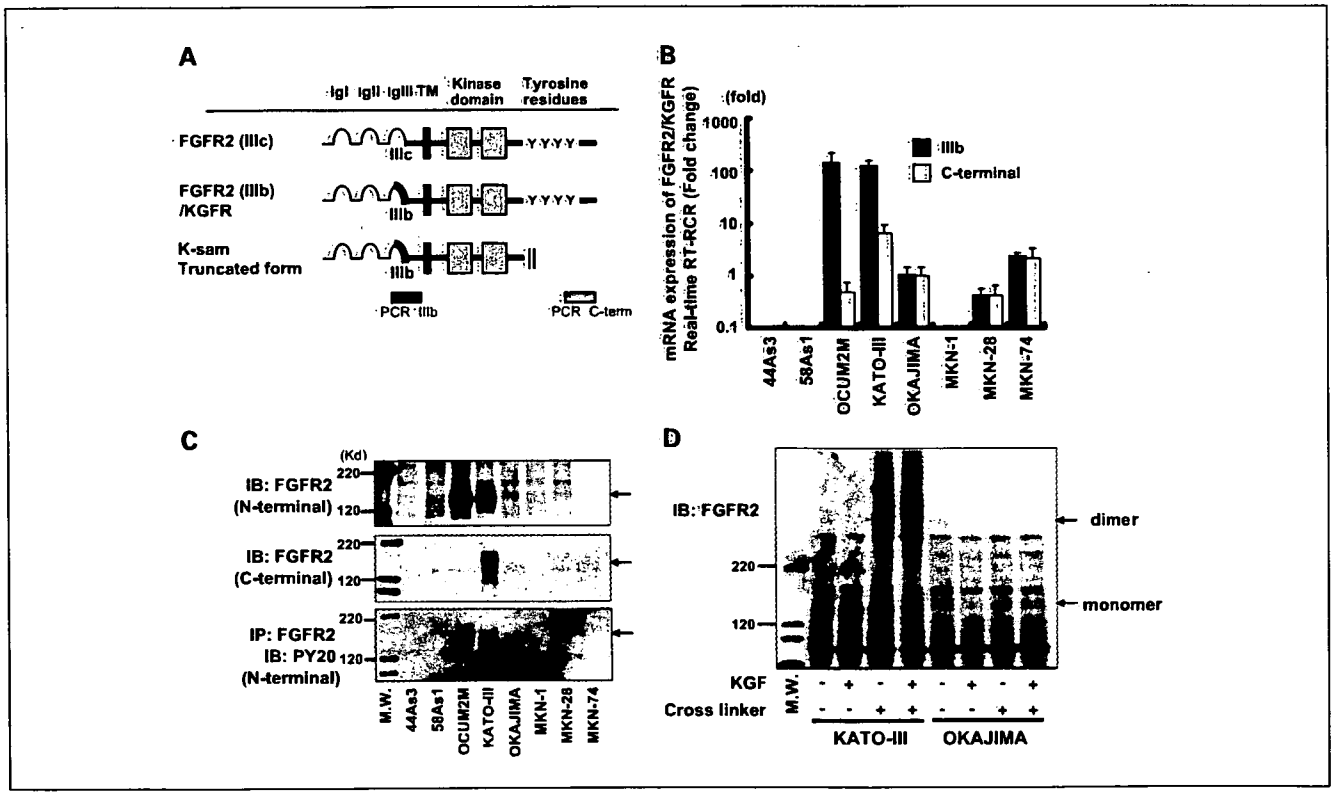


Fig. 2. *A*, schematic representation of FGFR2 and regions amplified by PCR. *B*, mRNA expression levels of FGFR2 were quantified by detecting the extracellular domain or COOH-terminal region by real-time reverse transcription-PCR. Expression in the cells is shown as a ratio to expression in OKAJIMA 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 $NH_2$ 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-(sulfosuccinimidyl) 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.* Predesigned 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, $2 \times 10^5$ 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; $5 \times 10^6$ 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 $cm^3$, 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 × width) × $\sqrt{}$(length × width) × ($\pi$/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.

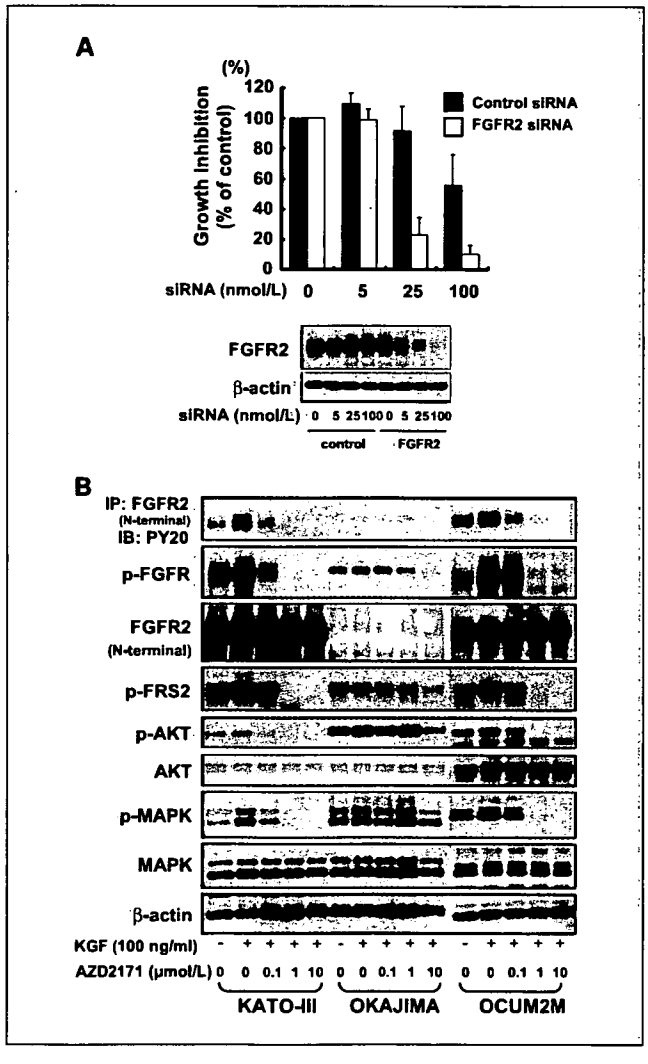


Fig. 3. *A*, FGFR2 targeting siRNA and cellular growth-inhibitory effect. KATO-III cells were plated on a 96-well plate and incubated in serum-containing medium for 24 h. After incubation, the cells were transfected with FGFR2-targeting or non-silencing siRNA and incubated for another 72 h. Cell growth was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. For immunoblotting, $2 \times 10^5$ cells per well were plated on a six-well plate and treated similarly. Marked inhibition of cell growth (~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 μmol/L AZD2171 for 3 h before adding 100 ng/mL KGF for 15 min. AZD2171 completely inhibited KGF-induced phosphorylation of FGFR2 at 1 μmol/L in the sensitive cell lines, compared with 10 μmol/L in the control cell line OKAJIMA. Similar results were observed for FRS-2, AKT, and mitogen-activated protein kinase (*MAPK*).

## 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 $IC_{50}$ of gefitinib for all cell lines was between 7 and 20 μmol/L. AZD2171 inhibited the growth of KATO-III cells and OCUM2M cells ($IC_{50}$, 0.15 and 0.37 μmol/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 $NH_2$ 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

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

| Cell line | $K_m$ | $K_i$ (μmol/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 μmol/L in KATO-III cells, compared with 10 μmol/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 μmol/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 μmol/L, respectively (Table 1). In contrast, the $K_i$ value of AZD2171 for recombinant VEGFR-2 was 0.0009 μmol/L (data not shown) and was consistent with previous reports (10). At the cellular level, phosphorylation of

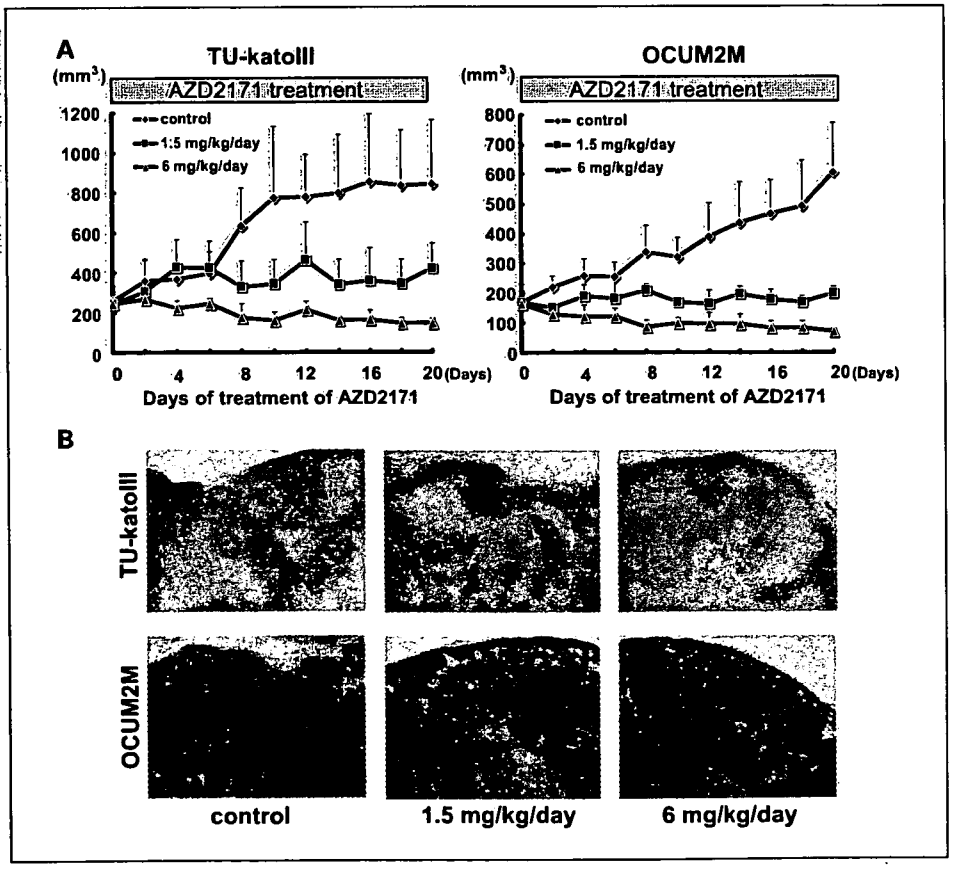


**Fig. 4.** *A, in vivo* growth-inhibitory effect of AZD2171 in a tumor xenograft model. After s.c. injecting $5 \times 10^6$ TU-kato-III or OCUM2M cells into both flanks of the mice, AZD2171 (1.5 or 6.0 mg/kg/d), or vehicle, was administered orally once daily for 3 wks. A marked tumor growth-inhibitory 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, ×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.

## Acknowledgments

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**Review Article**

# Genes Regulating the Sensitivity of Solid Tumor Cell Lines to Cytotoxic Agents: A Literature Review

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In order to review gene alterations associated with drug responses *in vitro* to identify candidate genes for predictive chemosensitivity testing, we selected from literature genes fulfilling at least one of the following criteria for the definition of '*in vitro* chemosensitivity associated gene': (i) alterations of the gene can be identified in human solid tumor cell lines exhibiting drug-induced resistance; (ii) transfection of the gene induces drug resistance; (iii) down-regulation of the gene increases the drug sensitivity. We then performed Medline searches for papers on the association between gene alterations of the selected genes and chemosensitivity of cancer cell lines, using the name of the gene as a keyword. A total of 80 genes were identified, which were categorized according to the protein encoded by them as follows: transporters ($n = 15$), drug targets ($n = 8$), target-associated proteins ($n = 7$), intracellular detoxifiers ($n = 7$), DNA repair proteins ($n = 10$), DNA damage recognition proteins ($n = 2$), cell cycle regulators ($n = 6$), mitogenic and survival signal regulators ($n = 7$), transcription factors ($n = 4$), cell adhesion-mediated drug resistance protein ($n = 1$), and apoptosis regulators ($n = 13$). The association between the gene alterations and chemosensitivity of cancer cell lines was evaluated in 50 studies for 35 genes. The genes for which the association above was shown in two or more studies were those encoding the major vault protein, thymidylate synthetase, glutathione S-transferase pi, metallothionein, tumor suppressor p53, and bcl-2. We conclude that a total of 80 *in vitro* chemosensitivity associated genes identified in the literature are potential candidates for clinical predictive chemosensitivity testing.

*Key words: chemotherapy – sensitivity – drug resistance – solid tumor*

## INTRODUCTION

Malignant neoplastic diseases remain one of the leading causes of death around the world despite extensive basic research and clinical trials. Advanced solid tumors, which account for most malignant tumors, still remain essentially incurable. For example, 80% of patients with non-small cell lung cancer have distant metastases either at the time of the initial diagnosis itself or at the time of recurrence after surgery for the primary tumor. Systemic chemotherapy against malignant tumors remains of limited efficacy in spite of the development in the recent past of several new chemotherapeutic agents; therefore, patients with distant metastases rarely live for long (1).

Tumor response to chemotherapy varies from patient to patient, and clinical objective response rates to standard chemotherapeutic regimens have been reported to be in the range of 20–40% for most common solid tumors. Thus, it would be of great benefit it became possible to predict chemosensitivity of various tumors even prior to therapy. DNA, RNA and protein-based chemosensitivity tests have

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been performed in an attempt to predict the clinical drug response, but the precise gene alterations that might be predictive of the chemosensitivity of the tumors are still unknown. Here we aimed to review the gene alterations that may be associated with the drug response *in vitro* (*in vitro* chemosensitivity associated genes) in order to identify candidate genes for predictive chemosensitivity testing in the clinical setting. The association between these gene alterations and clinical chemosensitivity in lung cancer patients has been reported elsewhere (2).

## METHODS

*In vitro* chemosensitivity associated genes were identified from the medical literature as described previously (2). Briefly, we conducted a Medline search for papers on tumor drug resistance published between 2001 and 2003. This search yielded 112 papers, including several review articles. Manual search of these papers led to identification of 134 genes or gene families that were potentially involved in drug resistance based on their function. We conducted a second Medline search for *in vitro* studies of the 134 genes or gene families using the name of the gene as a keyword. Genes that fulfilled at least one of the following criteria for the definition of *in vitro* chemosensitivity associated gene were selected from the 134 genes: (i) alterations of the gene can be identified in a human solid tumor cell lines exhibiting drug-induced resistance; (ii) transfection of the gene induces drug resistance; (iii) down-regulation of the gene or of the protein encoded by it increases the drug sensitivity. For this last category, we included studies in which the gene expression or function was suppressed by antisense RNA, hammerhead ribozyme, or antibody against the gene product. Finally, a Medline search for papers on the association between gene alterations and chemosensitivity of solid tumor cell lines was performed using the name of the gene as a keyword. Papers in which the association was evaluated in 20 or more cell lines were included in this study. The name of each gene was standardized according to the Human Gene Nomenclature Database of National Center for Biotechnology Information (NCBI).

## RESULTS

Of the 134 genes or gene families, gene alterations were found in cells exhibiting drug-induced resistance, transfection of the gene increased or decreased the drug resistance,

**Table 1.** Transporters and *in vitro* evidence of association with chemosensitivity

| Gene symbol | Alterations in DIRC | Sensitivity of | | Drugs | Association with chemosensitivity (cancer, drug) | Reference no. |
|---|---|---|---|---|---|---|
| | | UCs | DCs | | | |
| *ABC*A2 | U | – | S | Estramustine | – | 1 |
| *ABC*B1 | U | R | S | DOX, PTX, VCR, VBL | Yes (lung, DOX) | 2–11 |
| | | | | | No (lung, DOX) | 12 |
| *ABC*B11 | – | R | – | PTX | – | 13 |
| *ABC*C1 | U | R | S | CPT, DOX, ETP, MTX, VCR | Yes (lung, CDDP, DOX) | 11,14–21 |
| | | | | | No (lung, PTX) | 22 |
| *ABC*C2 | U | R | S | CDDP, DOX, MTX, VCR | No (lung, DOX) | 18, 21, 23–25 |
| *ABC*C3 | NC, U | R | – | ETP, MTX | Yes (lung, DOX) | 21, 25–28 |
| *ABC*C4 | NC, U | NC, R | – | MTX | No (lung, DOX) | 12, 25, 29–31 |
| *ABC*C5 | NC, U | NC | – | DOX, MIT | Yes (lung, ETP) | 12, 25, 31–34 |
| *ABC*G2 | M, U | R | – | DOX, MIT, MTX, SN38, TOP | – | 35–43 |
| *MVP* | U | – | NC | DOX | Yes (brain, CDDP, DOX) | 44–47 |
| | | | | | Yes (lung, DOX) | 10 |
| *ATP7*A | U | – | – | CDDP | – | 48 |
| *ATP7*B | U | R | – | CDDP | – | 48–52 |
| *SLC*29A1 | U | – | – | 5-FU | No (NCI-panel) | 52, 53 |
| *SLC*28A1 | – | S | – | 5′-DFUR | No (NCI-panel) | 53, 54 |
| *SLC*19A1 | D | S | – | MTX | Yes (NCI-panel) | 55–58 |

Alterations in drug-induced resistance cells (DIRC): D, down-regulated; M, mutated; NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): NC, no change; R, resistant; S, sensitive.
Drugs: CDDP, cisplatin; CPT, irinotecan; DOX, doxorubicin; ETP, etoposide; MIT, mitoxantrone; MTX, methotrexate; PTX, paclitaxel; SN38, irinotecan metabolite; TOP, topotecan; VBL, vinblastine; 5-FU, 5-fluorouracil; 5′-DFUR, 5′-deoxy-5-fluorouridine, capecitabine metabolite.

**Table 2.** Drug targets, the associated proteins, and *in vitro* evidence of association with chemosensitivity

| Gene symbol | Alterations in DIRC | Sensitivity of | | Drugs | Association with chemosensitivity (cancer, drug) | Reference no. |
|---|---|---|---|---|---|---|
| | | UCs | DCs | | | |
| *TUBB* | IEC, M | – | – | PTX | – | 59–63 |
| *TUBB4* | U | – | S | PTX | Yes (NCI-panel, PTX) | 59, 60, 63–66 |
| *TUBA* | IEC, M | R | – | PTX | – | 64, 67, 68 |
| *TYMS* | U | R | S | 5-FU | Yes (renal cell, 5-FU) | 69–74 |
| | | | | | No (NCI-panel, 5-FU) | 75 |
| | | | | | Yes (lung, DOX) | 10 |
| *TOP1* | M | R* | – | CPT | – | 76–84 |
| *TOP2A* | M, D | – | – | ETP, DOX | No (lung, DOX) | 10, 82–91 |
| *TOP2B* | D | – | – | ETP | – | 86, 87 |
| *DHFR* | M, U | R* | – | MTX | – | 92–96 |
| *MAP4* | – | S | – | PTX | – | 97 |
| *MAP7* | – | S | – | PTX | – | 98 |
| *STMN1* | U | R | – | PTX | – | 99, 100 |
| *KIF5B* | – | R | R | ETP, PTX | – | 101, 102 |
| *HSPA5* | – | R | – | ETP | – | 103 |
| *PSMD14* | – | R | – | CDDP, DOX, VBL | – | 104 |
| *FPGS* | D | – | – | 5-FU | – | 105 |

Alterations in drug-induced resistance cells (DIRC): D, down-regulated; IEC, isoform expression change; M, mutated; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): R, resistant; S, sensitive. Drugs: CDDP, cisplatin; CPT, irinotecan; DOX, doxorubicin; ETP, etoposide; MTX, methotrexate; PTX, paclitaxel; VBL, vinblastine; 5-FU, 5-fluorouracil.
*Over-expression of the mutant gene.

and down-regulation of the gene altered the drug sensitivity for 45, 57 and 32 genes, respectively, and a total of 80 genes fulfilled the criteria for the definition of an '*in vitro* chemosensitivity associated gene'. The genes were categorized according to the protein encoded by them as follows: transporters ($n = 15$, Table 1), drug targets ($n = 8$, Table 2), target-associated proteins ($n = 7$, Table 2), intracellular detoxifiers ($n = 7$, Table 3), DNA repair proteins ($n = 10$,

**Table 3.** Intracellular detoxifiers and *in vitro* evidence of association with chemosensitivity

| Gene symbol | Alterations in DIRC | Sensitivity of | | Drugs | Association with chemosensitivity (cancer, drug) | Reference no. |
|---|---|---|---|---|---|---|
| | | UCs | DCs | | | |
| *GSTP1* | U | – | S | CDDP, DOX, ETP | Yes (lung, DOX) | 10, 106, 107 |
| | | | | | Yes (NCI-panel) | 108 |
| *GPX* | – | R, NC | – | DOX | Yes (lung, CDDP) | 109–112 |
| *GCLC* | U | R | S | CDDP, DOX, ETP | Yes (NCI-panel) | 106, 108, 113–121 |
| *GGT2* | U | R | – | CDDP, OXP | – | 114, 117, 122, 123 |
| *MT* | U, NC | R | – | CDDP | Yes (urinary tract, CDDP) | 118, 124–130 |
| | | | | | Yes (lung, DOX) | 10, 131 |
| *RRM2* | U | R | – | 5-FU, GEM, HU | – | 71, 132–134 |
| *AKR1B1* | U | – | – | DNR | – | 135 |

Alterations in drug-induced resistance cells (DIRC): NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): NC, no change; R, resistant; S, sensitive. Drugs: CDDP, cisplatin; DNR, daunorubicin; DOX, doxorubicin; ETP, etoposide; GEM, gemcitabine; HU, hydroxyurea; OXP, oxaliplatin; 5-FU, 5-fluorouracil.

**Table 4.** DNA damage recognition and repair proteins and *in vitro* evidence of association with chemosensitivity

| Gene symbol | Alterations in DIRC | Sensitivity of | | Drugs | Association with chemosensitivity (cancer, drug) | Reference no. |
|---|---|---|---|---|---|---|
| | | UCs | DCs | | | |
| *HMGB1* | U | – | – | CDDP | – | 136 |
| *HMGB2* | – | S | – | CDDP | – | 137 |
| *ERCC1* | U | R | S | CDDP | – | 138–140 |
| *XPA* | U | R | – | CDDP | No (NCI-panel) | 141–143 |
| *XPD* | – | R | – | CDDP | Yes (NCI-panel) | 142–144 |
| *MSH2* | D, NC | – | – | CDDP | – | 145, 146 |
| *MLH1* | D, NC | – | – | CDDP | – | 145–147 |
| *PMS2* | D, NC | – | – | CDDP | – | 146, 147 |
| *APEX1* | – | R | – | BLM | – | 148 |
| *MGMT* | – | R | S | CPM, ACNU | Yes (lung, DOX) | 10, 149–152 |
| *BRCA1* | U | S | R | PTX | – | 153–155 |
| *GLO1* | – | R | – | DOX | – | 156 |

Alterations in drug-induced resistance cells (DIRC): D, down-regulated; NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): R, resistant; S, sensitive. Drugs: ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea; BLM, bleomycin; CDDP, cisplatin; DOX, doxorubicin; PTX, paclitaxel.

Table 4), DNA damage recognition proteins ($n = 2$, Table 4), cell cycle regulators ($n = 6$, Table 5), mitogenic and survival signal regulators ($n = 7$, Table 6), transcription factors ($n = 4$, Table 6), cell adhesion-mediated drug resistance protein ($n = 1$, Table 6), and apoptosis regulators ($n = 13$, Table 7).

The association between the gene alterations and *in vitro* chemosensitivity was evaluated in one study for 25 genes, in two studies for seven genes, in three studies for two genes, and in five studies for one gene, and in a total of 50 studies for 35 genes (Table 8). Significant association was found between chemosensitivity and alterations of genes encoding transporters, drug targets and intracellular detoxifiers (Table 8). Genes for which such association was shown in two or more studies were those encoding the major vault protein/lung resistance-related protein (*MVP*) (Table 1), thymidylate synthetase (*TYMS*) (Table 2), glutathione S-transferase pi (*GSTP1*), metallothionein (*MT*) (Table 3), tumor suppressor protein p53 (*TP53*), and B-cell CLL/lymphoma 2 (*BCL2*) (Table 7).

## DISCUSSION

We identified a total of 80 *in vitro* chemosensitivity associated genes. These genes have been the subject of considerable research, and of numerous scientific publications. In addition, we may also have to expect the existence of many other genes associated with chemosensitivity

**Table 5.** Cell cycle regulators and *in vitro* evidence of association with chemosensitivity

| Gene symbol | Alterations in DIRC | Sensitivity of | | Drugs | Association with chemosensitivity (cancer, drug) | Reference no. |
|---|---|---|---|---|---|---|
| | | UCs | DCs | | | |
| *RB1* | – | R | – | DOX | Yes (lung, DOX) | 157–159 |
| | | | | | No (lung, CDDP, DOX) | 160 |
| *GML* | | S | – | MMC, PTX | Yes (lung, CDDP) | 161–163 |
| *CDKN1A* | U | R, S | S | CDDP, BCNU, PTX | – | 164–171 |
| *CCND1* | – | R, S | S | CDDP, MTX, PTX | No (lung, DOX) | 10, 172–176 |
| *CDKN2A* | – | S, R | – | CDDP, 5-FU, PTX, TOP | Yes (brain, 5-FU) | 177–184 |
| *CDKN1B* | – | R | – | DOX | – | 185 |

Alterations in drug-induced resistance cells (DIRC): U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): R, resistant; S, sensitive. Drugs: BCNU, carmustine; CDDP, cisplatin; DOX, doxorubicin; MMC, mitomycin C; MTX, methotrexate; PTX, paclitaxel; TOP, topotecan; 5-FU, 5-fluorouracil.

**Table 6.** Mitogenic and survival signal regulators, integrins, transcription factors and *in vitro* evidence of association with chemosensitivity

| Gene symbol | Alterations in DIRC | Sensitivity of | | Drugs | Association with chemosensitivity (cancer, drug) | Reference no. |
|---|---|---|---|---|---|---|
| | | UCs | DCs | | | |
| *ERBB2* | – | R, NC | S | CDDP, PTX | Yes (lung, DOX) | 10, 22, 186–191 |
| *EGFR* | – | R | – | DOX | No (lung, CDDP, DOX, PTX) | 10, 22, 112, 192 |
| *KRAS2* | – | R* | – | CDDP | – | 193 |
| *HRAS* | – | R*, NC | – | Ara-C, DOX, PTX | No (lung, DOX) | 10, 193–197 |
| *RAF1* | – | R | – | DOX | – | 198 |
| *AKT1* | – | NC, R | S | CDDP, DOX, PTX | – | 199–201 |
| *AKT2* | – | R | S | CDDP | – | 200, 202 |
| *ITGB1* | – | – | S | ETP, PTX | – | 203, 204 |
| *JUN* | – | R | – | CDDP | No (lung, DOX) | 10, 205 |
| *FOS* | U | R | S | CDDP | No (lung, DOX) | 10, 206–208 |
| *MYC* | NC, U | S, R | R, S, NC | CDDP, DOX | No (lung, DOX) | 10, 209–216 |
| *NFKB1* | U | – | S | 5-FU, DOX, ETP | – | 217–222 |

Alterations in drug-induced resistance cells (DIRC): NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs)): NC, no change; R, resistant; S, sensitive. Drugs: Ara-C, 1-beta-D-arabinofuranosylcytosine; CDDP, cisplatin; DOX, doxorubicin; ETP, etoposide; PTX, paclitaxel; 5-FU, 5-fluorouracil.
*Up-regulated with mutated K-ras gene.

**Table 7.** Apoptosis regulators and in vitro evidence of association with chemosensitivity

| Gene symbol | Alterations in DIRC | Sensitivity of | | Drugs | Association with chemosensitivity (cancer, drug) | Reference no. |
|---|---|---|---|---|---|---|
| | | UCs | DCs | | | |
| *TP53* | – | S, R* | R, S | CDDP, DOX | Yes (brain) | 223–229 |
| | | | | | Yes (NCI-panel) | 230 |
| | | | | | No (breast, DOX) | 231 |
| | | | | | No (breast, DOX, PTX) | 232 |
| | | | | | No (lung, PTX) | 22 |
| *MDM2* | – | S, R | S | CDDP, DOX, PTX | – | 169, 233–238 |
| *TP73* | – | – | R | CDDP, ETP | – | 239, 240 |
| *BCL2* | U, D | R | – | CDDP, CPT, DOX | Yes (breast, DOX) | 164, 198, 231, 241–244 |
| | | | | | Yes (lung, PTX) | 22 |
| | | | | | No (breast, DOX) | 232 |
| *BCL2L1* | NC | R | S | CDDP, PTX | – | 243–251 |
| *MCL1* | – | – | S | DTIC | – | 252 |
| *BAX* | NC | S | R | CDDP, ETP, 5-FU | No (breast, DOX) | 231, 244, 253–260 |
| | | | | | No (lung, PTX) | 22 |
| *BIRC4* | – | NC | S | PTX | – | 261, 262 |
| *BIRC5* | – | R | S | CDDP, ETP | – | 263–265 |
| *TNFRSF6* | NC | – | S | CDDP | Yes (lung, DOX) | 10, 242 |
| *CASP3* | – | S | – | CDDP, DOX, ETP | No (lung, DOX) | 10, 266–268 |
| *CASP8* | – | – | R | CDDP | – | 261 |
| *HSPB1* | C | R | S | DOX | – | 52, 269–273 |

Alterations in drug-induced resistance cells (DIRC): D, down-regulated; NC, no change; U, up-regulated. Sensitivity of up-regulating cells (UCs) and down-regulating cells (DCs): NC, no change; R, resistant; S, sensitive. Drugs: CDDP, cisplatin; CPT, irinotecan; DOX, doxorubicin; DTIC, dacarbazine; ETP, etoposide; PTX, paclitaxel; 5-FU, 5-fluorouracil.
*Resistant in mutant *TP53* over-expressed cells.

**Table 8.** Gene categories and association with in vitro chemosensitivity

| Category | No. of genes | Total no. of studies | No. of studies showing association (%) |
|---|---|---|---|
| Transporter | 15 | 13 | 7 (54) |
| Drug target | 8 | 5 | 3 (69) |
| Target associated protein | 7 | 0 | 0 (0) |
| Intracellular detoxifier | 7 | 6 | 6 (100) |
| DNA repair | 10 | 3 | 2 (67) |
| DNA damage recognition protein | 2 | 0 | 0 (0) |
| Cell cycle | 6 | 5 | 3 (60) |
| Mitogenic signal | 5 | 3 | 1 (33) |
| Survival signal | 2 | 0 | 0 (0) |
| Transcription factor | 4 | 3 | 0 (0) |
| Cell adhesion-mediated drug resistance protein | 1 | 0 | 0 (0) |
| Apoptosis | 13 | 12 | 5 (42) |
| Total | 80 | 50 | 22 (44) |

but not selected in the current study, because they have never caught the scientific eye for some reasons. Thus, the results of this study may be significantly influenced by publication bias. Nonetheless, we do believe that these genes have been selected reasonably carefully, and that they may be helpful for establishing a clinical predictive chemosensitivity test.

While the association between alterations of the 80 genes and the chemosensitivity of various cell lines was evaluated in 50 studies, significant association was observed in only 22 (44%) (Table 8). The cellular functions of a gene vary among cell types and experimental conditions. The evaluation of the gene functions, however, was conducted under only limited cellular contexts in these studies, as expected. Thus, for example, the conditions of a gene transfection experiment may differ from those of an experiment to evaluate the chemosensitivity for many cell lines. The gene functions may not necessarily be examined under all possible conditions, but the evaluation must be conducted under conditions similar to those in the clinical setting in order to develop clinical chemosensitivity testing using these genes.

The other possibility for the poor correlation to *in vitro* chemosensitivity may be that more than one gene alterations are involved in the chemosensitivity of tumors. This may be discussed from the standpoint of the signal transduction pathway and from the cellular standpoint. From the standpoint of the signal transduction pathway, more than one gene may be involved in the reaction to a cytotoxic agent. One of the best examples is cooperation of *TP53* with another member of the p53 family, p73 (*TP73*), in the response to both DNA damage and chemosensitivity (3,4). From the cellular standpoint, several pathways may work additively, antagonistically, or complementally in determining the chemosensitivity of the cell. This can be understood well from the context of induction and inhibition of apoptosis being controlled by pro-apoptotic and anti-apoptotic pathways. Thus, it would be important to study several pathways at the same time, or to evaluate the net effect of the involvement of various pathways.

Complex factors influencing the cellular chemosensitivity may be operative on a tumor *in vivo*, in such a way that the tumor may exhibit highly heterogeneous gene alterations; that the tumor cells may interact with various host cells, including immune cells, fibroblasts and vascular endothelial cells; and that the differences in the distance between each tumor cell and blood vessels may affect the exposure level of tumor cells to a drug. No systematic approach has been developed to include this complex interplay of factors in the study of cellular chemosensitivity, although studies on cell adhesion-mediated drug resistance may be partly helpful.

Among the six genes for which the association was shown in two or more *in vitro* studies, four encode classical drug resistance proteins which are known to inhibit the drug–target interaction. These proteins are relatively specific for the drug as well as the cell type; e.g. *TYMS* is critical for 5-fluorouracil sensitivity. Thus, *TYMS* is a good candidate for chemosensitivity testing in patients with colorectal cancer who are treated with 5-fluorouracil (Table 2). *MVP* is involved in the transport of doxorubicin, therefore, it would be of interest to examine the association between the expression of *MVP* and the drug response in patients with breast cancer; the association of *MVP* with chemosensitivity has been evaluated only for brain tumor and lung cancer cell lines, to date (Table 1). However, the remaining two of the six genes, *TP53* and *BCL2*, are associated with apoptosis, and therefore may be relatively cell-type specific. Since all the three *in vitro* studies using breast cancer cell lines failed to show any associations between alterations of these genes and the chemosensitivity, the association should be evaluated in other tumor types in the clinical setting (Table 7).

The recently developed cDNA microarray technique allows analysis of the mRNA expression of more than 20 000 genes at once, and as many as 100–400 genes have been statistically shown as potentail chemosensitivity-related genes in various studies (5–7). The 80 genes in the current study were selected theoretically based on their known functions, and their contribution to *in vitro* chemosensitivity was shown in the experiments. Thus, it would be of interest to evaluate the expression profiles of these genes by cDNA microarray analysis, even if the difference in expression between sensitive and resistant cell lines does not reach statistical significance.

In conclusion, 80 *in vitro* chemosensitivity associated genes were identified from a review of the literature, which

may be considered to be future candidates for clinical predictive chemosensitivity testing.

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### Conflict of interest statement

None declared.

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