



SHORT COMMUNICATION

# Sequential occurrence of non-small cell and small cell lung cancer with the same *EGFR* mutation

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## KEYWORDS

EGFR mutation;  
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**Summary** We report a case of small cell lung cancer (SCLC) developing after prolonged treatment (more than 2 years) for primary adenocarcinoma of the lung, and we show that both the SCLC and non-small cell lung cancer (NSCLC) tissues obtained from the same site share the same deletion in exon 19 of *EGFR*. This case suggests that the activating *EGFR* mutations may confer the pathogenesis of a subset of SCLC.

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## 1. Introduction

The identification of somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) in patients with NSCLC and the association of such mutations with the clinical response to EGFR tyrosine kinase inhibitors such as gefitinib and erlotinib have had a substantial impact on the treatment of this disease [1,2]. To date,

however, only a few *EGFR* mutations have been detected in other solid tumors including SCLC.

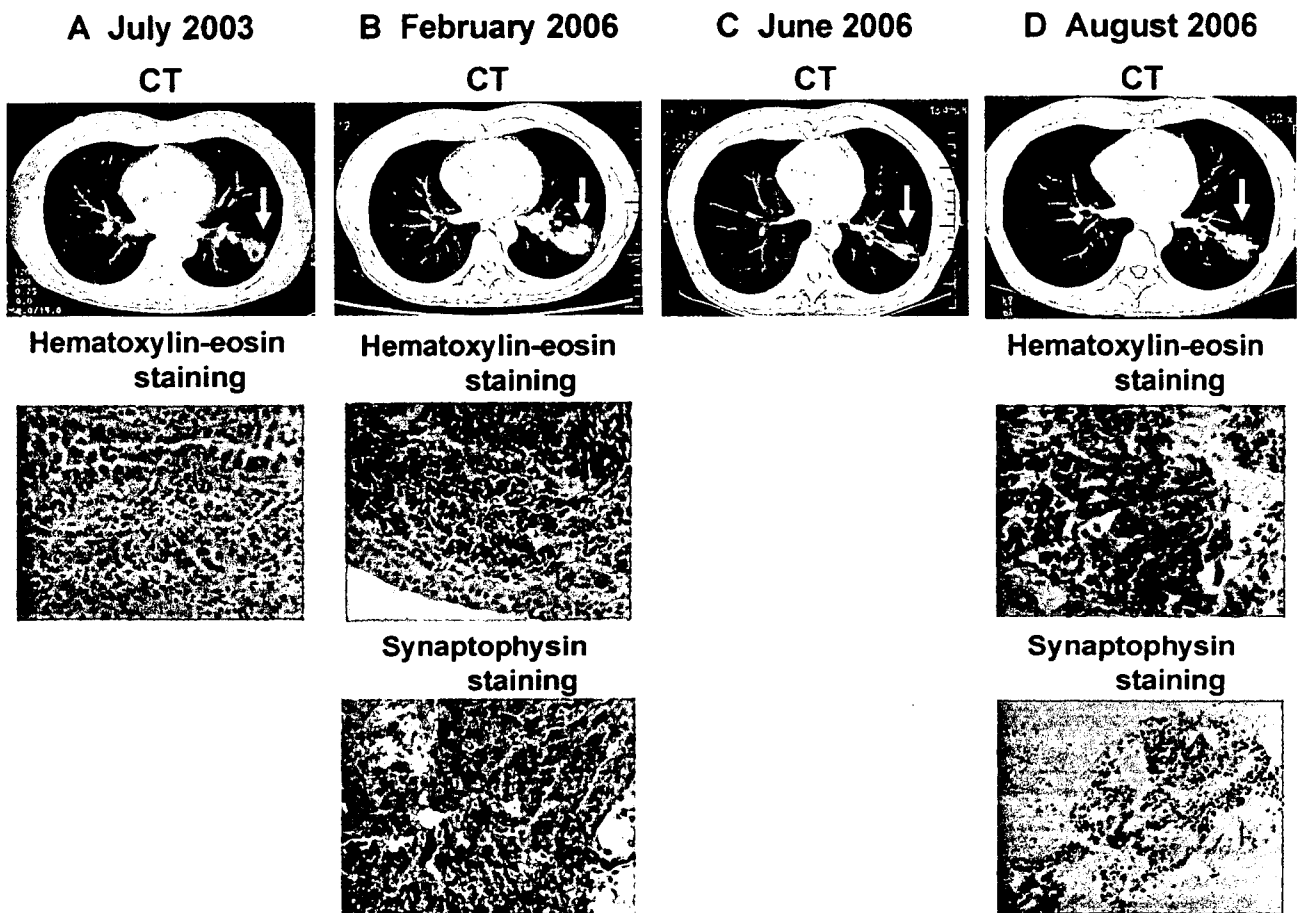
## 2. Case report

A 46-year-old Japanese woman with no smoking history was diagnosed in July 2003 with stage IIIB adenocarcinoma (acinar type) of the lung, with a primary tumor in the left lower lobe and pleural disseminations. A computed tomography (CT) scan showing the tumor (arrow) and hematoxylin–eosin (HE) staining of a tumor biopsy specimen are shown (Fig. 1A). The patient received first-line treatment with cisplatin and vinorelbine and showed a brief partial response. She

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**Fig. 1** Chest CT scan: (A) before treatment and HE staining of a tumor biopsy specimen; (B) before second lung biopsy and HE and synaptophysin stainings of a tumor biopsy specimen; (C) after four cycles of cisplatin and irinotecan; (D) before third lung biopsy and HE and synaptophysin stainings of a tumor biopsy specimen.

subsequently underwent combination chemotherapy with gemcitabine and paclitaxel, manifesting a minor response on radiographic examination. In September 2004, the mass in the left lower lobe had progressed and treatment with gefitinib (250 mg daily) was initiated. After 10 months of treatment with gefitinib alone and transient disease stabilization, a repeat evaluation in July 2005 showed progression of the primary lung tumor. Gefitinib was discontinued, and the patient was enrolled in a phase I clinical trial of new agents. The primary tumor showed no evidence of regression on radiological examination. A magnetic resonance imaging (MRI) scan in December 2005 revealed multiple brain metastases in both hemispheres, which were accompanied by symptoms including headache, nausea, and visual disturbances. After surgical resection of the largest tumor in the right parietal lobe, the patient was exposed to 10 fractions of 3 Gy whole-brain radiotherapy. Her symptoms improved markedly, and MRI scans after radiotherapy revealed almost complete regression of the brain metastases. Histological examination of the resected brain tumor revealed a synaptophysin-positive small cell cancer. The patient provided informed consent to repeated lung biopsies for histological examination. A biopsy specimen of the progressive mass in the left lower lobe in February 2006 revealed SCLC by HE staining and was positive for synaptophysin by immunohistochemical analysis (Fig. 1B). A second lung biopsy

specimen was microdissected for extraction of genomic DNA and analysis of *EGFR* mutations. A heterozygous in-frame 15-bp deletion in exon 19 of *EGFR* was detected with the use of the amplification refractory mutation system (ARMS); the genomic DNA of the patient was thus subjected to amplification by the polymerase chain reaction with primers specific for the wild-type (Fig. 2A, left panel) or mutant (Fig. 2A, right panel) versions of exon 19. The deletion was confirmed to be delE746–A750 by nucleotide sequencing. On the basis of the histological diagnosis of SCLC, the patient was treated with four cycles of cisplatin and irinotecan, and she achieved a partial response (Fig. 1C). A repeat chest CT evaluation in August 2006 showed progression of the primary lung tumor (Fig. 1D). A new lung biopsy specimen revealed nests of adenocarcinoma cells forming small tubular structures, the same subtype of the adenocarcinoma at initial diagnosis on July 2003, and was negative for synaptophysin staining (Fig. 1D). In addition, ARMS analysis of the adenocarcinoma specimen detected the same in-frame 15-bp deletion in exon 19 of *EGFR* that had been identified in the previous SCLC specimen (Fig. 2B).

### 3. Discussion

*EGFR* mutations are more frequent in women, Asians, individuals with adenocarcinoma, or those who have never

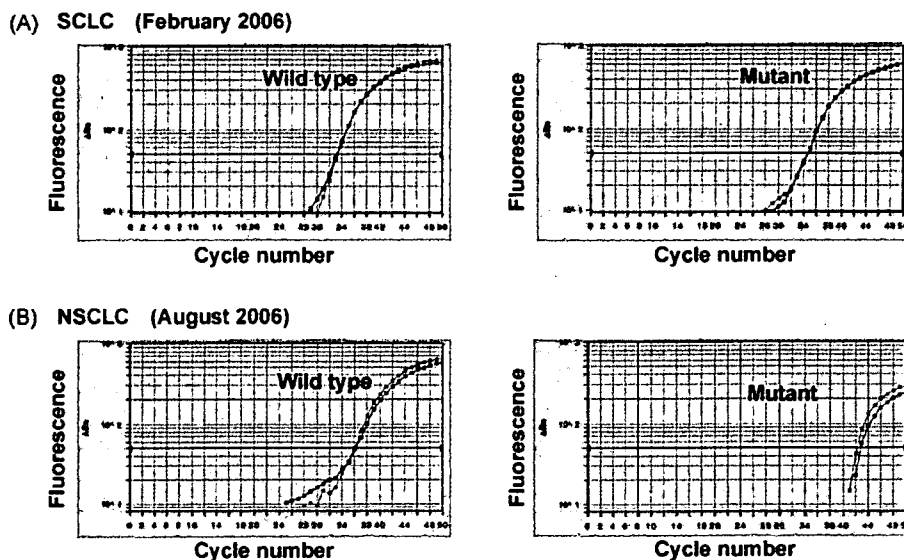


Fig. 2 Results of ARMS analysis of (A) the SCLC. Ascending curves, performed in duplicate (green and red), indicate that wild type (left panel) and deletion mutation in exon 19 (right panel) were detected; (B) the adenocarcinoma. Ascending curves, performed in duplicate (green and red), indicate that wild type (left panel) and deletion mutation in exon 19 (right panel) were detected.

smoked [3–5]. However, EGFR expression has been shown to be low or undetectable in SCLC, and screening of SCLC for EGFR mutations has yielded negative results [5]. We previously described the first case of SCLC with a deletion in exon 19 of EGFR in a nonsmoking Japanese woman [6]. Another case of SCLC with an 18-bp deletion in exon 19 of EGFR in a nonsmoking woman was also recently reported [7]. All reported cases of SCLC with EGFR mutations, including the present case, have thus been in women who have never smoked, even though SCLC occurs almost exclusively in smokers. Furthermore, all three of these SCLC cases were initially diagnosed as adenocarcinoma. In the present case, SCLC developed after prolonged treatment (>2 years) for primary adenocarcinoma, and both SCLC and NSCLC (adenocarcinoma) tissues obtained from the same site shared the same EGFR mutation. Small cell carcinoma of the prostate, which shares histological similarities with SCLC, has been shown to arise during the course of treatment for prostatic adenocarcinoma, suggesting that prostatic small cell carcinoma may originate from multipotent stem cells of the prostate that have the ability to differentiate into either epithelial or neuroendocrine type carcinoma [8–10]. It remains unclear whether the primary tumor of the present patient originally had a minor SCLC component or whether SCLC arose from transdifferentiation of the adenocarcinoma. Our finding that SCLC and NSCLC developed at the same site in the lung and shared the same somatic EGFR mutation suggests, however, that different types of lung cancer may arise from a common stem cell with multiple potential pathways of differentiation.

### Conflict of interest

We, all authors, indicate no potential conflicts of interest.

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# Skeletal metastases in non-small cell lung cancer: A retrospective study

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Bisphosphonate

## Summary

**Background:** The skeleton is one of the most common sites of metastasis in patients with advanced cancer. Bone metastases often cause SREs (skeletal-related events). Despite advances in the treatment of primary lung cancer, SREs still affect many patients. Therefore, we planned a retrospective study to investigate the clinical impact of SREs, and to compare differences in the therapeutic outcome between patients with and without skeletal metastases or SRE.

**Patients and methods:** We retrospectively investigated the charts of all 259 patients with non-small cell lung cancer (NSCLC) who consulted the Department of Medical Oncology at Kinki University School of Medicine between February 2002 and January 2005. We assessed their TNM stage, presence of skeletal metastases (on bone scintigraphy, MRI, and plain X-ray films), and outcome parameters such as SREs, analgesic use, and survival.

**Results:** A total of 70 patients (30.4%) were found to have skeletal metastases during their clinical course and 35 patients (50%) out of all 70 patients had SREs. Among 135 stage IV patients, a total of 56 (41%) had skeletal metastases, and 25 of these 56 patients (45%) had SREs. The most common SREs were the need for radiotherapy (34.3%) and hypercalcemia (20%). Patients with SREs tended to have worse survival, while no significant difference of survival was observed between patients with and without skeletal metastases.

**Conclusion:** It seems to be important to prevent SREs during the treatment of NSCLC, so further studies evaluating bisphosphonates in combination with chemotherapy are warranted.

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## 1. Introduction

Most patients with advanced cancer develop skeletal metastases during the course of their disease, and these are often associated with significant morbidity [1]. The major-

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ity of bone metastases arise from primary tumors of the breast, prostate, thyroid, or lung among others. In Western countries, it has been reported that the incidence of bone metastases in lung cancer patients is approximately 30–40%, and the median survival time (MST) of patients with such metastases is 7 months [2]. A more recent retrospective review of 435 patients with non-small cell lung cancer (NSCLC) revealed an incidence of 24% for skeletal metastases. In this review, the majority of skeletal metastases (66%) were detected at the time of initial staging [3]. Bone is a common site of cancer spread, ranking only behind the liver and the lungs in frequency.

Despite advances in the treatment of primary lung cancer, skeletal-related events (SREs) still affect many patients during their clinical course. Common complications of skeletal metastasis include bone pain, symptomatic pathologic fracture, spinal cord compression, and hypercalcemia of malignancy (HCM). These complications often require surgery to correct fractures or spinal deformities and/or radiation therapy to control the severe pain that is a hallmark of bone metastases. Pain due to bone metastases is the most frequent form of pain reported by cancer patients [4]. Thus, SREs have a negative impact on the quality of life, performance status, and function of cancer patients.

Although skeletal metastases due to lung cancer have already attracted attention in Western countries, little is known about the incidence of bone metastases arising from lung cancer in Japan. Therefore, we planned a retrospective study to investigate the clinical impact of SREs and to explore the therapeutic outcome of patients with or without skeletal metastases and/or SREs.

## 2. Patients and methods

### 2.1. Study population

We retrospectively investigated 259 patients with NSCLC who consulted the Department of Medical Oncology at Kinki University School of Medicine between February 2002 and January 2005.

The TNM stage, the presence of skeletal metastases (on bone scintigraphy, MRI, and plain X-ray films), and outcome parameters such as SREs, analgesic use, and survival were investigated.

In this study, SREs were defined as pathologic fracture, spinal cord compression, hypercalcemia, bone radiation therapy (palliative therapy for pain, or treatment/prevention of pathologic fractures and spinal cord compression), and bone surgery (stabilization or decompression).

### 2.2. Statistical analysis

The characteristics of stages III and IV patients were compared using the  $\chi^2$ -test. Survival curves were calculated and drawn by using the Kaplan–Meier method, and differences between stage IV patients with or without SREs were assessed by the log–rank test. All analyses were two-sided. Statistical software (Statistical Package SAS Software release 8.2) was used for statistical analysis, and  $p < 0.05$  was considered statistically significant.

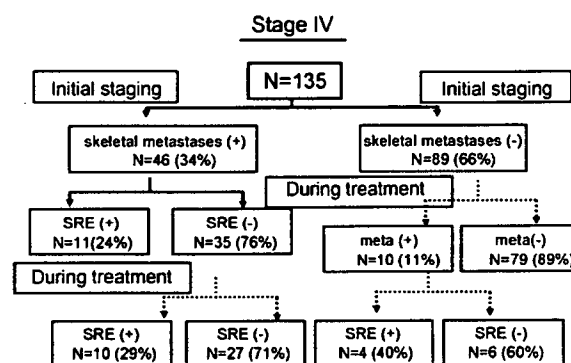


Fig. 1 Incidence of skeletal metastases and SREs in patients presenting with stage IV disease.

## 3. Results

### 3.1. Patients

We retrospectively investigated 259 NSCLC patients who visited and consulted the department of Medical Oncology, Kinki University School of Medicine, between February 2002 and January 2005. A total of 29 patients were excluded because of early stage disease, so the total number of patients assessed was 230. Among them, 156 patients (68%) were men. The pathologic diagnosis was adenocarcinoma in 140 patients (61%) and most patients had a good performance states (PS 0/1 in 193 patients, or 84%). The median age was 65 years. There were no obvious difference of these characteristics between patients in stage III and stage IV, although statistical analysis was not done.

### 3.2. Incidence of skeletal metastases

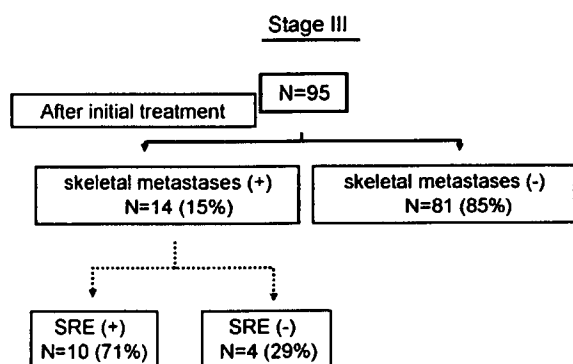
A total of 70 patients (30.4%) were found to have skeletal metastases during their clinical course. Among them, 46 patients (65.7%) had skeletal metastases at the time of initial diagnosis. Thirty-five (50%) of the 70 patients suffered from SREs. Eleven (31%) of the 35 patients had SREs at the time of initial staging, and 24 (69%) of the 35 patients developed SREs due to recurrence of their disease after treatment.

Of the 135 patients who were initially in stage IV, 56 patients (41%) had skeletal metastases, and 25 (45%) of these 56 patients suffered from SREs. Among the 56 patients with skeletal metastases, 46 patients (82%) had these metastases at the time of initial staging, while 11 (44%) of the 25 patients with SREs already had them at initial staging (Fig. 1).

A total of 95 patients were initially in stage III. After treatment of their cancer, 14 patients developed skeletal metastases and 10 (71%) of them suffered from SREs (Fig. 2).

### 3.3. Sites of skeletal metastasis

Table 1 shows the sites of skeletal metastasis. The spine was the most common site (50%), followed by the ribs (27.1%).



**Fig. 2** Incidence of skeletal metastases and SREs in patients presenting with stage III disease.

**Table 1** Sites of skeletal metastasis (n = 70)

Spine	35 (50.0%)
Ribs	19 (27.1%)
Ilium	7 (10.0%)
Sacrum	5 (7.1%)
Femur	4 (5.7%)
Skull	4 (5.7%)
Humerus	2 (2.9%)
Scapula	2 (2.9%)
Sternum	2 (2.9%)

### 3.4. Complications of skeletal metastasis

Table 2 shows the details of SREs. Among 70 patients with skeletal metastases, a total of 35 (50%) developed SREs. The most common SRE was bone radiation therapy in 24 patients (34.3%), followed by hypercalcemia in 14 patients (20%). Spinal cord compression occurred in 11 patients (15.7%).

### 3.5. Analgesic use

Among the 70 patients with skeletal metastases, 55 (78.6%) had localized bone pain, and 39 (70%) of the 55 patients required NSAIDs for pain relief, while 40 (73%) of them required opioids.

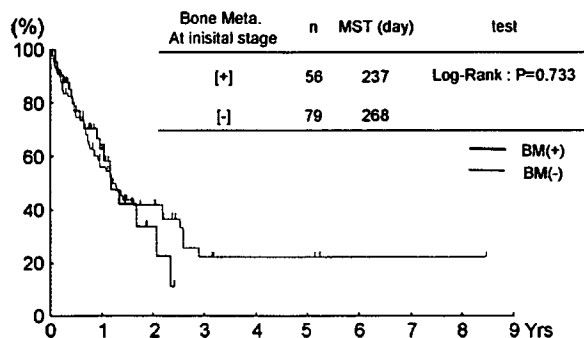
### 3.6. Survival

The MST was 679 days for stage III patients and 447 days for those with stage IV disease.

Fig. 3 shows survival of the patients who had stage IV disease with or without skeletal metastases. For patients with

**Table 2** Individual SREs in 35 out of 70 patients with skeletal metastases

1	Radiation to bone	24 cases: 34.3%
2	Hypercalcemia	14 cases: 20.0%
3	Spinal cord compression	11 cases: 15.7%
4	Pathologic fracture	5 cases: 7.1%
5	Surgical stabilization/decompression	0 cases: 0%
	Localized bone pain	55 cases: 78.6%



**Fig. 3** Overall survival of stage IV patients with and without skeletal metastases.

**Table 3** MST of patients presenting in stages III or IV

	N	MST (days)
<b>Stage IV</b>		
BM (-)	79	268
BM (+)	56	237
<b>BM (+)</b>		
SRE (+)	25	187
SRE (-)	31	366
<b>Stage III</b>		
BM (-)	81	314
BM (+) <sup>a</sup>	14	298
<b>BM (+)</b>		
SRE (+)	10	240
SRE (-)	4	255

<sup>a</sup> Following initial treatment.

skeletal metastases, MST was 237 days, while it increased to 268 days for those without such metastases. However, there was no statistically significant difference of survival between patients with and without skeletal metastases ( $p=0.733$ ). Interestingly, the MST was 187 days for stage IV patients with SREs, while it was 366 days for patients without SREs (Table 3), but this difference also failed to reach statistical significance. The median time to the first SRE was 182 days for patients presenting with stage III disease and 93 days for those presenting with stage IV disease, while the MST from the date of the first SRE was 40 days for stage III patients and 138 days for stage IV patients. The MST from the date of the first SRE was 92 days for all patients with SRE.

## 4. Discussion

As far as we know, this is the first report about the frequency and influence on survival of skeletal metastases and SREs in Japanese lung cancer patients. Our results were similar to 1997 data from the USA [2]. A total of 70 patients (30.4%) were noted to have skeletal metastases during their clinical course, as did 56 patients (41%) with stage IV disease at the time of diagnosis.

Our study also showed that the most common SRE was radiation therapy for bone metastases (34.3%), followed by hypercalcemia (20%), and spinal cord compression (15.7%).

On the other hand, the major SREs in western countries were radiation therapy for bone metastases in 49.9%, hypercalcemia in 8.0%, cord compression in 5.8%, and bone surgery in 12.6%. Compared with Japanese data, the frequency of surgery seems to be higher.

In our analysis, the MST of patients with skeletal metastases was 7.9 months and there was no significant difference of survival between the patients with and without skeletal metastases ( $p=0.733$ ). A total of 50% of the patients with skeletal metastases had SREs according to this retrospective study, which is similar to the results from Western countries [5–7].

Interestingly, the MST of our lung cancer patients with SREs was only half of that for patients without SREs (6.2 months for patients with SREs versus 12.2 months for patients without SREs). The lack of a significant difference may have been attributable to our small sample size. There seem to be two main reasons why the prognosis of lung cancer patients with SREs is poor. One is that they are unable to receive appropriate or sufficiently aggressive treatment because of deterioration of PS due to SREs. The other is that tumors associated with SREs may be chemoresistant because these lesions have progressed more, even if it is possible to treat them. Therefore, it is more important to prevent SREs than to treat them. In this study, the median time to the first SREs was 3.1 months in patients with stage IV disease. If this period could be extended, more patients would be able to receive second-line and third-line treatment for lung cancer. It is a well-known fact that bisphosphonates is effective in the treatment for hypercalcemia which is one of SREs [8,9]. Recently, bisphosphonates have been shown to be effective for the prevention of SREs in patients with various tumors, particularly breast cancer, prostate cancer, and multiple myeloma [10–13]. Saad et al. conducted a placebo-controlled trial of a new bisphosphonate (zoledronic acid) in patients with hormone-refractory metastatic prostate carcinoma and reported that it significantly reduced SREs and also significantly increased the time to the first SRE [14]. With regard to NSCLC, there has only been one study that evaluated the influence of bisphosphonates on SREs and the time to the first SRE [6]. However, as half of the subjects did not have NSCLC in that study and it was the only trial done previously, no consensus about the value of bisphosphonates for skeletal metastases of NSCLC has been obtained.

The present retrospective study revealed that the prognosis of NSCLC patients with SREs was worse than that of patients without SREs, while there was no survival difference between patients with and without skeletal metastases. Although it is unclear whether it is important to delay the time to the first SRE in NSCLC patients because of their poor prognosis, our data may support the clinical development of bisphosphonates. These drugs have also demonstrated antitumor activity in preclinical models through inhibition of angiogenesis and by the inhibition of cell proliferation and cell adhesion [15]. Therefore, the West Japan Thoracic Oncology Group is planning a randomized clinical trial to compare the combination of chemotherapy and zoledronic acid with chemotherapy alone for NSCLC patients with skeletal metastases. The aim is to examine

whether this combination has a survival benefit through delaying SREs and through the antitumor activity of zoledronic acid.

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# The novel microtubule-interfering agent TZT-1027 enhances the anticancer effect of radiation *in vitro* and *in vivo*

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TZT-1027 is a novel anticancer agent that inhibits microtubule polymerisation and manifests potent antitumour activity in preclinical models. We have examined the effect of TZT-1027 on cell cycle progression as well as the anticancer activity of this drug both *in vitro* and *in vivo*. With the use of tsFT210 cells, which express a temperature-sensitive mutant of Cdc2, we found that TZT-1027 arrests cell cycle progression in mitosis, the phase of the cell cycle most sensitive to radiation. A clonogenic assay indeed revealed that TZT-1027 increased the sensitivity of H460 cells to  $\gamma$ -radiation, with a dose enhancement factor of 1.2. Furthermore, TZT-1027 increased the radiosensitivity of H460 and A549 cells in nude mice, as revealed by a marked delay in tumour growth and an enhancement factor of 3.0 and 2.2, respectively. TZT-1027 also potentiated the induction of apoptosis in H460 cells by radiation both *in vitro* and *in vivo*. Histological evaluation of H460 tumours revealed that TZT-1027 induced morphological damage to the vascular endothelium followed by extensive central tumour necrosis. Our results thus suggest that TZT-1027 enhances the antitumour effect of ionising radiation, and that this action is attributable in part to potentiation of apoptosis induction and to an antivascular effect. Combined treatment with TZT-1027 and radiation therefore warrants investigation in clinical trials as a potential anticancer strategy.

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**Keywords:** TZT-1027; radiosensitisation; microtubule; mitotic arrest; apoptosis; antivascular effect

The combination of modalities of cancer treatment offers improvements in the survival of cancer patients compared with individual therapeutic approaches. Such therapeutic benefit has been achieved with combinations of chemo- and radiotherapy in a variety of cancers. The cytotoxicity of most chemotherapeutic agents as well as that of radiation is highly dependent on the phase of the cell cycle. Although various types of anticancer drugs are able to arrest cells at specific cell cycle checkpoints, the ability of antimicrotubule agents to block cell cycle progression in G<sub>2</sub>-M phase is the biological basis for combination of these agents with radiation (Pawlik and Keyomarsi, 2004). Microtubule-interfering agents have been shown to increase the radiosensitivity of tumour cells in preclinical and clinical studies (Liebmann *et al*, 1994; Choy *et al*, 1995; Edelstein *et al*, 1996; Vokes *et al*, 1996; Kim *et al*, 2001, 2003; Hofstetter *et al*, 2005; Simoens *et al*, 2006).

TZT-1027 (Soblidotin), a novel microtubule-interfering agent synthesised from dolastatin 10 (Figure 1), exhibits greater antitumour activities and a reduced toxicity compared with its

parent compound (Miyazaki *et al*, 1995). TZT-1027 inhibits microtubule assembly by binding to tubulin (Kobayashi *et al*, 1997; Natsume *et al*, 2000). *In vitro*, it inhibits the growth of various human cancer cells at low concentrations (Watanabe *et al*, 2000). *In vivo*, TZT-1027 also manifests a broad spectrum of activity against various murine tumours as well as human tumour xenografts, without inducing a pronounced reduction in body weight (Kobayashi *et al*, 1997; Watanabe *et al*, 2000, 2006a; Natsume *et al*, 2003, 2006). Furthermore, the drug exhibited a potent antivascular effect on existing vasculature in an advanced-stage tumour model (Otani *et al*, 2000). TZT-1027 is currently undergoing clinical evaluation, with a reduction in tumour size and disease stabilisation having been observed in a subset of patients (Schoffski *et al*, 2004; de Jonge *et al*, 2005; Greystoke *et al*, 2006; Tamura *et al*, 2007).

Despite its demonstrated efficacy against solid tumours, the effects of TZT-1027 in combination with radiation have not been examined. As an initial step in determining the antitumour activity of TZT-1027 in combination with radiation, we investigated the effect of this agent on cell cycle progression in synchronised tsFT210 cells (Osada *et al*, 1997), which harbour a temperature-sensitive mutant of Cdc2. We found that TZT-1027 induces arrest of cells in mitosis, the phase of the cell cycle most sensitive to radiation. We then studied the radiosensitising properties of TZT-1027 *in vitro* and *in vivo* with a human lung cancer model and elucidated the mechanism of radiosensitisation by this agent.

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## MATERIALS AND METHODS

### Cell lines and reagents

tsFT210 mouse mammary carcinoma cells, which express a temperature-sensitive mutant of Cdc2, were kindly provided by H Kakeya (Antibiotics Laboratory, Discovery Research Institute, RIKEN, Saitama, Japan) and were maintained under a humidified atmosphere of 5% CO<sub>2</sub> in air at 32.0°C in RPMI 1640 (Sigma, St Louis, MO, USA) supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin. H460 human lung large cell carcinoma and A549 human lung adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA, USA) and were maintained as for tsFT210 cells with the exception that the culture temperature was 37°C. TZT-1027 (Figure 1) was provided by Daiichi Pharmaceutical Co. Ltd (Tokyo, Japan). Nocodazole and roscovitine were obtained from Sigma.

### Cell cycle analysis

Cells were harvested, washed with phosphate-buffered saline (PBS), fixed with 70% methanol, washed again with PBS, and stained with propidium iodide (0.05 mg ml<sup>-1</sup>) in a solution containing 0.1% Triton X-100, 0.1 mM EDTA, and RNase A (0.05 mg ml<sup>-1</sup>). The stained cells (~1 × 10<sup>6</sup>) were then analysed for DNA content with a flow cytometer (FACScalibur; Becton Dickinson, San Jose, CA, USA).

### Measurement of mitotic index and apoptotic cells

Cells were harvested, washed with PBS, fixed with methanol:acetic acid (3:1, v/v), washed again with PBS, and stained with 4',6-diamidino-2-phenylindole (DAPI) (0.5 μg ml<sup>-1</sup>). The stained cells (~1 × 10<sup>6</sup>) were observed with a fluorescence microscope (IX71; Olympus, Tokyo, Japan). To determine the proportion of mitotic or apoptotic cells, we scored at least 300 cells in each of at least three randomly selected microscopic fields for each of three slides per sample. Cells with condensed chromosomes and no obvious nuclear membrane were regarded as mitotic cells, and the mitotic index was calculated as the percentage of mitotic cells among total viable cells. Cells with fragmented and uniformly condensed nuclei were regarded as apoptotic cells.

### Clonogenic assay

Exponentially growing H460 cells in 25-cm<sup>2</sup> flasks were harvested by exposure to trypsin and counted. They were diluted serially to appropriate densities and plated in triplicate in 25-cm<sup>2</sup> flasks containing 10 ml of medium. The cells were treated with 1 nM TZT-1027 or vehicle (dimethyl sulfoxide, or DMSO; final concentration, 0.1%) for 24 h and then exposed to various doses of γ-radiation with a <sup>60</sup>Co irradiator at a rate of ~0.82 Gy min<sup>-1</sup> and at room temperature. The cells were then washed with PBS, cultured in drug-free medium for 10–14 days, fixed with methanol:acetic acid (10:1, v/v), and stained with crystal violet. Colonies containing >50 cells were counted. The surviving fraction was calculated as: (mean number of colonies)/(number of inoculated cells × plating

efficiency). Plating efficiency was defined as the mean number of colonies divided by the number of inoculated cells for nonirradiated controls. The surviving fraction for combined treatment was corrected by that for TZT-1027 treatment alone. The dose enhancement factor (DEF) was calculated as the dose (Gy) of radiation that yielded a surviving fraction of 0.1 for vehicle-treated cells divided by that for TZT-1027-treated cells (after correction for drug toxicity).

### *In vivo* antitumour activity of TZT-1027 with or without radiation

All animal studies were performed in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kyoto University. The ethical guidelines followed meet the requirements of the UKCCCR guidelines (Workman *et al*, 1998). Tumour cells (2 × 10<sup>6</sup>) were injected subcutaneously into the right hind leg of 7-week-old female athymic nude mice. Tumour volume was determined from caliper measurement of tumour length (*L*) and width (*W*) according to the formula  $LW^2/2$ . Treatment was initiated when tumours in each group achieved an average volume of ~200–250 mm<sup>3</sup>. Treatment groups consisted of control, TZT-1027 alone, radiation alone, and the combination of TZT-1027 and radiation. Each treatment group contained six to eight mice. TZT-1027 was administered intravenously in a single dose of 0.5 mg kg<sup>-1</sup> of body weight; mice in the control and radiation-alone groups were injected with vehicle (physiological saline). Tumours in the leg were exposed to 10 Gy of γ-radiation with a <sup>60</sup>Co irradiator at a rate of ~0.32 Gy min<sup>-1</sup> immediately after drug treatment. Growth delay (GD) was calculated as the time for treated tumours to achieve an average volume of 500 mm<sup>3</sup> minus the time for control tumours to reach 500 mm<sup>3</sup>. The enhancement factor was then determined as:  $(GD_{\text{combination}} - GD_{\text{TZT-1027}})/(GD_{\text{radiation}})$ .

### TUNEL staining

Mice were killed 14 days after treatment initiation and the tumours were removed and preserved in 10% paraformaldehyde. Apoptosis in tumour sections was determined by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL) assay with the use of an apoptosis detection kit (Chemicon, Temecula, CA, USA). The number of apoptotic cells was counted in 10 separate microscopic fields (×100) for three sections of each tumour of each group.

### Histological analysis

A single dose of TZT-1027 (2.0 mg kg<sup>-1</sup>) or vehicle (physiological saline) was administered intravenously to mice when H460 tumours had achieved a volume of ~400 to 600 mm<sup>3</sup>. Tumour tissue was extirpated 4 or 24 h after drug administration, and half of the tissue was fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. The other half of the tumour tissue was fixed for 12–48 h in zinc fixative

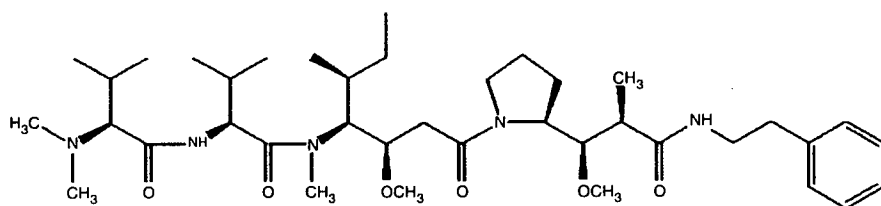


Figure 1 Chemical structure of TZT-1027.

(BD Biosciences, San Jose, CA, USA), embedded in paraffin, sectioned, and immunostained for CD31. Endogenous peroxidase activity was blocked by incubation of the latter sections for 20 min with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol, and nonspecific sites were blocked with antibody diluent (Dako Japan, Kyoto, Japan). Sections were then incubated overnight at 4°C with a 1:50 dilution of a rat monoclonal antibody to mouse CD31 (BD Biosciences), washed with PBS, and processed with a Histfine Simple Stain PO (M) kit (Nichirei, Tokyo, Japan) for detection of immune complexes. Sections were counterstained with Mayer's hematoxylin, covered with a coverslip with the use of a permanent mounting medium, and examined with a light microscope (CX41; Olympus, Tokyo, Japan).

### Statistical analysis

Data are presented as means  $\pm$  s.d. or s.e. and were compared by the unpaired Student's *t*-test. A *P* value of <0.05 was considered statistically significant.

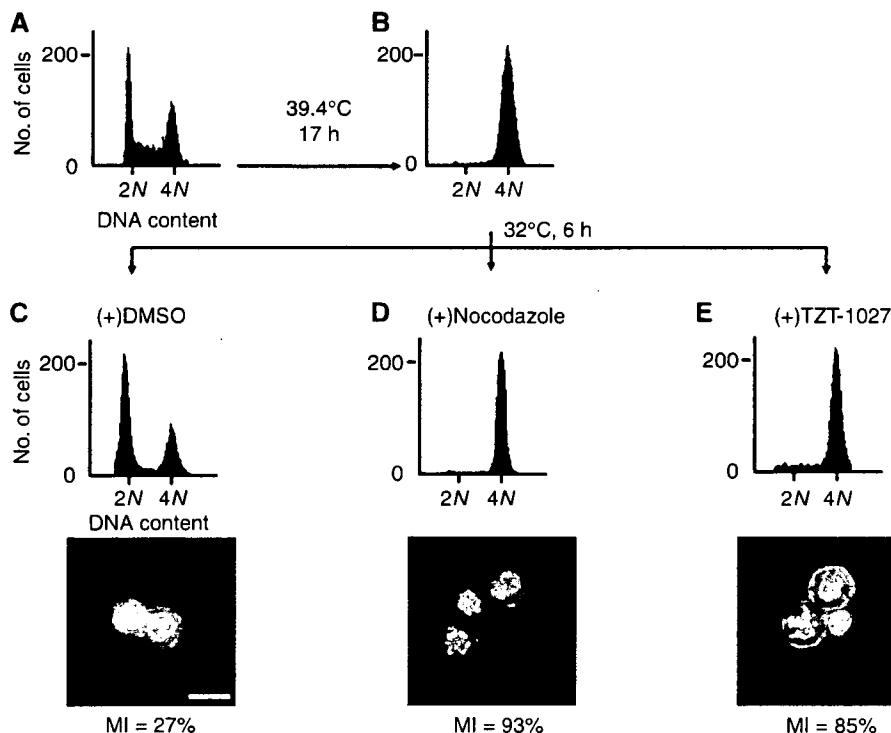
## RESULTS

### Induction of cell cycle arrest at M phase but not at G<sub>1</sub>-S in tsFT210 cells by TZT-1027

To examine the effect of TZT-1027 on cell cycle progression, we performed flow cytometric analysis of tsFT210 cells, which express a temperature-sensitive mutant of Cdc2. These mammary carcinoma cells exhibit a normal cell cycle distribution when incubated at the permissive temperature of 32.0°C, but they arrest at G<sub>2</sub> phase as a result of Cdc2 inactivation when incubated at the

nonpermissive temperature of 39.4°C (Figure 2A and B). We synchronised tsFT210 cells at G<sub>2</sub> phase by incubation at 39.4°C for 17 h and then cultured them at 32.0°C for 6 h in the presence of nocodazole (an inhibitor of microtubule polymerisation), TZT-1027, or vehicle (DMSO). In the presence of vehicle alone, the number of cells in G<sub>2</sub> phase decreased markedly and there was a corresponding increase in the number of cells in G<sub>1</sub> phase, indicative of re-entry of cells into the cell cycle (Figure 2C). In contrast, treatment with nocodazole or TZT-1027 prevented the cells from passing through G<sub>2</sub>-M phase (Figure 2D and E). Given that flow cytometric analysis did not distinguish between cells in M phase and those in G<sub>2</sub> phase, we determined the mitotic index of cells by DAPI staining and fluorescence microscopy. Most of the cells released from temperature-induced arrest in the presence of nocodazole manifested condensed chromosomes without a nuclear membrane, yielding a mitotic index of 93%; most of the cells had thus arrested in mitosis (Figure 2D). Most of the cells released from temperature-induced arrest in the presence of TZT-1027 showed similar mitotic figures, yielding a mitotic index of 85% (Figure 2E) and demonstrating that TZT-1027 also inhibits cell cycle progression at mitosis.

We next examined whether TZT-1027 affects the G<sub>1</sub>-S transition. We arrested tsFT210 cells at G<sub>2</sub> phase by incubation at 39.4°C, released the cells into G<sub>1</sub> phase by shifting to the permissive temperature for 6 h, and then incubated them for an additional 6 h in the presence of roscovitine (an inhibitor of CDK2 that prevents cell cycle progression at G<sub>1</sub> phase), TZT-1027, or vehicle (Figure 3). The cells incubated with vehicle passed through G<sub>1</sub> phase and yielded a broad S-phase peak (Figure 3D), whereas those treated with roscovitine did not pass through G<sub>1</sub> phase (Figure 3E). In contrast, TZT-1027 had no effect on passage of the synchronised tsFT210 cells through the G<sub>1</sub>-S transition (Figure 3F). Together,



**Figure 2** Inhibition of tsFT210 cell cycle progression through G<sub>2</sub>-M by TZT-1027. Cells were cultured at the permissive temperature of 32.0°C (A) and then incubated for 17 h at the nonpermissive temperature of 39.4°C (B). They were subsequently released from G<sub>2</sub> arrest by incubation at 32.0°C for 6 h in the presence of DMSO (C), 1  $\mu$ M nocodazole (D), or 2 nM TZT-1027 (E). At each stage of the protocol, cells were analysed for DNA content by staining with propidium iodide and flow cytometry. The 2N and 4N peaks indicate cells in G<sub>0</sub>-G<sub>1</sub> and G<sub>2</sub>-M phases of the cell cycle, respectively. The cells were also stained with DAPI and examined by fluorescence microscopy after treatment with DMSO, nocodazole, or TZT-1027 (lower panels), and the mitotic index (MI) was determined; scale bar, 20  $\mu$ m. Data are representative of at least three independent experiments.

these results indicate that the effect of TZT-1027 on cell cycle progression is specific to M phase.

### Induction of cell cycle arrest at M phase in asynchronous H460 cells by TZT-1027

We next examined whether TZT-1027 induced mitotic arrest in asynchronous H460 human non-small cell lung cancer cells. Flow cytometric analysis revealed that treatment of H460 cells with TZT-1027 for 24 h induced a threefold increase in the proportion of cells with a DNA content of 4N compared with that apparent for

vehicle-treated cells (29.1 vs 8.7%) (Figure 4A and B). Furthermore, DAPI staining revealed that TZT-1027 induced a significant increase in the mitotic index of H460 cells compared with that for the control cells (23.3 vs 4.6%) (Figure 4C and D), indicating that most of the TZT-1027-treated cells with a DNA content of 4N were arrested in M phase rather than in G<sub>2</sub> phase. These observations thus showed that TZT-1027 also induced mitotic arrest in asynchronous H460 cells.

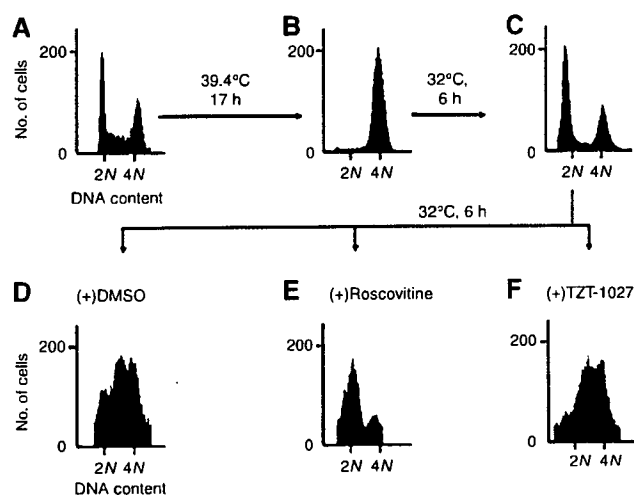
### Radiosensitisation of H460 cells by TZT-1027 *in vitro*

Cells in M phase are more sensitive to radiation than are those in other phases of the cell cycle. Given that exposure of H460 cells to TZT-1027-induced mitotic arrest, we next examined whether this agent might sensitise H460 cells to  $\gamma$ -radiation with the use of a clonogenic assay. H460 cells were incubated for 24 h with 1 nM TZT-1027 or vehicle (DMSO) and then exposed to various doses (0, 2, 4, or 6 Gy) of  $\gamma$ -radiation. The cells were then allowed to form colonies in drug-free medium for 10–14 days. Survival curves revealed that TZT-1027 increased the radiosensitivity of H460 cells, with a DEF of 1.2 (Figure 5A).

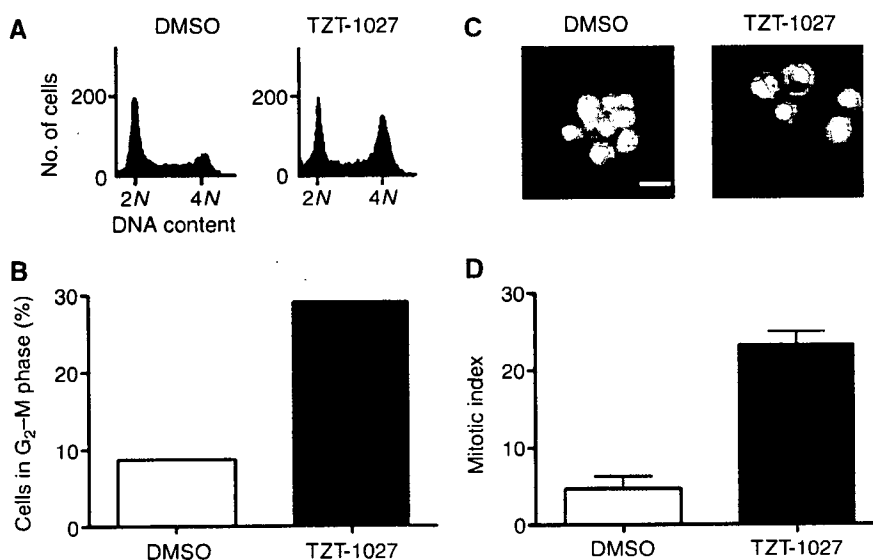
To determine whether radiosensitisation by TZT-1027 was reflected by an increase in the proportion of apoptotic cells, we exposed H460 cells to 1 nM TZT-1027 or vehicle for 24 h, treated the cells with various doses (0, 2, 4, or 6 Gy) of radiation, and then incubated them in drug-free medium for an additional 24 h before quantification of apoptosis. Combined treatment with TZT-1027 and 4 or 6 Gy of radiation resulted in a significant increase in the number of apoptotic cells compared with the sum of the values for treatment with drug alone or radiation alone (Figure 5B). TZT-1027 thus promoted radiation-induced apoptosis in H460 cells.

### Radiosensitisation of H460 cells and A549 cells by TZT-1027 *in vivo*

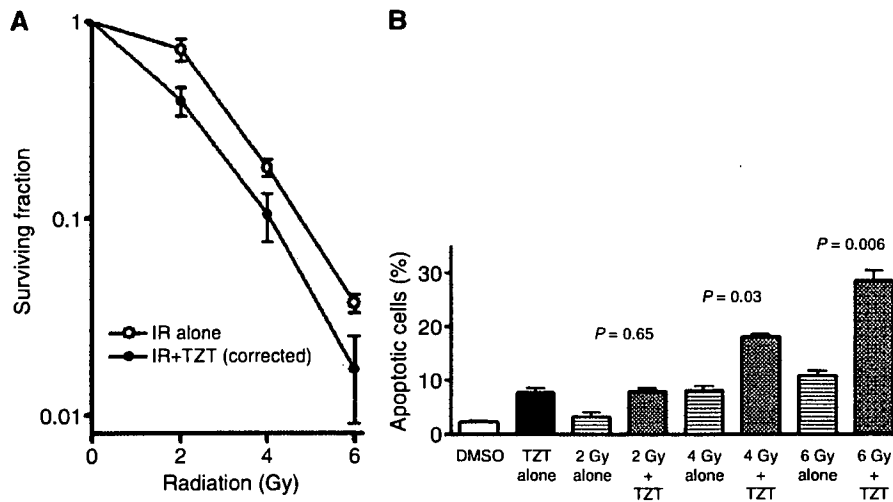
To determine whether the TZT-1027-induced increase in the radiosensitivity of tumour cells observed *in vitro* might also be apparent *in vivo*, we injected H460 cells or A549 human lung



**Figure 3** Lack of effect of TZT-1027 on tsFT210 cell cycle progression through G<sub>1</sub>-S. Exponentially growing tsFT210 cells (A) were arrested in G<sub>2</sub> phase by incubation for 17 h at 39.4°C (B). The cells were incubated at 32.0°C first for 6 h to allow progression to G<sub>1</sub> phase (C) and then for an additional 6 h in the presence of DMSO (D), 50  $\mu$ M roscovitine (E), or 2 nM TZT-1027 (F). At each stage of the protocol, cells were analysed for DNA content by flow cytometry. Data are representative of at least three independent experiments.



**Figure 4** Induction of cell cycle arrest at M phase in H460 cells by TZT-1027. H460 cells were incubated in the presence of 1 nM TZT-1027 or vehicle (DMSO) for 24 h, after which DNA content was measured by flow cytometry (A) and the fraction of cells in G<sub>2</sub>-M phase was determined (B). The cells were also stained with DAPI and examined by fluorescence microscopy (C) and the mitotic index was determined (D). Data in (A) through (C) are representative of at least three independent experiments; data in (D) are means  $\pm$  s.d. of values from three independent experiments. Scale bar in (C), 20  $\mu$ m.



**Figure 5** Sensitisation of H460 cells to  $\gamma$ -radiation by TZT-1027 *in vitro*. **(A)** Clonogenic assay. Cells were incubated with 1 nM TZT-1027 or vehicle (DMSO) for 24 h, exposed to the indicated doses of  $\gamma$ -radiation, and then incubated in drug-free medium for 10–14 days for determination of colony-forming ability. Survival curves were generated after correction of colony formation observed for combined treatment with ionising radiation (IR) and TZT-1027 by that apparent for treatment with TZT-1027 alone. **(B)** Assay of apoptosis. Cells were incubated with 1 nM TZT-1027 or vehicle (DMSO) for 24 h, exposed to various doses (0, 2, 4, or 6 Gy) of  $\gamma$ -radiation, and then incubated for 24 h in drug-free medium. Cells were then fixed and stained with DAPI for determination of the proportion of apoptotic cells by fluorescence microscopy. Data in **(A)** and **(B)** are means  $\pm$  s.d. of values from three independent experiments. *P* values in **(B)** are for comparison of the value for combined treatment with TZT-1027 and radiation vs the sum of the corresponding values for each treatment alone, after correction of all data by the control (DMSO) value.

adenocarcinoma cells into nude mice in order to elicit the formation of solid tumours. The mice were then treated with TZT-1027, radiation, or both modalities. Treatment with TZT-1027 alone (single dose of 0.5 mg kg<sup>-1</sup>) or with radiation alone (single dose of 10 Gy) resulted in relatively small inhibitory effects on tumour growth, whereas combined treatment with both TZT-1027 and radiation exerted a markedly greater inhibitory effect (Figure 6A and B). The tumour GDs induced by treatment with TZT-1027 alone, radiation alone, or both TZT-1027 and radiation were 1.0, 2.6, and 8.8 days, respectively, for H460 cells and 1.4, 4.9, and 12.4 days, respectively, for A549 cells (Table 1). The enhancement factor for the effect of TZT-1027 on the efficacy of radiation was 3.0 for H460 cells and 2.2 for A549 cells, revealing the effect to be greater than additive. No pronounced tissue damage or toxicities such as diarrhoea or weight loss of >10% were observed in mice in any of the four treatment groups (Table 2).

We examined the effects of the treatment protocols on apoptosis in H460 tumours by TUNEL staining of tumour sections. Quantification of the number of apoptotic cells revealed that the combined treatment with radiation and TZT-1027 induced a significant increase in this parameter compared with treatment with radiation or TZT-1027 alone (Figure 6C).

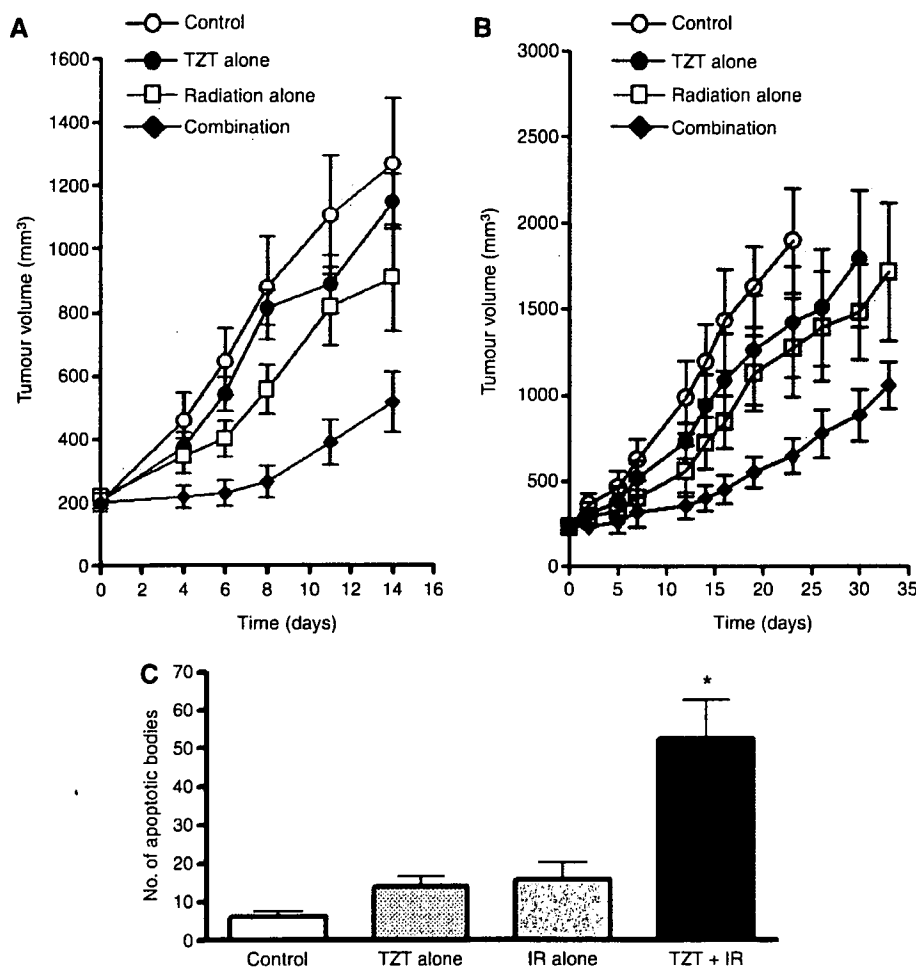
#### Histological appearance of H460 tumours after administration of TZT-1027

Finally, we examined whether an effect of TZT-1027 on tumour vasculature might contribute to the antitumour activity of this drug *in vivo*. Mice harbouring H460 tumours were injected with TZT-1027, and the tumours were excised 4 or 24 h thereafter and examined by hematoxylin-eosin staining (Figure 7A–C) or by immunostaining for the endothelial cell marker CD31 (Figure 7D and E). Tumour capillaries appeared congested, with thrombus formation, and showed a loss of endothelial cells 4 h after administration of TZT-1027 (Figure 7B and E), whereas vessels within viable areas of control tumours were generally not congested and showed an intact normal endothelium (Figure 7A and D). The effects of TZT-1027 on the tumour vasculature appeared selective, given that neither loss of CD31 staining nor

vessel congestion was apparent in the vasculature of surrounding normal tissue after drug treatment (Figure 7E). Extensive necrosis was apparent at the tumour core, with a characteristic thin rim of viable tumour cells remaining at the periphery, 24 h after TZT-1027 administration (Figure 7C). These results were thus indicative of a characteristic antivascular effect of TZT-1027 in the H460 tumour model.

#### DISCUSSION

TZT-1027 is a novel antitumour agent that inhibits microtubule polymerisation and exhibits potent antitumour activity in preclinical models (Miyazaki *et al*, 1995; Kobayashi *et al*, 1997; Natsume *et al*, 2000, 2003, 2006; Otani *et al*, 2000; Watanabe *et al*, 2000, 2006a). We investigated the effect of TZT-1027 on cell cycle progression with the use of tsFT210 cells, which can be synchronised in G<sub>2</sub> phase by incubation at 39.4°C and consequent inactivation of Cdc2 (Osada *et al*, 1997; Tamura *et al*, 1999). The use of these cells allows cell synchronisation without the need for agents that prevent DNA synthesis (such as hydroxyurea or thymidine) or that inhibit formation of the mitotic spindle (such as nocodazole). Although such agents halt cell cycle progression in specific phases of the cycle, they are also toxic and kill a proportion of the treated cells. The tsFT210 cell system is thus suited to sensitive analysis of the effects of new compounds on cell cycle progression without loss of cell viability. We have now shown that tsFT210 cells released from G<sub>2</sub> arrest by incubation at 32.0°C failed to pass through M phase in the presence of TZT-1027. Although previous flow cytometric analysis of exponentially growing tumour cells revealed that TZT-1027 induced a marked increase in the proportion of cells in G<sub>2</sub>-M (Watanabe *et al*, 2000), it was uncertain whether the drug arrested cell cycle progression in G<sub>2</sub> or in mitosis. Our morphological data now indicate that, similar to the effect of nocodazole, TZT-1027 arrested tsFT210 cells in M phase rather than in G<sub>2</sub>, consistent with the mode of action of this new compound. Given that microtubules contribute to various cellular functions in addition to cell division, including intracellular transport and signal transduction (Mollinedo and Gajate,



**Figure 6** Sensitisation of H460 and A549 cells to  $\gamma$ -radiation by TZT-1027 *in vivo*. (**A** and **B**) Evaluation of tumour growth. Nude mice with H460 (**A**) or A549 (**B**) tumour xenografts ( $\sim 200$  to  $250$  mm<sup>3</sup>) were treated with a single intravenous dose of TZT-1027 ( $0.5$  mg kg<sup>-1</sup>), a single dose of  $\gamma$ -radiation ( $10$  Gy), or neither (control) or both modalities, and tumour volume was determined at the indicated times thereafter. Data are means  $\pm$  s.e. for six to eight mice per group. (**C**) Quantification of apoptotic cells in H460 tumour sections by TUNEL staining 14 days after the initiation of treatment as in (**A**). Data are means  $\pm$  s.d. \* $P < 0.05$  vs mice treated with TZT-1027 alone or radiation alone.

**Table 1** Tumour growth delay value

Treatment	H460		A549	
	Days <sup>a</sup>	GD <sup>b</sup>	Days	GD
Control	4.5		5.5	
TZT-1027 alone	5.5	1	6.9	1.4
Radiation alone	7.1	2.6	10.4	4.9
TZT-1027 + Radiation	13.3	8.8	17.9	12.4
Enhancement factor	3		2.2	

<sup>a</sup>Days, the period needed for the sizes of xenografts in each group to reach  $500$  mm<sup>3</sup>;  
<sup>b</sup>GD, the additional periods needed for the sizes of xenografts in each group to reach  $500$  mm<sup>3</sup> in addition to the period needed for controls to reach  $500$  mm<sup>3</sup>.

2003), TZT-1027 might also be expected to affect tumour cells in interphase. With the use of synchronised tsFT210 cells, however, we found that TZT-1027 had no effect on progression of cells through the G<sub>1</sub>-S transition of the cell cycle. The effect of TZT-1027 on cell cycle progression thus appears to be specific to M phase.

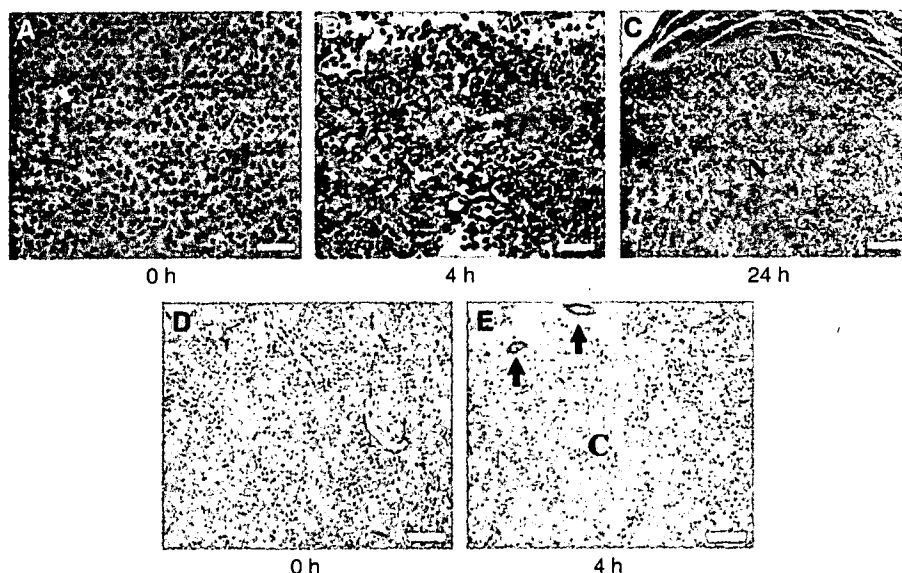
Given that cells are most sensitive to radiation during mitosis (Sinclair and Morton, 1966; Sinclair, 1968; Pawlik and Keyomarsi,

**Table 2** Body weight loss

Treatment	% of B.W.L. <sup>a</sup>	
	H460	A549
Control	3.6	1.2
TZT-1027 alone	9.9	5.2
Radiation alone	9.7	5.5
TZT-1027+Radiation	8.7	9.9

<sup>a</sup>% of B.W.L. relative body weight loss 7 days after the initiation of the treatment.

2004), we next investigated the possible interaction between TZT-1027 and ionising radiation in human lung cancer cell lines. We found that TZT-1027 increased the sensitivity of H460 cells to  $\gamma$ -radiation *in vitro*. The proportion of H460 cells in mitotic phase at the time of irradiation was increased by TZT-1027 treatment, consistent with the notion that this effect contributes to the observed radiosensitisation induced by this drug. TZT-1027 was previously shown to induce apoptosis in several tumour cell lines (Watanabe *et al*, 2000). Although the relation between apoptosis and radiosensitivity is controversial (Lawrence *et al*, 2001; Pawlik and Keyomarsi, 2004), we showed that treatment of H460 cells with



**Figure 7** Histological analysis of H460 tumours after treatment with TZT-1027. Mice bearing H460 tumour xenografts were treated with a single dose of TZT-1027 ( $2.0 \text{ mg kg}^{-1}$ ), and the tumours were excised at various times thereafter and either stained with hematoxylin-eosin (**A–C**) or immunostained for CD31 (**D** and **E**). (**A** and **D**) Control sections of an untreated tumour showing normal capillaries with an intact endothelium and viable tumour cells. (**B** and **E**) Sections of a tumour removed 4 h after administration of TZT-1027. Vascular congestion, with pink deposits of fibrin, and loss of endothelial cells as well as diffuse tumour cell degeneration are apparent in (**b**). Dark immunostaining of intact endothelium (arrows) is apparent in surrounding normal connective tissue, whereas little staining of endothelial cells was observed in the core (**C**) of the tumour (**E**). (**C**) Section of a tumour removed 24 h after TZT-1027 administration, showing extensive central necrosis (N) and a rim of viable cells (V). Scale bars:  $50 \mu\text{m}$  (**A** and **B**),  $100 \mu\text{m}$  (**C**), and  $200 \mu\text{m}$  (**D** and **E**).

TZT-1027 before irradiation induced a marked increase in the proportion of apoptotic cells compared with that apparent with radiation alone. These results thus suggested that potentiation of apoptosis contributed to radiosensitisation by TZT-1027.

Combined treatment with radiation and a single administration of TZT-1027 also inhibited the growth of tumours formed by H460 or A549 cells *in vivo* to a greater extent than did either treatment alone. Tumour microenvironmental factors, such as the vascular supply, are important determinants of sensitivity to radiation therapy *in vivo*. The ability of microtubule-targeting agents to induce a rapid shutdown of the existing tumour vasculature has been recognised by their designation as vascular-targeting agents (VTAs) (Jordan and Wilson, 2004). Treatment with VTAs such as ZD6126 and combretastatin A-4-P typically results in the destruction of large areas of a tumour, with surviving cells remaining only at the tumour periphery (Dark *et al*, 1997; Blakey *et al*, 2002). These peripheral viable tumour cells presumably derive their nutritional support from nearby normal blood vessels that are not responsive to VTA treatment (Li *et al*, 1998; Siemann and Rojiani, 2002). Such support together with a rapid upregulation of angiogenic factors such as vascular endothelial growth factor may directly facilitate the growth and expansion of the remaining tumour cells (Wachsberger *et al*, 2003; Thorpe, 2004). Given that these residual tumour cells are likely well oxygenated (Wachsberger *et al*, 2003), they are an ideal target for radiation therapy. Several studies have recently shown that treatment with VTAs enhances the therapeutic effect of radiotherapy (Li *et al*, 1998; Siemann and Rojiani, 2002, 2005; Horsman and Murata, 2003; Masunaga *et al*, 2004), consistent with the idea that the components of such combination therapy act in a complementary manner, with VTAs attacking the poorly oxygenated cell population in the central region of tumours and radiation killing the well-oxygenated proliferating cells at the tumour periphery (Li *et al*, 1998; Siemann and Rojiani, 2002; Wachsberger *et al*, 2003). TZT-1027 was previously shown to increase vascular permeability and to induce a decrease in tumour blood flow followed by a marked increase in tissue necrosis in the central

region of tumour xenografts (Otani *et al*, 2000; Watanabe *et al*, 2006b). We have now shown that TZT-1027 treatment resulted in congestion and occlusion of tumour blood vessels followed by extensive necrosis of the tumour core, with only a thin rim of viable tumour cells remaining, in the H460 tumour model, suggesting that TZT-1027 acts as a VTA. The action of TZT-1027 as a VTA might thus contribute to the radiosensitising effect observed *in vivo* in the present study.

The clinical use of microtubule-interfering agents such as taxanes in combination with radiation has been successful in improving local tumour control. However, taxanes are often of limited efficacy because of the development of cellular resistance such as that mediated by P-glycoprotein-dependent drug efflux (Goodin *et al*, 2004). The action of TZT-1027 has been suggested to be less affected by multidrug resistance factors, including overexpression of P-glycoprotein, than that of other tubulin inhibitors (Watanabe *et al*, 2006a), suggesting that TZT-1027 may be effective in the treatment of taxane-refractory tumours. Further investigations are thus warranted to examine the combined effects of TZT-1027 and ionising radiation on drug-resistant tumour cells. Whether TZT-1027 enhances the tumour response to clinically relevant fractionated doses of radiation such as 2 Gy per fraction also warrants further study.

In conclusion, we have found that the inhibitory effect of TZT-1027 on cell cycle progression is highly specific to M phase. Moreover, TZT-1027 enhanced the effects of radiation on human cancer cells both *in vitro* and in animal models *in vivo*. These preclinical results provide a rationale for future clinical investigations of the therapeutic efficacy of TZT-1027 in combination with radiotherapy.

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ORIGINAL ARTICLE

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## Oxaliplatin/fluorouracil/leucovorin (FOLFOX4 and modified FOLFOX6) in patients with refractory or advanced colorectal cancer: post-approval Japanese population experience

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### Abstract

**Background.** The oxaliplatin/fluorouracil/leucovorin (FOLFOX regimen) is an effective and generally well-tolerated regimen in Western clinical studies of advanced colorectal cancer. In Japan, oxaliplatin was approved in April 2005.

**Methods.** To evaluate the objective tumor responses and feasibility (toxicities) of FOLFOX regimens (FOLFOX4 and modified FOLFOX6, mFOLFOX6) in a predominantly Japanese population with refractory or advanced colorectal cancer in Japan, 51 consecutive patients with histologically confirmed metastatic colon or rectum cancer who were treated between April 2005 and March 2006 were enrolled in a retrospective study. FOLFOX4 was used for treatment in 39% (first-line, 45%) of these patients, and mFOLFOX6 was used for treatment in 61% (first-line, 61%). Tumor responses were assessed radiologically, and toxicities were graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 regarding toxicities other than peripheral sensory neuropathy.

**Results.** The objective response rates (in those who underwent first- or second-line therapy) were 50.0% and 8.7%, respectively. The tumor control rate (partial response [PR] + stable disease [SD]) was 80.4%. There were no toxicity-related deaths. Neutropenia grade 3 was experienced in 20% of patients, and often caused delay in the subsequent treatment course. Mild to moderate cumulative peripheral sensory neuropathy affected 78% of patients. The incidence of hypersensitivity reactions to oxaliplatin in our study was lower than that in reported in Western countries.

**Conclusion.** Both FOLFOX regimens have good efficacy in refractory or advanced colorectal cancer in a Japanese population, with an acceptable overall toxicity profile.

**Key words** Oxaliplatin · FOLFOX · Colorectal cancer · Japanese population

### Introduction

In 2000, it has reported that colorectal cancer (CRC) was diagnosed in more than 90000 patients per year in Japan, resulting in 36000 deaths per year.

Colorectal cancer accounts for 10% to 15% of all cancers and is the third leading cause of cancer-related death in Western countries. Approximately one-half of all patients develop metastatic disease. The prognosis for these patients is poor, although palliative chemotherapy has been shown to be able to prolong survival and improve the quality of life over best supportive care. For many years, the treatment of metastatic colorectal cancer was restricted to 5-fluorouracil (5FU) and the biomodulation of this agent.<sup>1</sup> Oxaliplatin and irinotecan, combined with continuous infusion of 5FU, significantly improved response rate, progression-free survival (PFS), and overall survival.<sup>2–4</sup> FOLFOX4 (oxaliplatin and leucovorin [LV] 5FU2) is more active than LV5FU2 alone, and has also shown superiority over IFL (irinotecan, FU bolus, leucovorin). Oxaliplatin (L-OHP), a new third-generation 1,2-DACH-platinum derivative, has a mechanism of action similar to that of other platinum derivatives.<sup>5–9</sup> However, its spectrum of antitumor activity in tumor models differs from those of cisplatin and carboplatin. In addition, it has also been observed to demonstrate activity against cisplatin-resistant colon carcinoma cell lines.<sup>10</sup> In addition, experimental data have shown synergistic activity of the oxaliplatin/FU combination. The clinical toxicity of oxaliplatin is also distinct from that of other platinum drugs: it has no renal toxicity and minimal hematotoxicity; it causes both a reversible acute, cold-related dysesthesia and a dose-limiting cumulative peripheral sensory neuropathy that usually regresses rapidly after treatment withdrawal. The recent availability of five active chemotherapeutic agents has doubled the median overall survival for metastatic CRC from 10 to 20 months.

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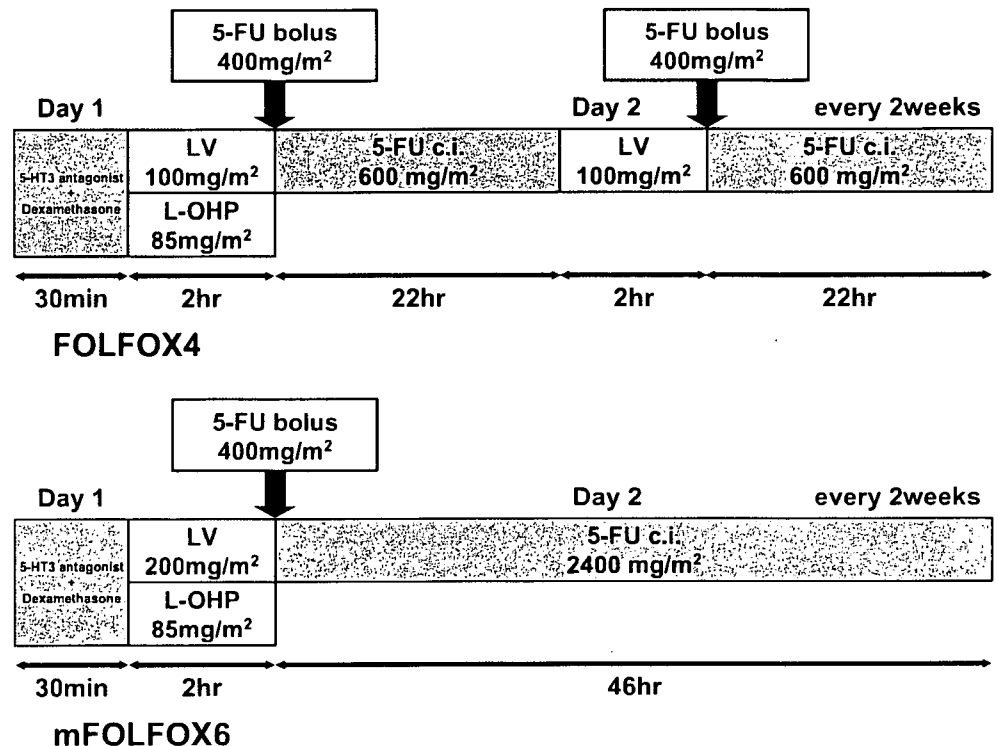
Three combinations have shown excellent first-line efficacy in phase III trials – IFL with bevacizumab, FOLFOX, and FOLFIRI – however, neither of these combinations is clearly superior. Our clinical practice in Japan has been guided in a major way by extrapolation from the results of clinical trials conducted mainly in Western countries. To evaluate the value of FOLFOX regimens in the treatment of refractory or advanced CRC, a retrospective analysis study was designed to assess the feasibility (toxicities) and efficacy of combining oxaliplatin with the LV5FU2 schedule in a Japanese population. We herein report our experience with two FOLFOX regimens (FOLFOX4 and modified [m] FOLFOX6) in patients with advanced CRC, specifically, the toxicities and objective tumor response rates obtained.

## Patients and methods

A retrospective analysis study was conducted at Kinki University Hospital in 51 consecutive patients with histologically confirmed metastatic colon or rectum cancer who were treated between April 2005 and March 2006. The primary objectives were to assess the feasibility (toxicities) and efficacy of two FOLFOX regimens (FOLFOX4 and mFOLFOX6) in a Japanese population. FOLFOX4 is a regimen comprising oxaliplatin 85 mg/m<sup>2</sup> as a 2-h infusion (day 1); LV 100/m<sup>2</sup> per day as a 2-h infusion (days 1 and 2); followed by a 5FU bolus 400 mg/m<sup>2</sup> per day and 5FU 600 mg/m<sup>2</sup> per day as a 22-h infusion (days 1 and 2). mFOLFOX6 is a regimen also comprising oxaliplatin 85 mg/m<sup>2</sup> as a 2-h infusion

(day 1), LV 200 mg/m<sup>2</sup> per day as a 2-h infusion (day 1), followed by a 5FU bolus 400 mg/m<sup>2</sup> (day 1) and 5FU 2400 mg/m<sup>2</sup> per day as a 46-h infusion (days 1 to 2). These therapies were administered on day 1 and repeated on day 2 of a 14-day treatment cycle. Routine antiemetic prophylaxis with a serotonin (5-HT<sub>3</sub>) antagonist (granisetron) and dexamethasone was given (Fig. 1). The use of implantable ports and infusion pumps allowed chemotherapy to be administered on an outpatient basis in some cases. Treatment was continued until either disease progression, the occurrence of unacceptable toxicity, or the patient refused further treatment. Toxicity was graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 regarding toxicities other than peripheral sensory neuropathy and by following the oxaliplatin-specific scale (DEB-NTC). The definitions in the oxaliplatin-specific scale, which was developed as a specific scoring scale for oxaliplatin-inducing peripheral sensory neuropathy, are as follows: grade 1, transient dysesthesia and/or paresthesia lasting for less than 7 days; grade 2, transient dysesthesia and/or paresthesia lasting for 7 days or longer; and grade 3, dysesthesia and/or paresthesia with pain or function impairment that interferes with activities of daily living (such as difficulty with fastening buttons and writing). The response of measurable target lesions to treatment was objectively evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria after each four cycles of treatment. Complete response (CR) was defined as the disappearance of all disease. Partial response (PR) was defined as at least a 30% reduction in the sum of the longest diameters of all measured lesions by at least 4 weeks. Progressive disease (PD) was defined as an increase in lesions by 20%

**Fig. 1.** Treatment schema for FOLFOX4 and mFOLFOX6 regimens. LV, leucovorin; 5-FU, 5-fluorouracil; L-OHP, oxaliplatin; c.i., continuous infusion



or greater, or the appearance of new lesions. Responses not falling into any of these categories were classified as stable disease (SD).

## Results

### Patient characteristics

A total of 51 patients with a median age of 61 years (range, 34–78 years) with refractory or advanced CRC were retrospectively analyzed. The patients' characteristics are listed in Table 1. Patients were treated with the FOLFOX4 (39%) or mFOLFOX6 regimens (61%). Twenty-eight patients (55%; FOLFOX4, 9; mFOLFOX6, 19) were treated in a first-line setting, and 23 patients (45%; FOLFOX4, 11; mFOLFOX6, 12) were treated in a second-line setting. Since April 2005, 4 months prior to beginning this study, we have used the FOLFOX4 regimen for inpatients, and from that time we selected the mFOLFOX6 regimen for all patients in the outpatient setting. The total number of chemotherapy cycles administered was 384, with a median of 8 cycles per patient (range, 1–12 cycles). The median dose intensity (actual/planned dose) was 93.4% for oxaliplatin and 100% for 5FU in the FOLFOX4 group and 91.2% for oxaliplatin and 94.8% for 5FU in the mFOLFOX6 group. The median dose intensity of oxaliplatin was 37 mg/m<sup>2</sup> per week (range, 31–42 mg/m<sup>2</sup> per week) in the FOLFOX4 group and 34 mg/m<sup>2</sup> per week (range, 23–42 mg/m<sup>2</sup> per week) in the mFOLFOX6 group.

### Hematological toxicity

Several pertinent hematological toxicities are listed in Table 2, shown with numbers of patients who experienced

them. The onset of neutropenia typically occurred between 10 and 14 days after treatment. Grade 3 and 4 neutropenia was observed in 20% of patients, with neutropenic fever being uncommon. Neutropenia often caused delay in the start of a subsequent treatment course. In all, 88 (23%) of 384 cycles were delayed due to toxicity, most commonly hematological: 64 (17%) for neutropenia and 24 (6%) for neurotoxicity. Using our administration schedule, no thrombocytopenia of over grade 3 was observed to develop. In addition, only one patient developed grade 3 anemia with transfusion.

### Nonhematological toxicity

The most common nonhematological adverse effects of the FOLFOX regimens were peripheral neuropathy and lethargy (fatigue). These effects are listed in Table 3. Two patients experienced grade 2 hypersensitivity reactions (rash/hives, erythema, and one patient also experienced vomiting) during the administration of oxaliplatin. The symptoms rapidly resolved, in a few minutes, on symptomatic treatment (termination of infusion, use of steroids, and antagonists of type 1 and 2 histamine receptors). In using successful strategies over the next treatment courses (slowing the infusion rate, increasing the doses of steroids, and dose reduction of oxaliplatin), both patients were able to tolerate rechallenge of oxaliplatin, and one patient achieved a partial response. Peripheral neurotoxicity, characterized by paresthesia in a symmetric, glove-and-stocking distribution, occurred in 40 (78%) patients and there was no grade over 3.

Whenever the number of treatment cycles increases, neuropathy, within grade 2 level, tends to increase. The incidence of neurotoxicity along with the number of treatment cycles is listed in Table 4. Cold-related dysesthesia

**Table 1.** Characteristics of the study patients

Characteristics	n = 51
Sex, male/female	28/23
Age, years, median (range)	61 (34–78)
Performance status (ECOG), 0/1/2	19/25/7
Primary tumor, colon/rectum/rectosigmoid	28/20/3
Adjuvant therapy, +/-	18/33
Previous irinotecan therapy, +/-	15/36
Site of metastases, lung/liver/LN/peritoneum	22/21/18/7
FOLFOX4/mFOLFOX6	20/31
First-line/second-line	28/23
Dose reduction: +/-	7/44

ECOG, Eastern Cooperative Oncology Group; LN, distant lymph nodes

**Table 2.** Hematological toxicity (CTCAE V3.0)

	n = 51					
	Grade 1	Grade 2	Grade 3	Grade 4	All grades	Grade $\geq$ 3
Leucocytopenia	17	16	1	0	67%	2%
Neutropenia	18	15	9	1	84%	20%
Anemia	19	8	1	0	55%	2%
Thrombocytopenia	23	5	0	0	55%	0%

**Table 3.** Nonhematological toxicity (CTCAE V3.0)<sup>a</sup>

	n = 51					
	Grade 1	Grade 2	Grade 3	Grade 4	All grades	Grade $\geq$ 3
Anorexia	16	2	2	0	39%	4%
Nausea	13	2	2	0	33%	4%
Vomiting	5	2	2	0	18%	4%
Mucositis	10	1	2	0	25%	4%
Febrile neutropenia	–	–	0	0	0%	–
Hand-foot syndrome	2	0	0	0	4%	–
Pigmentation	4	0	–	–	7%	–
Allergy	0	2	–	–	4%	–
Lethargy	13	4	0	0	33%	–
AST/ALT elevation	22	2	0	0	47%	–
Diarrhea	8	2	2	0	23%	4%
Sensory neuropathy	31	9	0	0	78%	–

<sup>a</sup>Other than sensory neuropathy

**Table 4.** Incidence of neurotoxicity in FOLFOX regimens

Grade	1–4 Cycles (n = 9)			5–8 Cycles (n = 28)			9–12 Cycles (n = 14)			All cycles (n = 51)		
	1	2	3	1	2	3	1	2	3	1	2	3
Sensory neuropathy	5	0	0	19	2	0	7	7	0	31	9	0
	56%	0%	0%	68%	7%	0%	50%	50%	0%	60%	18%	0%

Grade	The oxaliplatin-specific scale (DEB-NTC)			
	0	1	2	3
Dysesthesia and/or paresthesia	No abnormality	Transient dysesthesia and/or paresthesia lasting less than 7 days	Transient dysesthesia and/or paresthesia lasting 7 days or more	Dysesthesia and/or paresthesia with pain or function impairment that interferes with activities of daily living

was reported in 31 patients (61%). Paresthesia lasting 7 days or longer (grade 2) occurred in 9 patients (18%). Peripheral neuropathy appeared in two forms. In the first form, an acute, transient, cold-exacerbated dysesthesia or paresthesia occurred shortly after the administration of oxaliplatin; it affected the hands, feet, perioral area, and throat; and typically lasted for several days after drug administration. In the second form, a delayed-onset, cumulative, dose-related peripheral neuropathy was characterized by paresthesias affecting the hands and feet that did not remit between cycles of treatment. Investigators also reported pharyngolaryngeal dysesthesia in only one patient; however, no patients had a laryngospasm-like syndrome.

Overall, 7 of the 51 patients (14%) required dose modification during treatment; dose reduction was required for oxaliplatin alone in 4 patients, for 5FU alone in 2 patients and for both agents in 1 patient. The majority of dose reductions were by one level (reduction to 65 mg/m<sup>2</sup> oxaliplatin and/or 75% of the starting dose of 5FU). No patients required a second-level dose reduction. The adverse events most commonly leading to dose reduction were neurotoxicity (1 patient in FOLFOX4 and 3 patients in mFOLF-FOX6) and diarrhea (2 patients in mFOLF-FOX6). In addition, 2 patients in the mFOLF-FOX6 setting underwent a dose reduction of oxaliplatin due to allergic reaction. The most common reason for treatment discontinuation was PD.

#### Antitumor activity

All 51 patients were able to be evaluated for response. Objective responses are listed in Table 5 and Table 6. There was no complete response. The overall objective response rates (in those who underwent first-line or second-line therapy) were 50.0% and 8.7%, respectively (Table 6). Stable disease was achieved in 49% of patients. The tumor control rate (PR + SD) was 80.4%.

#### Discussion

The recent advent of several new agents for the treatment of metastatic CRC has markedly enhanced the therapeutic armamentarium for this disease. Oxaliplatin in combination with infusional 5FU in the FOLFOX regimens has been shown to be effective in achieving improved response rate, time to progression, and survival time compared with 5FU/LV. In addition, recent large clinical phase III studies (N9741, EFC4584, GERCOR) showed that combination chemotherapy regimens, including irinotecan and oxaliplatin, markedly improved response rates and prolonged median survival over those seen with 5FU/LV,<sup>11–13</sup> and these combination chemotherapy regimens have supplanted 5FU/LV as a standard systemic approach for metastatic CRC. The median survival time (MST) has been gradually pro-

**Table 5.** Objective responses – (1)

FOLFOX4 ( <i>n</i> = 20)		SD	PD	NE
CR	PR			
0	5 (25%) First-line, 3; second-line, 2	12 (60%)	3 (15%)	0
mFOLFOX6 ( <i>n</i> = 31)				
0	11 (35.5%) First-line, 11; second-line, 0	13 (41.9%)	7 (22.6%)	0

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable

**Table 6.** Objective responses – (2)

First-line ( <i>n</i> = 28)		SD	PD	NE
CR	PR			
0	14 (50%) FOLFOX4, 3; mFOLFOX6, 11	11 (39.3%)	3 (10.7%)	0
Second-line ( <i>n</i> = 23)				
0	2 (8.7%) FOLFOX4, 2	14 (60.9%)	7 (30.4%)	0

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable

longed through the use of 5FU/LV with irinotecan and oxaliplatin. Currently, with the addition of molecular targeted agents, an MST of over 20 months has been reported.

Since April 2005, and the approval of oxaliplatin in Japan, clinical practice in this country has been conducted in a major way by extrapolation from the results of clinical trials conducted mainly in large Western phase III studies. The results of the present retrospective study demonstrate the efficacy and feasibility of FOLFOX regimens (FOLFOX4 and mFOLFOX6) as treatment for patients with advanced CRC in the Japanese population, as has been shown in Western populations. In this retrospective analysis study in a Japanese population, neutropenia grade 3/4 occurred in 20% of the patients who were assigned to receive oxaliplatin, but it was nonfebrile, whereas grade 3/4 vomiting and mucositis affected only 4% of the patients, while diarrhea affected 4%.

Lethargy has been described as the most frequent adverse event of the mFOLFOX6 regimen in a recent report by Braun et al.<sup>14</sup> In our study, 17 (33%) patients experienced lethargy similar to general fatigue symptoms.

The cumulative dose-limiting toxicity of oxaliplatin is peripheral sensory neuropathy, which reportedly occurs in about 70%–80% of patients; it typically resolves a few months after discontinuation of treatment, and may be exacerbated by cold stimulation. In our series, paresthesia lasting 7 days or longer was observed in 18% of patients and led to an oxaliplatin dose reduction for four patients after they had received a minimum of seven cycles (or at least 4 months) of chemotherapy.

The mechanism of this neurotoxicity has been elucidated to be as follows: the increased neuronal excitability is due to the action of oxaliplatin on voltage-gated sodium channels through the chelation of calcium by the oxaliplatin

metabolite. The prevention of this neurotoxicity is a major goal, taking in to account the wide indications of this drug. Various different approaches have been either previously studied or are now being evaluated, based on pathogenic or practical concepts: (1) modification of the administration schedule; (2) substances acting upon sodium channels, such as calcium-magnesium, carbamazepine, gabapentine, venlafaxine; (3) detoxifying agents and antioxidants, such as glutathione, amifostine, alphasipoic acid, tocopherol; (4) substances used in other kinds of neuropathy, such as glutamine and alphasipoic acid; (5) neurotrophic factors, such as nerve growth factor (NGF), LIF; and (6) oxaliplatin analogs, with a DACH platin, without oxalate. Calcium-magnesium infusion appears to be an efficient and safe approach.

In this study, after September 2005, 32 patients (63%) were administered calcium-magnesium infusion for the prevention of the oxaliplatin-related neurotoxicity. Further studies are necessary for a better understanding and prevention of this potentially severe neurotoxicity.

In terms of antitumor activity, although the response rate (RR) in our population was slightly lower in comparison to that in previous Western clinical studies,<sup>11–13</sup> both of the oxaliplatin-based regimens demonstrated a promising objective RR in the first-line setting (50.0%) and in the tumor control rate (80.4%).

In a GERCOR study, the median survival was 21.5 months in 109 patients allocated to FOLFIRI then FOLFOX6 versus 20.6 months in 111 patients allocated to FOLFOX6 then FOLFIRI (*P* = 0.99). In first-line therapy, FOLFIRI achieved a 56% RR and 8.5-month median PFS, versus FOLFOX6, which achieved a 54% RR and 8.0-month median PFS (*P* = 0.26). Second-line FOLFIRI achieved a 4% RR and 2.5-month median PFS, versus