expression of the phosphorylated band of NDRG1/Cap43 in SaOS2x2 and SaOS2x3 compared to SaOS2 and SaOS2x1 (Fig. 4).

Cell differentiation and NDRG1/Cap43 knockdown. In order to verify the relationship between the NDRG1/Cap43 expression and cell differentiation, we established two human osteosarcoma cell lines constitutively expressing NDRG1/Cap43 siRNA, U2OS/si-1 and U2OS/si-8, derived from human osteosarcoma U2OS cells. The expression of the NDRG1/Cap43 protein was markedly down-regulated in both the U2OS/si-1 and U2OS/si-8 cells compared to their parental counterpart, U2OS/mock (Fig. 5A). The U2OS/mock cells showed relatively the same levels of NDRG1/Cap43 as the U2OS cells (data not shown). Furthermore, the expression of the p21 protein was also down-regulated in both the NDRG1/Cap43 knockdown cell lines (Fig. 5A). Cell proliferation rates were found to be significantly increased in U2OS/si-1 and U2OS/si-8, compared to U2OS/mock (Fig. 5B).

We also compared the differentiation between the NDRG1/Cap43-knockdown cell lines and their parental counterpart. As shown in Fig. 5C, the production of osteocalcin was significantly down-regulated by the NDRG1/Cap43 knockdown in U2OS/si-1 ($p<0.05$) and U2Os/si-8 ($p<0.01$), indicating a close correlation between cell differentiation and NDRG1/Cap43 in osteosarcoma cells.

Discussion

In this study, we demonstrated that the NDRG1/Cap43 expression is increased concomitantly with the promotion of cell differentiation by vitamin D_3 in the two osteosarcoma cell lines, MG63 and U2OS. However, the NDRG1/Cap43 expression was not affected by vitamin D_3, which failed to induce cell differentiation in SaOS2. Mahonen *et al*, have reported the differences between MG63 and SaOS2 in the response of type I collagen synthesis to vitamin D_3 (25). Zehenter *et al*, have also reported that BMP-2 increases Sox9 mRNA levels in MG63 but not in other osteosarcoma cell lines (26). The failure in the expression of both NDRG1 and osteocalcin in the SaOS2 cells could be due to the loss of function of the vitamin D_3 signaling, pathway including its cognate receptor. However, the precise mechanisms causing these three cell lines to show different responses to vitamin D_3, is not clear.

We have previously reported that the gelatinolytic activity by matrix metalloproteinase-9 and the invasive ability during the Matrigel invasion, are both decreased in pancreatic cancer cell lines with a high NDRG1/Cap43 expression (27). In this study, during the acquirement of the invasion activity by SaOS2, the expression of the NDRG1/Cap43 protein was markedly decreased, suggesting a close correlation between NDRG1/Cap43 and the invasive potential of osteosarcoma cells. However, Matrigel did not affect the invasion of MG63 at all (data not shown). This response was possibly influenced by the differences in the differentiation state of these cells: MG63 cells are considered to be early osteoblast-like cells, whereas SaOS2 cells are not as well differentiated as the MG63 cells. Furthermore, the upper band, corresponding to the phosphorylated NDRG1/Cap43, was markedly decreased in the SaOS2x2 and SaOS2x3 cells. The phosphorylation of NDRG1/Cap43 is mediated through SGK1 and GSK3, essential Ser/Thr kinase family proteins (28). Further study is required in order to determine how the phosphorylation by these enzymes could be modified during the acquisition of invasiveness of osteosarcoma cells in Matrigel.

Kurdistani *et al*, have previously reported that the NDRG1/Cap43 expression was induced by wild-type-p53, and demonstrated a parallel increase in the expression of NDRG1/Cap43 and p21, and the CdK inhibitor in SaOS2 cells (4). Based on these findings, we established two osteosarcoma cell lines, derived from U2OS, with a decreased NDRG1/Cap43 expression, in order to examine the relationship between the NDRG1/Cap43 and p21 expression and cell differentiation. p21 is involved in cell differentiation via the p53-independent pathway in various cell lineages including osteosarcoma (29-31). In U2OS/si-1 and U2OS/si-8 with a decreased NDRG1/Cap43 expression, the p21 expression was down-regulated, resulting in enhanced cell proliferation compared to the parental cells. The production of osteocalcin was suppressed by the NDRG1/Cap43 knockdown. On the whole, our present study strongly suggests that NDRG1/Cap43 plays a pivotal role in the cell differentiation of osteosarcoma cells in close association with p21.

In conclusion, we demonstrated that NDRG1/Cap43 contributed to the regulation of cell differentiation as well as cell proliferation by osteosarcoma cells. Furthermore, NDRG1/Cap43 also affects cell motility and invasion by osteosarcoma cells. NDRG1/Cap43 could be useful as a molecular target for the development of novel therapeutics against osteosarcoma.

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Expression of selected gene for acquired drug resistance to EGFR-TKI in lung adenocarcinoma

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ABSTRACT

Background: Individualized treatment is an attractive challenge that may allow for more effective and safer treatment of human disease. Activating mutations in the epidermal growth factor receptor (EGFR) gene in lung adenocarcinoma are associated with a dramatic clinical response to EGFR-tyrosine kinase inhibitors (TKIs). However, patients often experience a relapse after treatment with EGFR-TKIs, even when the tumors are initially highly sensitive. However, the "whole picture" regarding acquired resistance remains unclear.
Methods: Tumor specimens were collected from 11 lung adenocarcinoma patients before and after treatment with gefitinib. The status of the EGFR and K-ras genes were investigated by PCR-based analyses. Immunohistochemistry and real-time PCR assays were used to evaluate the MET gene in terms of its tyrosine phosphorylation and amplification, respectively. The expression of HGF, PTEN, and EGR-1, and changes in the epithelial–mesenchymal transition (EMT) status including the expression of E-cadherin and gamma-catenin as epithelial markers, and vimentin and fibronectin as mesenchymal markers, were evaluated by immunohistochemistry.
Results: Seven (64%) of the gefitinib refractory tumors exhibited a secondary threonine-to-methionine mutation at codon 790 in *EGFR* (T790M). All of the tumors had wild type K-ras gene expression. No MET amplification was detected in any of the samples, nor was there phosphorylation of MET detected in any of the resistant samples. Neither MET gene amplification, nor the overexpression of HGF was observed in samples without the T790M mutation. A strong expression of HGF was detected in 6 of 8 specimens with the T790M mutation. Three (38%) of 8 cases showed a loss of PTEN in samples with the T790M mutation. A loss of EGR-1 was detected in 2 (29%) of 7 cases, including one tumor without PTEN. Four (57%) of 7 cases showed positive expression of phosphorylated Akt (p-Akt). A change in the EMT status between pre-and post-treatment was observed in 4 (44%) of 9 cases. In all examined samples cases, some alterations of gene or proteins were observed.
Conclusions: The current results showed that these alterations in gene or protein expression can account for all resistant mechanisms. This phenomenon suggests the existence of complicated relationships among acquired resistance-related genes.

1. Introduction

Currently, sensitive somatic mutations in the EGFR gene in lung adenocarcinoma are associated with a dramatic clinical response to EGFR-TKIs [1,2]. Thus, molecular targeted drug therapy has been promoted because the selection of patients by genetic makers can increase the therapeutic response for patients with NSCLC [3–5]. However, despite an initial response to the treatment with EGFR-TKIs in such patients, the majority of patients eventually experience a progression of the disease [2,6]. Identifying and understanding the mechanisms of treatment resistance can provide a method for blocking or reversing the mechanism of resistance.

Abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; EMT, epithelial–mesenchymal transition; T790M, threonine-to-methionine mutation at codon 790 of EGFR; p-MET, phosphorylation of MET; p-Akt, phosphorylation of Akt; IHC, immunohistochemical; RECIST, response evaluation criteria in solid tumors; CT, computed tomography; PS, proportion scores; IS, intensity score; TS, total score; CR, complete response; PR, partial response; SD, stable disease; TTP, time to progression; L858R, a substitution of arginine for leucine at codon 858; PI 3-kinase, phosphatidylinositol-3-kinase.

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Table 1
Summary of the patients showing acquired resistance to gefitinib.

Case	Sex	Age[a]	Smoking	Stage[b]	Stage[c]	Specimen[d]	Specimen[e]	Chemotherapy[f]	Response[g]	TTP[h] (days)
1	M	58	Never	IIIB	IIIB	Primary lung tumor	Pulmonary metastasis	Yes	PR	191
2	M	55	Never	IIIB	IIIB	Primary lung tumor	Pulmonary metastasis	No	PR	174
3	F	54	Never	IB	IIIB	Primary lung tumor	Lymph node metastasis	Yes	SD	368
4	F	70	Never	IA	IA	Primary lung tumor	Liver metastasis	Yes	PR	60
5	F	65	Current	IIIB	IIIB	Lymph node	Pleural effusion	No	PR	110
6	M	53	Current	IIIA	IIIA	Primary lung tumor	Lymph node metastasis	Yes	PR	352
7	F	84	Never	IB	IIB	Primary lung tumor	Pleural effusion	No	PR	295
8	F	57	Never	IIIA	IIA	Primary lung tumor	Lymph node metastasis	No	SD	210
9	F	76	Never	IV	IV	Primary lung tumor	Primary lung tumor	No	SD	221
10	F	85	Never	IIIA	IIIA	Primary lung tumor	Skin metastasis	No	CR	210
11	F	52	Never	IIIB	IIIB	Lymph node	Lymph node metastasis	No	PR	233

[a] Age at beginning of gefitinib therapy.
[b] Clinical stage.
[c] Stage at first presentation.
[d] Analyzed specimen of the tumor before treatment with gefitinib.
[e] Specimens at post-treatment.
[f] Previous chemotherapy.
[g] Response to gefitinib.
[h] Time to progression after gefitinib therapy.

Explanations for the EGFR-TKI resistance include the T790M mutation in exon 20 of EGFR, MET amplification, overexpression of HGF, and changes in the EMT status [6–10]. We also recently reported that gefitinib-resistant cell lines showed a marked down-regulation of PTEN expression and increased Akt phosphorylation. Furthermore, nuclear translocation of the EGR1 transcription factor, which regulates PTEN expression, was shown to be suppressed in resistant clones and restored in their revertant clones [11]. However, the "whole picture" regarding acquired resistance remains unclear, and few studies have so far investigated the resistance-related genes in EGFR-TKI resistant specimens from a translational viewpoint. Therefore, a detailed study using matched specimens from both pre- and post-treatment is essential. This study was a retrospective analysis of the acquired resistance-related gene profile in refractory tumors (recurrent and metastatic) after an initial response to treatment that was performed to elucidate the mechanism(s) underlying acquired resistance to EGFR-TKIs. To our knowledge, this is the first comprehensive analysis of the acquired resistance-related gene profile in such pre- and post-treatment study.

2. Materials and methods

2.1. Patients and their characteristics

The characteristics of the 11 patients are listed in Table 1. There were 3 male and 8 female patients. Nine patients were non-smokers and 2 patients were current smokers. The tumor stage was classified according to the International Union against Cancer tumor-node-metastasis classification of malignant tumors [12]. Eight patients from 1995 to 2007 developed recurrent disease after surgery for primary tumors. Three patients were non-surgical cases (cases 5, 9, and 11). Therefore, the pathological stage was adopted for the surgical cases, and the clinical stage for the three non-surgical cases.

The tumor samples were collected from surgically resected specimens from eight primary tumors, two metastatic lymph nodes, and one from the biopsy specimen from an endobronchially invading tumor by a transbronchial biopsy. The institutional review board's approved informed consent for the use of the tumor tissue specimens was obtained either from all the patients or from the patient's legal guardians. All patients received 250 mg gefitinib per day. The treatment was continued until the disease progressed. All of the tumors were pathologically confirmed to be adenocarcinoma. Prior chemotherapy had been administrated in 4 patients.

The objective response of the patients was evaluated using the response evaluation criteria in solid tumors (RECIST) criteria, and routine clinical and laboratory assessments and chest X-rays were performed biweekly and computed tomography (CT) scans were performed 1 month after the start of gefitinib and every 3 months thereafter. Imaging studies (bone scans and brain imaging) were performed every 3 months after the initiation of gefitinib treatment. Refractory tumors were obtained from lymph node metastases (4 cases), and pulmonary metastases (2 cases), and from endobronchially invading tumor, skin metastasis, and liver metastasis (patient No. 4 by autopsy). Four separate metastatic pulmonary tumors located in segments of 4, 5, 6, and 8 of the left lung were obtained from patient No. 1 by video-assisted thoracoscopic surgery [9]. Therefore, 12 gefitinib-refractory specimens were analyzed. All of the specimens were stained with hematoxylin and eosin for the histopathological diagnosis.

2.2. Analyses of EGFR and K-ras mutations

The genomic DNA was extracted from each tumor by previously described methods [1]. The EGFR mutations in exons 19–21 were examined by sequencing [1]. The K-ras mutations were investigated by PCR-based analyses [13].

2.3. Detection of MET amplification and immunohistochemical staining for phosphorylation of MET and HGF

The MET gene copy numbers were determined by real-time PCR assays. PCR was performed for each primer set in triplicate, and the mean value was calculated [13]. Amplification was defined as more than 1.31 copies, which was calculated by the mean of the MET gene copy number measured plus 2 times the standard deviation [13]. The status of MET phosphorylation was examined by IHC staining using phosphor-specific antibodies, and was evaluated using previously described methods [13]. The status of HGF was also investigated by previously described methods [13]. The positive and the negative controls were processed by the normal gallbladder harboring over expression of HGF and the exclusion of the primary antibody, respectively [13].

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2.4. Immunohistochemical (IHC) staining for PTEN and ERG-1

The sections were placed in 0.1% sodium azide buffer (pH 9.0) (Nichirei, Tokyo, Japan) and autoclaved at 121 °C for 10 min. They were treated with 3% H_2O_2 for 5 min to block the endogenous peroxidase activity. The primary antibody reaction used a mouse monoclonal anti-PTEN antibody (Dako, M3627 Cytomation, Glostrup, Denmark) or EGR-1 antibody (SC-101033, Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:100 and 1:50, respectively, in PBS. Reactions were incubated for 18 h at 4 °C following a previously described method [14]. Thereafter, IHC staining was performed by the labeled polymer method (Histofine Simple Stain MAX-PO kit, Nichirei, Tokyo, Japan) according to the manufacturer's instructions. The positive controls for PTEN and EGR-1 used HeLa cells [15]. The negative controls were processed by the exclusion of the primary antibody. Initially, 4 groups for the proportional score (PS) for the positive staining cells were assigned according to the frequency of positive tumor cells (0, none; 1, <25%; 2, <25–50%; 3, >50%). Thereafter, 4 groups for the intensity score (IS) were assigned according to the intensity of the staining (0, none; 1, weak; 2, moderate; and 3, strong). The proportional score and the intensity score were then added together to obtain a total score (TS), which ranged from 0 to 6. The expression status of the cytoplasm of tumor was categorized as negative expression when the score was 0–3 and a positive expression when the score was 4–6 for PTEN [16]. Staining was considered positive if nuclear localization of EGR-1 was present and negative if nuclear staining was absent [17]. The slides were independently examined by two of the investigators (H. U. and H. S.) who were blinded to the clinicopathological data. When a discrepancy was found between the 2 investigators, a consensus was reached via their simultaneous examination using a double-headed microscope.

2.5. Immunohistochemical staining for phosphorylation of Akt- or EMT-related molecules

The status of p-Akt was examined by IHC staining using previously described methods [18]. The positive and the negative controls were processed by the breast cancer specimen harboring over expression of p-Akt and the exclusion of the primary antibody, respectively. The EMT status was also examined using a previously described method. Briefly, IHC staining was used to analyze the protein expression of E-cadherin and gamma-catenin as epithelial markers, and vimentin and fibronectin as mesenchymal markers. The up-regulation of mesenchymal markers or down-regulation of epithelial markers in acquired samples was defined as a change in the EMT [10] (Fig. 1).

3. Results

3.1. Response to gefitinib and EGFR and K-ras mutations

All of the tumors exhibited EGFR mutations. Five showed a deletion in exons 19 and 6 had a substitution of arginine for leucine at codon 858 (L858R) in exon 21 of EGFR in the gefitinib pre-treated tumors (Table 2). The response to the initial gefitinib treatment was a complete response (CR) in 1 case, a partial response (PR) in 7 cases, and stable disease (SD) in 3 cases. The time to progression (TTP) ranged from 60 to 368 days. The mean follow-up periods from the date of administration of gefitinib to the date of death or last known contact was 790 days, which ranged from 167 to 1499 days. Seven (64%) of the gefitinib refractory tumors exhibited a secondary T790M mutation, which was not detected in the tumors before the gefitinib treatment. There were no other novel secondary mutations of *EGFR* at exons 19–21. All of the tumors showed wild type

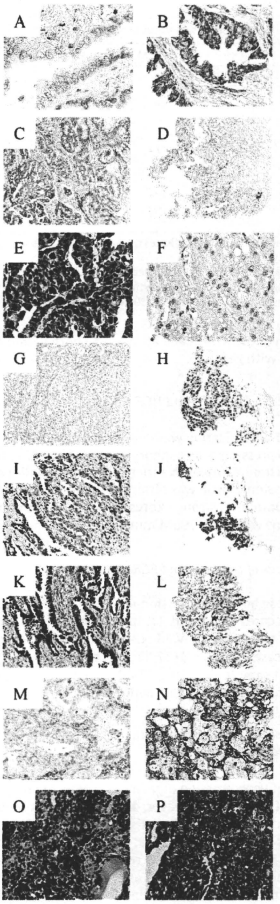

Fig. 1. Representative IHC staining. (A) A weak expression of HGF of pre-treatment tumor in case 3. (B) A strong expression of HGF with brown stained cytoplasm of post-treatment tumor is shown in case 3. (C) A positive expression of PTEN with brown stained cytoplasm of a pre-treatment tumor in case 6. (D) A negative expression of PTEN of post-treatment tumor in case 6. (E) A positive expression of EGR-1 with brown stained nuclei of pre-treatment tumor in case 4. (F) A negative expression of EGR-1 of post-treatment tumor in case 4. (G) A negative expression of p-Akt of pre-treatment tumor in case 6. (H) A positive expression of p-Akt with brown stained nuclei and of post-treatment tumor in case 6. (I) A positive expression of E-cadherin with brown stained membranes of pre-treatment tumor in case 1. (J) A positive expression of E-cadherin of post-treatment tumor in case 1. (K) A positive expression of gamma-catenin with brown stained cytoplasm of a pre-treatment tumor in case 1. (L) A negative expression of gamma-catenin of post-treatment tumor in case 1. (M) A negative expression of fibronectin of pre-treatment tumor in case 10. (N) A positive expression of fibronectin with brown stained membrane of post-treatment tumor in case 10. (O) A negative expression of vimentin of pre-treatment tumor in case 2. (P) A positive expression of vimentin with brown stained membrane and cytoplasm of post-treatment tumor in case 2.

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Table 2
Summary of the gene expression status of resistance-related molecules in sensitive and resistant tumors.

Case	EGFR[a]	T790M	K-ras[b]	MET	p-MET	HGF[c]	PTEN	EGR-1	p-Akt	EMT[d]
1	19/19	−/+	w/w	−/−	+/−	W/S	+/n.e.	n.e.	n.e.	+
2	19/19	−/+[e]	w/w	−/−	−/−	W/S	+/−	+/+	+/+	+
3	21/21	−/+	w/w	−/−	−/−	W/S	+/+	−/+	−/+	−
4	21/21	−/−	w/w	−/−	−/−	W/W	+/+	+/−	−/−	+
5	19/19	−/+	w/w	−/−	−/n.e.	W/n.e.	+/n.e.	n.e.	n.e.	n.e.
6	19/19	−/+	w/w	−/−	−/−	W/S	+/−	−/+	−/+	−
7	21/21	−/−	w/w	−/−	−/n.e.	W/n.e.	+/n.e.	n.e.	n.e.	n.e.
8	21/21	−/+	w/w	−/−	−/−	W/S	−/−	+/+	−/+	−
9	19/19	−/+	w/w	−/−	−/−	W/S	+/−	n.e.	n.e.	−
10	21/21	−/−	w/w	−/−	−/−	W/W	−/+	+/+	−/+	+
11	21/21	−/−	w/w	−/−	−/−	S/W	+/−	+/−	−/−	−

[a] Before and after the slash indicates the gene expression status of sensitive and resistant tumors, respectively. 19: Exon 19 deletion, 21: Exon 21 L858R.
[b] w: wild type.
[c] W/S: weak/strong, n.e.: not evaluated.
[d] The change in EMT status from the tumor before to the lesion after treatment with gefitinib.
[e] One of the four tumors showed a T790M mutation (Onitusuka).

expression of the K-ras gene at codon 12 both before and after the treatment with gefitinib.

3.2. Analyses of the MET and HGF status

No MET amplification was detected in any of these samples. No positive expression of phosphorylated MET (p-MET) was detected in any resistant samples. Neither MET gene amplification, nor an overexpression of HGF was observed in samples without the *EGFR* T790M mutation. A strong expression of HGF was detected in 6 of 8 specimens with the T790M mutation.

3.3. Analyses of the PTEN and EGR-1 status

All cases showed expression of PTEN in the sensitive samples, except for cases 8, 10, and 11. Interestingly, both pre- and post-treatment specimens showed positive and negative expressions of PTEN in 4 cases. In total, 3 (37.5%) of 8 cases showed a loss of PTEN in resistant samples with the T790M mutation. Loss of EGR-1 was detected in 2 (29%) of 7 cases without PTEN.

3.4. Analyses of the phosphorylation of Akt and changes in EMT-related molecules

No positive expression of p-Akt was detected in sensitive samples except for case 2. However, 4 (57%) of 7 cases showed positive expression of p-Akt in resistant samples. Similarly, change in the EMT status between pre-and post-treatment were found in 4 (44%) of 9 cases (Fig. 2). No significant relationships were found between EGFR sensitive mutations, such as exon 19 deletion or L858R, and the molecular alterations described above (Fig. 3).

4. Discussion

In addition to the well-known T790 mutation, we previously reported the overexpression of HGF and changes in the EMT status as a cause of acquired resistance [9,10]. We also reported that gefitinib-resistant cell lines showed a marked down-regulation of PTEN expression and increased Akt phosphorylation. Furthermore, nuclear translocation of the EGR1 was shown to be suppressed in resistant clones and restored in their revertant clones *in vitro* [11]. However, the "whole picture" and interactions among these molecules with regard to acquired resistance remains unclear. The current results have helped fill in the missing information about acquired resistant mechanism, because alterations in gene or protein expression or phosphorylation were detected in every case.

Interestingly, a loss of PTEN co-occurred in only limited tumors with strong expression of HGF. Thus, PTEN loss might represent an additional mechanism of acquired resistance in HGF overexpressing tumors. In fact, there is evidence of direct interactions between HGF and PTEN in glioblastoma cells [19], and PTEN has a lipid phosphatase-dependent antagonistic effect on phosphatidylinositol-3-kinase (PI 3-kinase), which is a prominent component of the signaling cascades activated by c-Met, the receptor of HGF [20].

The loss of PTEN was not necessarily correspondent to loss of EGR-1 and positive expression of p-Akt. Di Loreto et al. also reported that there was discordance between PTEN and EGR-1 in thyroid proliferative lesions [17]. This discrepancy between the *in vitro* and *in vivo* results may be due to (1) disparities among experimental systems, (2) differences in microenvironments, such as the presence of tumor-related macrophages and fibroblasts, and (3) other unknown mechanisms. Furthermore, up-regulation of p-Akt occurred only in tumors without loss of PTEN except for case 6. These results might indicate the existence of a separate mechanism of resistance. Yamasaki et al. reported that down-regulation of PTEN may represent an early step in resistance, while the subse-

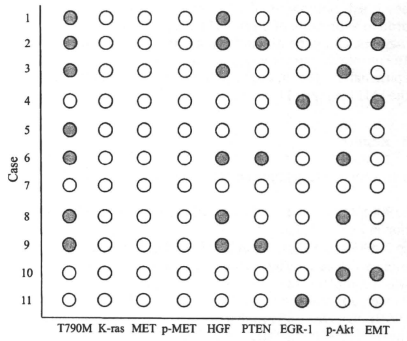

Fig. 2. The changes in genes or proteins in resistant tumors. The dark circle shows the alterations of genes or proteins in the resistant tumors. T790M, an acquired T790M mutation in the sample; K-ras, an acquired K-ras mutation; MET, an amplification of MET; p-MET, positive expression of p-MET; HGF, positive expression of HGF; PTEN, loss of PTEN in the sample; EGR-1, loss of EGR-1; p-Akt, positive expression of p-Akt in the sample; EMT, the change in the EMT status in the sample.

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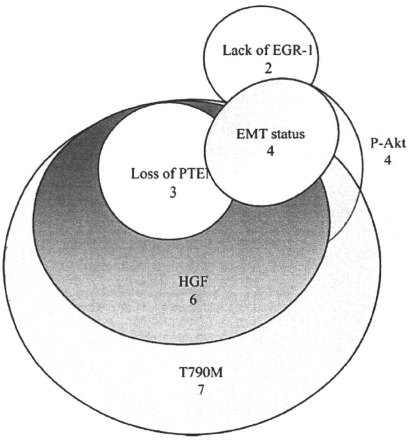

Fig. 3. A Venn diagram illustrating the relationship among T790M mutation, p-MET overexpression, HGF overexpression, a loss of PTEN expression, a loss of EGR-1 expression, and the change in the EMT status in patients with lung adenocarcinoma ($n = 11$) after treatment with gefitinib. The diameters of each circle are roughly proportional to the number of alterations.

quent up-regulation of p-Akt by other mechanisms could increase the extent of resistance [21]. Sos et al. found cooccurrence of homozygous deletion of PTEN and EGFR mutation in 1 out of 24 samples with EGFR mutations. Therefore, EGFR-mutation and loss of PTEN might be intrinsically coexisting in rare cases [22]. High levels of HGF immunoreactivity were detected in tumors from patients who showed intrinsic resistance with no relation to T790M mutation by previous report [23]. However, their finding was with small number of patients with limited samples. Thus, a study with large scale is needed. Furthermore, we evaluated the influence of the biomarker on the patients' TTP. No significant difference in TTP was observed except for EMT status. Interestingly, the TTP of the group without change in the EMT status was significantly better than that with change and the 6 months TTP rates were 85.7% and 50.0%, respectively ($p = 0.016$).

In summary, these findings suggest that combinations of the T790M mutation, and alterations in the expression of HGF, PTEN, EGR-1, p-Akt, and changes in the EMT status can underlie acquired resistance. Imbalances in the patient's characteristics cannot be excluded given the small number of patients with limited biopsies because EGFR-TKI-treated recurrent tumors are not readily biopsied, and are frequently unavailable via autopsy. Nevertheless, the results may represent an important issue and should be taken into account in designing future clinical trials based on anti-EGFR therapies, since understanding the mechanisms of treatment resistance offers the possibility to define other methods for intervening by overcoming the acquired resistance. Further studies with a larger number of patients and more samples are warranted.

Conflicts of interest

None.

Acknowledgements

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Invest New Drugs
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PHASE I STUDIES

Feasibility study of two schedules of sunitinib in combination with pemetrexed in patients with advanced solid tumors

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Summary *Background* Sunitinib is an oral multitargeted tyrosine kinase inhibitor of vascular endothelial growth factor and platelet-derived growth factor receptors, as well as of other receptor types. We have performed a feasibility study to investigate the safety of sunitinib in combination with pemetrexed for treatment of advanced refractory solid tumors. *Methods* Sunitinib was administered once daily on a continuous daily dosing (CDD) schedule (37.5 mg/day) or a 2-weeks-on, 1-week-off treatment schedule (50 mg/day, Schedule 2/1) in combination with pemetrexed at 500 mg/m^2 on day 1 of repeated 21-day cycles. *Results* Twelve patients were enrolled in the study: six on the CDD schedule and six on Schedule 2/1. None of the treated patients experienced a dose-limiting toxicity. Toxicities were manageable and similar in type to those observed in monotherapy studies of sunitinib and pemetrexed. Pharmacokinetic analysis did not reveal any substantial drug–drug interaction. One patient with squamous cell lung cancer showed a partial response and five patients had stable disease. *Conclusions* Combination therapy with sunitinib administered on Schedule 2/1 (50 mg/day) or a CDD schedule (37.5 mg/day) together with standard-dose pemetrexed (500 mg/m^2) was well tolerated in previously treated patients with advanced solid tumors.

Keywords Sunitinib · Pemetrexed · Feasibility study · Solid tumors

Introduction

Progress in the molecular biology of solid tumors has established the important role of tumor angiogenesis and the multiple signaling pathways underlying this process in tumor development [1]. Moreover, antiangiogenic therapy that targets signaling by the vascular endothelial growth factor (VEGF) pathway represents a key advance in clinical oncology [2, 3]. Sunitinib (SUTENT®) is an oral multi-targeted tyrosine kinase inhibitor of VEGF receptors (VEGFR1 to VEGFR3), platelet-derived growth factor receptors (PDGFRα and PDGFRβ), and other receptor tyrosine kinases [4–6]. It has shown single-agent activity and acceptable tolerability in phase I/II studies of patients with a variety of advanced refractory solid tumors [4]. The clinical benefits observed with sunitinib have resulted in multinational approval for its use in the treatment of patients with advanced renal cell carcinoma or imatinib-resistant or -intolerant gastrointestinal stromal tumors [7, 8].

As targeted agents such as sunitinib enter into clinical practice, there is interest in assessment of the efficacy and safety of these agents administered in combination with chemotherapy in cancer patients, including those with treatment-refractory tumors. Preclinical studies indicate that the combination of sunitinib with chemotherapeutic agents results in increased antitumor activity [9]. One chemotherapeutic agent tested, pemetrexed, is an antimetabolite that suppresses cell replication by inhibiting multiple enzymes in

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the folate pathway and which shows clinical activity against a broad range of solid tumors, including non-small cell lung cancer (NSCLC) and mesothelioma [10–12]. The adverse effects of sunitinib are largely nonoverlapping with those of pemetrexed, making the latter an appropriate agent, in terms of safety, for combination with sunitinib. We have now performed a feasibility study to assess the safety and tolerability of two dosing schedules of sunitinib (continuous daily dosing [CDD] schedule and 2 weeks on treatment followed by 1 week off treatment [Schedule 2/1]) in combination with fixed-dose (500 mg/m^2) pemetrexed.

Patients and methods

Study population

Patients with histologically proven advanced solid tumors and who were 20 years of age or older were enrolled in the study. Other key inclusion criteria included: prior treatment with one or more chemotherapy regimens; an Eastern Cooperative Oncology Group performance status of ≤1; resolution of acute toxicities resulting from prior therapy; adequate organ function; and a life expectancy of ≥3 months. Key exclusion criteria included: prior treatment with pemetrexed or sunitinib or irradiation of≥25% of bone marrow; hemoptysis (≥5 mL per episode or ≥10 mL/day) occurring ≤4 weeks before the onset of study treatment; chemotherapy, surgery, or radiation therapy instituted <4 weeks before the start of the study (with the exception of palliative radiotherapy for nontarget lesions); symptomatic or uncontrolled brain metastases, spinal cord compression, carcinomatous meningitis, or leptomeningeal disease; a history of cardiac disease, cerebrovascular events, or pulmonary embolism within the 12 months prior to the onset of study treatment; ongoing cardiac dysrhythmias of National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) grade≥2, atrial fibrillation (any grade), or a prolonged QTc interval; hemorrhage of CTCAE grade 3 within the 4 weeks before the start of the study treatment; or hypertension that could not be controlled with standard antihypertensive agents.

Study design and treatment

The study was a randomized, open-label study (NCT00732992) of sunitinib in combination with pemetrexed in patients with advanced solid tumors. The primary objective was assessment of overall safety, including dose-limiting toxicities (DLTs), for two treatment regimens of sunitinib plus pemetrexed. Secondary endpoints included plasma pharmacokinetic evaluations and preliminary antitumor activity.

Sunitinib was administered orally, once daily, according to either the CDD schedule or Schedule 2/1. Pemetrexed (500 mg/m^2) was administered as a 10-min infusion on day 1 of a 21-day cycle. Patients were instructed to take 500 μg of folate daily, beginning 1 week before day 1 of cycle 1 until study discontinuation. Vitamin B_{12} (1 g) was injected intramuscularly 1 week before day 1 of cycle 1 and again every 9 weeks until study discontinuation. A phase I dose-escalation trial of sunitinib in combination with pemetrexed conducted outside of Japan had demonstrated tolerability of the combination of sunitinib at 37.5 mg (CDD schedule) or 50 mg (Schedule 2/1) with pemetrexed at 500 mg/m^2 [13]. On the basis of these results, we selected the starting doses of sunitinib in the present study as 37.5 mg for the CDD schedule cohort and 50 mg for the Schedule 2/1 cohort. Doses were interrupted or reduced if adverse events of grade 3 or 4 were observed. Doses were delayed if a patient did not meet the following criteria on the first day of each subsequent cycle: absolute neutrophil count of ≥2,000 cells/μL, platelet count of ≥100,000 cells/μL, and calculated creatinine clearance of ≥45 mL/min. Patients were allowed to undergo a maximum of two dose reductions of either drug; the minimum dose for pemetrexed was 250 mg/m^2 and that for sunitinib was 25 mg/day. Treatment was repeated in a 21-day (3-week) cycle until disease progression, unacceptable toxicity, or withdrawal of patient consent occurred. DLTs were assessed during the first treatment cycle and were used to determine whether the dose or schedule was feasible. They were defined as drug-related toxicities of grade 3 or 4, including neutropenia (grade 3 with infection, grade 4 for ≥7 days, or febrile for >24 h), thrombocytopenia (grade≥3 with bleeding or grade 4 for ≥7 days), lymphopenia accompanied by an opportunistic infection, or any nonhematologic toxicity of grade 3 or 4 for ≥7 days. Initially, six patients were randomized to each dosing schedule (three patients each). If no more than one of the three patients experienced a DLT by day 21 of cycle 1, then an additional six patients (three patients each) were randomized for treatment at the same dose. If ≥2/3 or ≥2/6 patients on a schedule experienced a DLT, the dose was reduced and three additional patients were enrolled.

All patients provided written informed consent. The study was approved by the institutional review board of Kinki University Hospital and was performed in accordance with the International Conference on Harmonization of Good Clinical Practice guidelines, as well as with applicable local laws and regulatory requirements.

Study assessments

Safety was assessed according to CTCAE version 3.0. In patients with measurable disease, objective response was

determined according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 [14].

Pharmacokinetic evaluations

For patients randomized to the CDD schedule, blood samples were collected on day 1 of cycle 2 (sunitinib, before as well as 2, 4, 6, 8, 10, and 24 h after dosing; pemetrexed, before as well as 10 min and 1, 2, 4, 6, 8, 10, and 24 h after dosing) to evaluate pharmacokinetic parameters. For patients randomized to Schedule 2/1, blood samples were collected on day 14 of cycle 1 to determine the trough level of sunitinib. The plasma concentrations of sunitinib, its active metabolite (SU12662), and pemetrexed were measured by validated high-performance liquid chromatography and tandem mass spectrometry, with the lower limits of detection being 0.1 ng/mL for sunitinib and SU12662 and 0.1 μg/mL for pemetrexed. Standard plasma pharmacokinetic parameters were estimated by noncompartmental methods. They included the maximum plasma concentration (C_{max}), plasma predose concentration (C_{trough}), time to C_{max} (T_{max}), area under the plasma concentration-time profile from time zero to 24 h after dosing (AUC_{0-24}), area under the plasma concentration-time profile from time zero to infinity ($AUC_{0-\infty}$), elimination half-life ($t_{1/2}$), oral clearance (CL/F), clearance (CL), and volume of distribution at steady state (V_{ss}).

Statistical analysis

Given the exploratory nature of the study, all analyses were descriptive, with no formal statistical test performed on the data.

Results

Patient characteristics

Twelve patients were enrolled in the study from August to November 2008: six patients for the CDD schedule and six for Schedule 2/1. The most common malignancy in the 12 treated patients was NSCLC (n=9, 75%). All patients received at least one dose of the study treatment. Patient demographic and baseline characteristics are summarized in Table 1.

Treatment delivery

A total of 66 cycles of treatment with sunitinib plus pemetrexed was completed, with a median number of cycles per patient of four for the CDD schedule and five for Schedule 2/1. All 12 patients were ultimately withdrawn from the study, the most common reason for which was disease progression (three patients on the CDD schedule and five patients on Schedule 2/1). Treatment was withdrawn because of adverse events in one patient on each schedule (hemoglobin decrease for the CDD schedule and febrile neutropenia for Schedule 2/1). Seven dose reductions each for sunitinib and pemetrexed were instituted (three for the CDD schedule and four for Schedule 2/1), mainly as a result of myelosuppression.

Safety

All 12 patients were evaluable for safety analysis. None of the patients treated on the CDD schedule or Schedule 2/1 experienced a DLT, whereas all individuals experienced at least one adverse event during the study. The major adverse events during the entire treatment period are shown in

Table 1 Patient characteristics according to dosing schedule

	CDD schedule (n=6)	Schedule 2/1 (n=6)
Median (range) age (years)	55.5 (48–69)	66.0 (57–69)
Male/female (n)	6/0	4/2
ECOG performance status 0/1 (n)	2/4	4/2
Primary malignancy (n)		
NSCLC	6	3
Pancreatic cancer	0	1
Pancreatic neuroendocrine tumor	0	1
Uterine sarcoma	0	1
Previous therapy (n)		
Surgery	2	2
Chemotherapy	6	6
Number of prior regimens (n)		
1	3	5
2	2	1
≥3	1	0

CDD schedule continuous daily dosing of sunitinib (37.5 mg) plus pemetrexed (500 mg/m^2) once every 3 weeks, *Schedule 2/1* 2-weeks-on and 1-week-off dosing of sunitinib (50 mg) plus pemetrexed (500 mg/m^2) once every 3 weeks, *ECOG* Eastern Cooperative Oncology Group, *NSCLC* non-small cell lung cancer

Table 2. The most common nonhematologic toxicities (any grade) across both schedules were fatigue (n=11), taste alteration (n=9), skin discoloration (n=8), anorexia (n=8), and fever (n=8). Nonhematologic toxicities of grade 3 included diarrhea (n=2) as well as fatigue, proteinuria, and dehydration (n=1 each) on the CDD schedule, and an increase in alanine aminotransferase and hypertension (n=1 each) on Schedule 2/1. No nonhematologic toxicities of grade 4 were observed for either schedule. The most common hematologic toxicity of grade 3 or 4 was a decrease in neutrophil number, with six patients (CDD schedule, n=4; Schedule 2/1, n=2) experiencing this

Table 2 Treatment-emergent (all-causality) adverse events (NCI CTCAE version 3.0) occurring with an incidence of ≥2 cases (or of special interest) in patients on either the CDD schedule or Schedule 2/1

Adverse event	CDD schedule (n=6)				Schedule 2/1 (n=6)				Total (n=12)
	Grade				Grade				All grades[a]
	1	2	3	4	1	2	3	4	
Nonhematologic									
Fatigue	3	1	1	0	6	0	0	0	11
Taste alteration	1	2	0	0	6	0	0	0	9
Skin discoloration	5	0	0	0	3	0	0	0	8
Anorexia	3	1	0	0	3	1	0	0	8
Fever	3	1	0	0	3	1	0	0	8
AST increased	4	0	0	0	1	1	0	0	6
Diarrhea	1	0	2	0	2	0	0	0	5
Stomatitis	1	1	0	0	2	1	0	0	5
ALT increased	2	1	0	0	1	0	1	0	5
Hypoalbuminemia	0	2	0	0	1	2	0	0	5
Hand–foot syndrome	2	1	0	0	2	0	0	0	5
Vomiting	2	0	0	0	2	0	0	0	4
Cough	0	3	0	0	1	0	0	0	4
Rash	2	1	0	0	1	0	0	0	4
Eyelid edema	1	0	0	0	2	0	0	0	3
Cheilitis	0	0	0	0	3	0	0	0	3
Nausea	1	0	0	0	1	1	0	0	3
Nasopharyngitis	1	0	0	0	2	0	0	0	3
Proteinuria	0	0	1	0	1	1	0	0	3
Constipation	1	0	0	0	1	0	0	0	2
Edema	2	0	0	0	0	0	0	0	2
Infection	0	0	0	0	0	2	0	0	2
Dehydration	0	1	1	0	0	0	0	0	2
Pain-joint	1	0	0	0	1	0	0	0	2
Headache	2	0	0	0	0	0	0	0	2
Neuropathy	0	0	0	0	2	0	0	0	2
Hypertension	0	1	0	0	0	0	1	0	2
Hypothyroidism[b]	0	0	0	0	0	1	0	0	1
TSH increased[b]	0	1	0	0	0	0	0	0	1
Epistaxis[b]	1	0	0	0	0	0	0	0	1
Hemorrhage[b]	0	1	0	0	0	0	0	0	1
Hematologic									
Platelets decreased	3	1	2	0	3	0	1	0	10
Leukocytes decreased	0	2	3	0	0	4	1	0	10
Neutrophils decreased	0	0	4	1	0	1	2	1	9
Hemoglobin decreased	0	2	1	0	1	1	0	0	5
Lymphopenia	0	0	1	0	0	0	1	0	2
Febrile neutropenia[b]	0	0	0	0	0	0	1	0	1

NCI CTCAE National Cancer Institute Common Terminology Criteria for Adverse Events, *CDD* continuous daily dosing, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *TSH* thyroid-stimulating hormone

[a] No adverse events of grade 5 occurred

[b] Adverse events of special interest occurring with an incidence of <2 on either the CDD schedule or Schedule 2/1

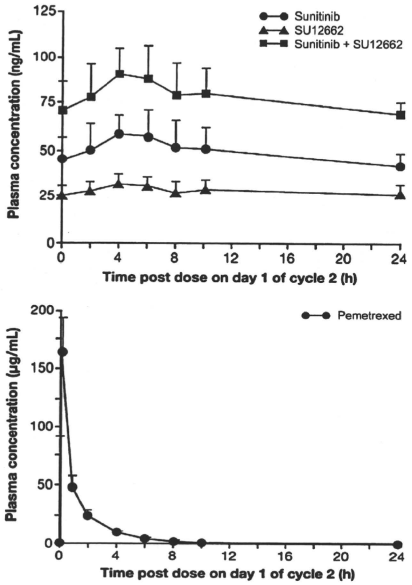

Fig. 1 Plasma concentration-time profiles for sunitinib, SU12662, total drug (sunitinib + SU12662), and pemetrexed on day 1 of cycle 2 for the CDD schedule. Data are means±standard deviation (SD) for three patients

adverse event at grade 3 and two patients (n=1 for each schedule) at grade 4. Other hematologic toxicities of grade 3 or 4 included a decrease in leukocytes of grade 3 in four patients (CDD schedule, n=3; Schedule 2/1, n=1), a decrease in platelets of grade 3 in three patients (CDD schedule, n=2; Schedule 2/1, n=1), and a decrease in hemoglobin level of grade 3 in one patient (CDD schedule).

Adverse events considered to be serious occurred in three patients (CDD schedule, n=2; Schedule 2/1, n=1): one patient on the CDD schedule had dehydration (grade 2), one patient on the CDD schedule had infectious enteritis and dehydration (both of grade 3), and one patient on Schedule 2/1 had pyrexia (grade 2), pneumothorax (grade 1), pleural effusion (grade 1), and febrile neutropenia (grade 3). There were no deaths during the study.

Pharmacokinetics

The mean plasma concentration-time profiles and pharmacokinetic parameters for sunitinib, its active metabolite (SU12662), total drug (sunitinib + SU12662), and pemetrexed for three patients who received the planned treatment on the CDD schedule are shown in Fig. 1 and Tables 3 and 4. The mean C_{trough} for day 1 of cycle 2 was 45.6 ng/mL for sunitinib, 25.1 ng/mL for SU12662, and 70.6 ng/mL for total drug, and each of the corresponding mean plasma concentration-time profiles showed relatively slow absorption and elimination, consistent with previous observations [15]. The pharmacokinetic parameters obtained for sunitinib (37.5 mg) on the CDD schedule with pemetrexed (500 mg/m^2) in the present study did not appear to differ substantially from the dose-normalized parameters previously obtained for single dosing of sunitinib at 25 or 50 mg [15, 16]. The plasma concentration of pemetrexed during sunitinib continuous dosing declined with a fast elimination rate (mean $t_{1/2}$ was 2.75 h), and the $t_{1/2}$, CL, and V_{ss} values were similar to those previously obtained for single dosing of pemetrexed at 500 mg/m^2 [17]. For Schedule 2/1, the mean C_{trough} for day 14 of cycle 1 in six patients who received the planned treatment was 78.5 ng/mL

Table 3 Pharmacokinetic parameters of sunitinib, SU12662, and total drug (sunitinib + SU12662) for the CDD schedule

Parameter	Sunitinib	SU12662	Total drug
C_{trough} (ng/mL)	45.6±11.7 (26)	25.1±5.08 (20)	70.6±13.9 (20)
	[41.3]	[28.0]	[69.3]
T_{max} (h)	4 (4–6)	4 (4–4)	4 (4–6)
C_{max} (ng/mL)	59.9±10.9 (18)	31.6±5.49 (17)	91.56±14.2 (15)
	[59.6]	[34.7]	[94.3]
AUC_{0-24} (ng·h/mL)	1,190±247 (21)	675±107 (16)	1,866±269 (14)
	[1,161]	[665]	[1,951]
CL/F (L/h)	32.4±6.65 (20)	ND	ND
	[32.3]		

CDD continuous daily dosing, *ND* no data, *SD* standard deviation

Data are arithmetic means±SD (coefficient of variation, (%) [median], with the exception of those for T_{max}, which are medians (range). Sampling was performed on day 1 of cycle 2

Table 4 Pharmacokinetic parameters of pemetrexed for the CDD schedule

Parameter	Value
T_{max} (h)	0.167 (0.167–0.167)
C_{max} (μg/mL)	1636±30.7 (19) [167]
$AUC_{0-\infty}$ (μg·h/mL)	1916±36.3 (19) [202]
$t_{1/2}$ (h)	2.7546±0.531 (19) [2.558]
CL (L/h)	4.976±1.38 (28) [4.30]
V_{ss} (L)	10.46±3.13 (30) [10.9]

CDD continuous daily dosing, *SD* standard deviation

Data are arithmetic means ± SD (coefficient of variation, (%) [median], with the exception of those for T_{max}, which are medians (range). Sampling was performed on day 1 of cycle 2

for sunitinib, 38.2 ng/mL for SU12662, and 117.0 ng/mL for total drug. The plasma concentration of sunitinib observed for both schedules was considered to have achieved a steady state on the basis of previous results [15]. The C_{trough} values of sunitinib, SU12662, and total drug observed for both the CDD schedule (sunitinib, 37.5 mg/day) and Schedule 2/1 (sunitinib, 50 mg/day) suggested that the plasma concentrations increased in a dose-dependent manner.

Tumor response

Eight of the 12 patients were evaluable for response by RECIST. A partial response was observed in one patient with NSCLC on the CDD schedule, whereas five patients (two on the CDD schedule and three on Schedule 2/1) had stable disease (Fig. 2a). Most patients showed a decrease in the size of the target lesion while on the study treatment.

Discussion

Our feasibility study investigated the overall safety of sunitinib administered on the CDD schedule or Schedule 2/1 in combination with pemetrexed for the treatment of subjects with advanced refractory solid tumors. Phase I studies of sunitinib monotherapy have been performed according to various schedules, including a 3-week cycle consisting of treatment for 2 weeks followed by a 1-week rest period (Schedule 2/1), a 4-week cycle comprising treatment for 2 weeks followed by 2 weeks off treatment (Schedule 2/2), or a 6-week cycle of treatment for 4 weeks followed by 2 weeks off treatment (Schedule 4/2) [18, 19]. Daily dosing with sunitinib at 50 mg resulted in a target

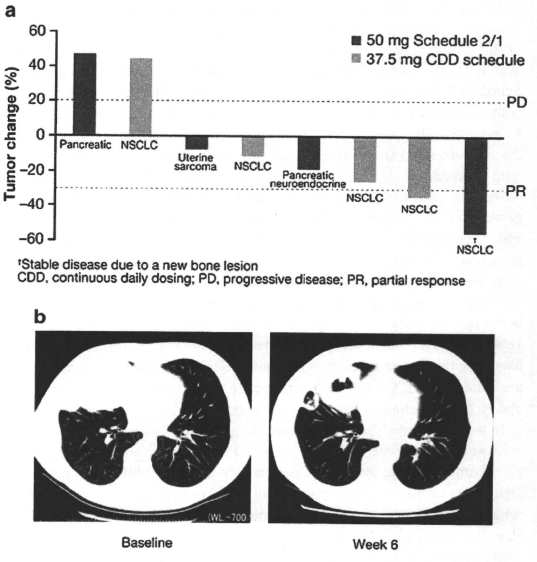

Fig. 2 Tumor response. **a** maximum percentage change in the size of the target lesion in the eight evaluable patients. *PD* progressive disease, *PR* partial response, †stable disease due to a new bone lesion. **b** computed tomography of a solid tumor in the right lung of a patient indicated by † in part **a** at baseline (left panel) and on day 14 of cycle 2 for the CDD schedule. The tumor showed marked central cavitation after treatment

plasma concentration greater than the 50 ng/mL required to inhibit PDGFR and VEGFR, and DLTs of fatigue, asthenia, and thrombocytopenia occurred at a dose of 75 mg on all schedules; a recommended dose of 50 mg was thus established for Schedules 2/1, 2/2, and 4/2 [4]. Preclinical and clinical studies showing tumor regrowth during the off-dosing period suggested that better tumor control might be achieved with sunitinib on a CDD schedule [20, 21]. Subsequent clinical trials demonstrated that CDD of sunitinib at 37.5 mg was well tolerated and showed clinical activity largely similar to that observed for administration on intermittent schedules, providing flexibility in dosing schedule [22–24].

Phase I studies have shown that myelosuppression is the predominant DLT of pemetrexed [25]. We previously found that the maximum tolerated dose of pemetrexed supplemented with folic acid and vitamin B_{12} was 1,200 mg/m^2, which was twice the previously determined such dose (600 mg/m^2) for administration without vitamin supplementation [17, 26]. The results of randomized trials comparing pemetrexed at 500 mg/m^2 versus 900 mg/m^2 or 1,000 mg/m^2 in patients with recurrent NSCLC showed that the higher doses did not exhibit a greater clinical efficacy than the lower dose, thereby establishing the clinically recommended dose of 500 mg/m^2 for pemetrexed supplemented with folic acid and vitamin B_{12} [27, 28].

Given the differences in metabolism and elimination between sunitinib and pemetrexed, we assessed the safety of the combination of recommended doses of these drugs. We initiated treatment with sunitinib at 50 mg/day on Schedule 2/1 or at 37.5 mg on the CDD schedule together with pemetrexed at 500 mg/m^2. There were no DLTs in the 12 patients of the present study who received both drugs at the recommended single-agent doses. Most toxicities were mild or moderate in extent, and similar in type to those observed in the monotherapy studies of sunitinib and pemetrexed. All toxicities of grade 3 or 4 were reversible and manageable with symptomatic treatment and dose reduction or interruption. Hypertension is often associated with treatment with angiogenesis inhibitors, including sunitinib, but this condition developed in only two patients in the present study and, in both cases, blood pressure was controlled with standard antihypertensive therapy. No patients experienced cardiac abnormalities, including electrocardiogram (ECG) changes or a decline in left ventricular ejection fraction to below the lower limit (50%).

In the present study, the full pharmacokinetic profile was evaluated at steady state only for the CDD schedule, given that pharmacokinetic interaction is generally assessed with high drug exposure. The concomitant administration of pemetrexed and sunitinib showed no marked effect on the pharmacokinetics of either drug, compared with previous single-dosing results. These findings suggest that there was no substantial pharmacokinetic interaction between sunitinib and pemetrexed, consistent with the differences in the pathways of metabolism and elimination for these drugs. Sunitinib is primarily metabolized by cytochrome P450-3A4 (CYP3A4) in hepatic microsomes, whereas pemetrexed is not metabolized to an appreciable extent, but is primarily eliminated renally [4, 29]. It is not likely that sunitinib or its metabolites inhibit the renal elimination of pemetrexed. In addition, *in vitro* studies with human liver microsomes suggested that pemetrexed administration is not likely to result in clinically relevant inhibition of the metabolic clearance of drugs metabolized by CYP3A [30]. The trough plasma concentrations for total drug (sunitinib + SU12662) in both treatment arms of the present study suggest that sufficient exposure was achieved with regard to target inhibition, according to the required inhibitory concentration values.

Although tumor evaluation was not the primary objective of the present study, and the small sample size precludes any definitive conclusions regarding treatment efficacy, antitumor activity data were suggestive of a potential clinical benefit. It is possible that further pemetrexed studies might be restricted to patients with non-squamous NSCLC because of the pemetrexed label indications [31]. However, the one partial response in the present study was observed in a patient with squamous NSCLC; the tumor cavitation apparent in this patient after study treatment (Fig. 2b) is characteristic of the antitumor effect of antiangiogenic therapy. Given that sunitinib has shown promising single-agent activity in patients with recurrent NSCLC [22, 32], further research is warranted to determine whether sunitinib might improve the effect of pemetrexed, not only in nonsquamous NSCLC, but also in squamous NSCLC.

In conclusion, combination therapy with sunitinib administered according to Schedule 2/1 (50 mg/day), or a CDD schedule (37.5 mg/day) together with standard-dose pemetrexed (500 mg/m^2), was well tolerated in previously treated patients with advanced solid tumors. In both dosing schedules, sunitinib exposure remained above the target plasma concentration in the presence of pemetrexed. Given that both sunitinib and pemetrexed have shown antitumor activity as single agents for various types of solid tumors including NSCLC, sunitinib in combination with pemetrexed is a viable therapeutic regimen that warrants future investigation.

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ORIGINAL ARTICLE

High-dose dexamethasone plus antihistamine prevents colorectal cancer patients treated with modified FOLFOX6 from hypersensitivity reactions induced by oxaliplatin

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Abstract
Background Oxaliplatin is a third-generation platinum compound and a key agent for the management of colorectal cancer. Patients treated with oxaliplatin are at risk for hypersensitivity reactions. We designed a modified premedication regimen to prevent oxaliplatin-related hypersensitivity reactions and assessed if this approach is effective.
Methods A retrospective cohort study of patients with advanced colorectal cancer who received modified FOLFOX6 (mFOLFOX6) was performed. Patients received routine premedication with dexamethasone 8 mg and granisetron 3 mg for the first five cycles of mFOLFOX6. From the sixth cycle onward, cohort 1 received the same premedication, and cohort 2 received modified premedication (diphenhydramine 50 mg orally, followed by dexamethasone 20 mg, granisetron 3 mg, and famotidine 20 mg). We compared the incidence of hypersensitivity reactions, duration of treatment, and reasons for treatment withdrawal between the two cohorts.
Results A total of 181 patients were studied (cohort 1, 81; cohort 2, 100). Hypersensitivity reactions developed in 16 patients (20%) in cohort 1 and 7 (7.0%) in cohort 2 ($P = 0.0153$). The median number of cycles increased from 9 in cohort 1 to 12 in cohort 2. Apart from progressive disease, neurotoxicity was the reason for discontinuing treatment in 20% of the patients in cohort 1, as compared with 53% in cohort 2.
Conclusion Increased doses of dexamethasone and antihistamine significantly reduced oxaliplatin-related hypersensitivity reactions. This effective approach should be considered for all patients who receive FOLFOX, allowing treatment to be completed as planned.

Keywords Colorectal cancer · FOLFOX · Hypersensitivity reaction · Oxaliplatin · Premedication

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Introduction

Oxaliplatin, a third-generation platinum derivative, in combination with fluorouracil and leucovorin (FOLFOX) is among the most effective chemotherapies for metastatic colorectal cancer. The increasing use of oxaliplatin for chemotherapy has led to an increased incidence of oxaliplatin-related hypersensitivity reactions. The MOSAIC trial, in which more than 1,100 patients with colorectal cancer received 5-fluorouracil with oxaliplatin in an adjuvant setting, reported a 10.3% incidence of hypersensitivity reactions, which were one of the major reasons for discontinuing treatment [1].

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Hypersensitivity is defined as an unexpected reaction inconsistent with a drug's usual toxicity profile. Such reactions usually occur during or immediately after treatment. Once sensitized, patients have recurrent hypersensitivity reactions on subsequent exposure to oxaliplatin. Desensitization protocols have been designed to prevent hypersensitivity reactions. Such protocols have allowed successful rechallenge with oxaliplatin [2, 3]. However, clinical criteria for rechallenge with oxaliplatin remain a matter of debate. Reliable methods for predicting the risk of severe hypersensitivity reactions to oxaliplatin have not been established. The potential risks of rechallenge with oxaliplatin after severe anaphylaxis should be weighed against the expected benefits according to the specific clinical situation.

Hypersensitivity reactions to platinum salts (cisplatin, carboplatin) are classically type I (i.e., immediate) reactions [4], the incidence of which increases with multiple cycles of therapy [5]. The symptoms can resolve after treatment with antihistamines and steroids. More recent series have documented a considerably higher incidence of hypersensitivity reactions, ranging between 8% and 19% [6–10]. Besides these reports, studies assessing the preventative effect of premedication on oxaliplatin-related hypersensitivity are scant.

We have designed a modified premedication regimen, which includes a higher dose of dexamethasone (20 mg) plus an antihistamine. This dose of dexamethasone has been shown to be safe and effective for the prophylaxis of paclitaxel-associated hypersensitivity reactions [11]. Dexamethasone (20 mg) can be administered intravenously for desensitization against oxaliplatin hypersensitivity [3, 12]. These findings suggested that the prophylactic use of dexamethasone (20 mg) would reduce the incidence or severity of hypersensitivity reactions. We gave our modified regimen for premedication to patients with advanced colorectal cancer after they had received five cycles of a modified regimen of FOLFOX6 (mFOLFOX6) with standard premedication. We retrospectively compared the frequencies of hypersensitivity reactions between patients who received this modified premedication regimen with those who received standard premedication for the duration of FOLFOX treatment to determine whether our regimen was effective.

Patients and methods

Patient selection

This investigation was a retrospective cohort study of patients with advanced colorectal cancer who received modified FOLFOX6 (mFOLFOX6: oxaliplatin 85 mg/m^2 plus concurrent leucovorin 400 mg/m^2 as a 2-h intravenous infusion on day 1, followed by a bolus injection of 5-fluorouracil 400 mg/m^2 and by a 46-h continuous intravenous infusion of 5-fluorouracil 2,400 mg/m^2, repeated every 2 weeks) at Kinki University Hospital from September 2005 through September 2009. Eligible patients had to have adenocarcinoma of the colon or rectum; unresectable metastases; adequate bone marrow, liver, and kidney functions; a World Health Organization performance status of 0–2; and an age of $\geq$18 years. Patients who received five cycles of mFOLFOX6 without any allergic reactions were eligible. Patients with central nervous system metastases, only bone metastases, second malignancies, bowel obstruction, peripheral neuropathy of grade 3 or higher, symptomatic angina pectoris, or disease confined to previous radiation fields were excluded.

Chemotherapy and premedication

The patients were divided into two cohorts. In cohort 1, patients received routine premedication for the first five and subsequent cycles of mFOLFOX6 from September 2005 through September 2007. In cohort 2, treated between October 2007 and September 2009, patients similarly received routine premedication for the first five cycles. The premedication included routine antiemetic prophylaxis with dexamethasone 8 mg and granisetron 3 mg in 50 ml 0.9% saline, given intravenously 15 min before oxaliplatin. To reduce the risk of hypersensitivity reactions associated with continued treatment, from the sixth cycle onward all patients in cohort 2 received a modified premedication regimen, consisting of diphenhydramine 50 mg given orally 30 min before oxaliplatin, followed by dexamethasone 20 mg, granisetron 3 mg, and famotidine 20 mg in 50 ml saline, given intravenously 15 min before oxaliplatin.

Definition of allergic reactions

A hypersensitivity reaction to oxaliplatin was defined as the development of at least one of the following signs or symptoms after treatment with oxaliplatin: palmar erythema, pruritus, urticaria, diffuse erythroderma, tachycardia, angina, wheezing, facial or tongue edema, dyspnea, hypertension, hypotension, respiratory arrest, anaphylaxis, seizure, or death. Clinically significant respiratory compromise (wheezing associated with hypoxia or hypercarbia, and respiratory arrest), clinically significant cardiovascular compromise (angina, symptomatic hypotension or hypertension, and cardiovascular collapse), anaphylaxis, seizure, and death were all considered manifestations of a severe allergic reaction.

Study objectives and outcome measures

The primary objective of this study was to evaluate whether the modified premedication regimen reduced the incidence of hypersensitivity reactions. The primary outcome measure was the reduction in such reactions as compared with routine premedication. Secondary objectives were to evaluate the safety of the modified premedication regimen and to compare the duration of treatment with mFOLFOX6 and the reasons for treatment discontinuation between the two cohorts. Progressive disease was excluded from the analysis of reasons for treatment discontinuation.

Statistical analysis

A primary analysis was performed to compare cohorts 1 and 2. To assess the effect of premedication on hypersensitivity reactions to oxaliplatin in cohorts 1 and 2, we calculated risk ratios and 95% confidence intervals (95% CI). In addition, we calculated adjusted risk ratios with 95% CI for covariates (age, sex, diagnosis, prior treatment) by performing a Poisson regression analysis. To assess the effect of treatment exposure to the premedication on hypersensitivity reactions to oxaliplatin in cohorts 1 and 2, we compared the number of cycles between the cohorts with the use of the Wilcoxon test. All tests were two-sided with a significance level ≤ 0.05.

Results

Patient characteristics

The characteristics of the 181 eligible patients are listed in Table 1 (81 in cohort 1 and 100 in cohort 2). The patients' characteristics were well balanced between the cohorts, except for bevacizumab, because bevacizumab was approved in July 2007 in Japan. In 2007, bevacizumab was introduced to Japan; we therefore assessed the number of cycles administered for mFOLFOX6 alone in cohort 1 ($n = 81$) and for mFOLFOX6 alone ($n = 49$) and mFOLFOX6 plus bevacizumab ($n = 51$) in cohort 2. No patient in cohort 1 received bevacizumab, whereas nearly half the patients in cohort 2 received bevacizumab. No patient had a known history of allergy to a platinum salt. Five patients had a history of drug allergy.

Incidence of hypersensitivity reactions to oxaliplatin

In cohort 1, hypersensitivity reactions developed in 16 (20%) of 81 patients who received routine premedication (Table 2). Six of these patients (7.4%) had manifestations of severe allergic reactions. In cohort 2, hypersensitivity reactions occurred in 7 (7.0%) of 100 patients who received modified premedication (Table 2). Three of these patients (3.0%) had manifestations of severe allergic reactions. The incidence of hypersensitivity reactions differed significantly between the cohorts (risk ratio, 0.3544; 95% CI, 0.1532–0.8196; $P = 0.0153$). Poisson regression analysis yielded a risk ratio of 0.3581 (95% CI, 0.1541–0.8324; $P = 0.0170$) (Table 2). None of the patients with a history of drug allergy had hypersensitivity reactions.

Table 1 Patient characteristics

	Routine premedication (cohort 1)	Modified premedication (cohort 2)
No. of patients	81	100
Median age, years (range)	62 (29–82)	62 (34–84)
Sex		
Male/female	53/28	66/34
Diagnosis		
Colon	44	51
Rectum	37	49
Line of therapy		
First-line therapy	43	50
Second-line therapy	27	42
Third-line or subsequent therapy	11	8
mFOLFOX6 + bevacizumab	0	51
Median cumulative oxaliplatin dose for the first five cycles (mg/m^2)	414	419

FOLFOX6 chemotherapy with oxaliplatin plus fluorouracil and leucovorin

Treatment exposure

The 81 patients in cohort 1 received a total of 382 cycles of mFOLFOX6 (Table 2). The median number of cycles of mFOLFOX6 was 9 (9 as first-line therapy, 9 as second-line or subsequent therapy) (Table 3). The 100 patients in cohort 2 received a total of 781 cycles (Table 2). The median number of cycles of mFOLFOX6 was 12 overall (Table 2). The number of cycles differed significantly between the cohorts on the Wilcoxon test ($P < 0.0001$) (Table 2). In cohort 2, the median number of cycles of mFOLFOX6 without bevacizumab was 11 (10 as first-line therapy, 11 as second-line or subsequent therapy) (Table 3). The median number of cycles of mFOLFOX6 plus bevacizumab was 12 (12 as first-line therapy, 12 as second-line or subsequent therapy) (Table 3). The number of cycles in patients who additionally received bevacizumab did not differ significantly on the Wilcoxon test. The reasons for treatment

Table 2 Effect of premedication on incidence of hypersensitivity reactions to oxaliplatin

	Incidence of hypersensitivity reactions/ total patients (%)	Risk ratio (95% CI) (*P* value)	Adjusted risk ratio (95% CI) (*P* value)	Incidence of hypersensitivity reactions/total cycles (%)	Median cycles	*P* value
Routine premedication (cohort 1)	16/81 (20)	0.3544 (0.1532–0.8196) ($P = 0.0153$)	0.3581 (0.1541–0.8324) ($P = 0.0170$)	16/382 (4.2)	9	<0.0001
Modified premedication (cohort 2)	7/100 (7.0)			7/781 (0.90)	12	

CI confidence interval

Table 3 Effect of modified premedication on median number of treatment cycles

Cohort	Regimen	Line of therapy	Median cycles of mFOLFOX6 (range)	No. of patients
Routine premedication (cohort 1)	mFOLFOX6	First-line therapy	9 (6–17)	43
		Second-line or subsequent therapy	9 (6–22)	38
Modified premedication (cohort 2)	mFOLFOX6	First-line therapy	10 (6–28)	27
		Second-line or subsequent therapy	11 (6–29)	22
	mFOLFOX6 + bevacizumab	First-line therapy	12 (7–31)	23
		Second-line or subsequent therapy	12 (7–31)	28

discontinuation differed between the cohorts (Table 4). The main reason for treatment discontinuation in cohort 1 was hypersensitivity reactions (53%). Hypersensitivity was the second reason for discontinuing treatment in 11% of the patients in cohort 2. The main reason for treatment discontinuation in cohort 2 was neurotoxicity (53%). Neurotoxicity was the second reason for discontinuing treatment in 20% of the patients in cohort 1.

Table 4 Reasons for treatment discontinuation

Reasons for discontinuation	Routine premedication (cohort 1) ($n = 30$)		Modified premedication (cohort 2) ($n = 62$)	
	No. of patients	%	No. of patients	%
Neurotoxicity	6	20	33	53
Hypersensitivity reactions	16	53	7	11
Fatigue	0	0	3	4.8
Vomiting	0	0	2	3.2
Thrombocytopenia	0	0	1	1.6
Febrile neutropenia	2	6.7	4	6.5
Liver dysfunction	2	6.7	0	0
Thrombosis	0	0	1	1.6
Diarrhea	0	0	1	1.6
Others	4	13	10	16

Safety

Modified premedication did not increase the incidence of adverse effects related to the high dose of dexamethasone, such as exacerbation of diabetes, osteoporosis, and compression fracture. Diphenhydramine was associated with mild somnolence in two patients, but this symptom resolved promptly.

Discussion

The incidence of hypersensitivity reactions in cohort 1 was similar to that in previous studies. Allergic reactions usually develop after several infusions of oxaliplatin [13]. In cohort 2 of our study, the use of modified premedication decreased the incidence of hypersensitivity reactions to 7.0%. Modified premedication with increased doses of dexamethasone and antihistamines thus reduced the incidence of hypersensitivity reactions by 14 percentage points as compared with cohort 1, treated with routine premedication. Gowda et al. [9] evaluated the incidence of hypersensitivity reactions to oxaliplatin and reported 32 hypersensitivity reactions in 169 patients (incidence, 18.9%) who received oxaliplatin preceded by dexamethasone (10 mg) and ondansetron (Zofran, 8 mg). Brandi et al. [7] reported that hypersensitivity reactions occurred in 18.1% of patients who received oxaliplatin preceded by ondansetron. Other than these reports, studies assessing the

preventative effect of premedication on oxaliplatin-related hypersensitivity are scant.

In our study, all patients received mFOLFOX6. Kim et al. retrospectively investigated 247 patients given oxaliplatin-containing regimens and reported that the incidences of hypersensitivity reactions did not depend on the oxaliplatin-containing regimen employed [6]. The modified premedication regimen used in the present study might thus be useful for the management of hypersensitivity reactions to other oxaliplatin-containing regimens.

The patient characteristics were well balanced between the cohorts. The median number of cycles increased from 9 to 12 when modified premedication was used instead of routine premedication. This three-cycle increase in the median number of cycles administered to patients who received modified premedication is particularly important, because prolonged therapy might contribute to improved survival. In cohort 2, patients could receive mFOLFOX6 plus bevacizumab, newly approved in Japan. The addition of bevacizumab to oxaliplatin-based, first-line chemotherapy has been shown to significantly improve progression-free survival in patients with metastatic colorectal cancer [14, 15]. We therefore examined if increasing the number of treatment cycles was associated with the inclusion of bevacizumab. The median number of cycles in patients who additionally received bevacizumab was similar to that in patients treated with mFOLFOX6 without bevacizumab. We found no association between bevacizumab and the number of cycles administered to cohort 2. Bevacizumab thus apparently did not contribute to a longer duration of treatment. Kim et al. [6] reported that anti-vascular epithelial growth factor (anti-VEGF) monoclonal antibody bevacizumab was not associated with hypersensitivity reactions when given with combination chemotherapy regimens. Consistent with their results, we found no difference in the frequency of hypersensitivity reactions according to the presence or absence of bevacizumab.

The major reasons for discontinuing treatment with mFOLFOX6 were neurotoxicity and hypersensitivity reactions. Neurotoxicity was the most remarkable as well as the most common dose-limiting factor. Treatment withdrawal was based on the highest grade adverse effects occurring during the previous cycle. Sensory neuropathy was treatment limiting in patients who received FOLFOX4 (85 mg/m^2 oxaliplatin) because it generally occurred after 8–10 cycles [16]. Tournigand et al. [17] reported that oxaliplatin was associated with grade 3 neuropathy in 20% of patients who received FOLFOX6 (100 mg/m^2 oxaliplatin) and in 34% of patients after 12 cycles. In our study, neurotoxicity was the reason for discontinuing treatment in 20% of the patients in cohort 1, as compared with 53% of those in cohort 2. These reports supported our results that a decreased frequency of hypersensitivity reactions was associated with an increased rate of treatment discontinuation caused by neurotoxicity.

If treatment is discontinued because of neurotoxicity, oxaliplatin-based therapy may be able to be resumed after this adverse effect resolves. This strategy enables treatment for longer periods. When oxaliplatin is used in an adjuvant setting, in which the median number of courses of treatment ranges from 10 to 12, it is important to note that the use of modified premedication reduced the frequency of hypersensitivity reactions from 20% to 7.0%, allowing treatment to be completed as planned. Completion of adjuvant treatment by our strategy may reduce the relapse rate, thereby contributing to improved survival.

The exact mechanism responsible for platinum-related hypersensitivity reactions is unknown, but several mechanisms may be involved. Hypersensitivity reactions have been linked to the release of histamine and other vasoactive substances and ascribed to type I hypersensitivity IgE-mediated reactions [9, 18]. Hypersensitivity reactions usually develop after multiple infusions of oxaliplatin (7 on average) [19], clearly showing that repeated exposure to the drug is prerequisite to the induction of an allergic immune response.

The optimal strategy for resuming treatment after discontinuation caused by an episode of hypersensitivity remains controversial. Because resumption of treatment can be fatal, several preventive procedures have been proposed. Patient desensitization is of interest because of its consistent efficacy but has been studied in only a small number of subjects [19]. Moreover, desensitization is cumbersome to implement. The prick test, using a concentration of 1 mg/ml oxaliplatin, appears not to be very sensitive. Skin tests are useful for detecting IgE-mediated reactions, but their sensitivity is not high enough. When hypersensitivity reactions to oxaliplatin do occur, symptoms generally subside on discontinuation of treatment and administration of steroids and antihistamines. Mild sensitivity reactions to oxaliplatin can be controlled by treatment with antihistamines, steroids, or both. Interestingly, all the patients in cohort 1 of our study received premedication with dexamethasone 8 mg and granisetron 3 mg as a part of a "standard antiemetic" regimen before the infusion of oxaliplatin. In cohort 2, we confirmed that modified premedication with an increased dose of dexamethasone plus an antihistamine effectively decreased hypersensitivity reactions. Premedication was not associated with any side effects. In particular, adverse events potentially associated with a high dose of dexamethasone, such as exacerbation of diabetes, osteoporosis, and compression fractures, did not occur.

In conclusion, our study showed that modified premedication with an increased dose of dexamethasone plus an antihistamine from the sixth cycle of mFOLFOX6