Table III. *Summary of toxicity during induction chemotherapy.*

	FP (n=14)		DCF (n=16)	
	Grade 3	Grade 4	Grade 3	Grade 4
Hematologic toxicity				
Leukopenia	1	0	9	1
Neutropenia	0	1	2	8
Febrile neutropenia	0	0	4	0
Anemia	1	0	0	1
Thrombocytopenia	0	0	1	0
Non-hematologic toxicity				
Nausea/vomiting	0	0	1	0
Diarrhea	0	0	0	0
Mucositis	0	0	2	0
Anorexia	0	0	1	0
Renal	0	0	0	0
Infection	1	0	1	0

FP: Cisplatin plus 5-fluorouracil; DCF: docetaxel plus 5-fluorouracil and cisplatin.

Table IV. *Postoperative complications.*

	FP (n=10)	DCF (n=12)
Pneumonia	2	3
Cardiovascular (pulmonary embolism, arrhythmia, venous embolism)	2	1
Laryngeal nerve palsy	1	1
Anastomotic leak	0	2
Wound infection	2	1
Hemorrhage	0	0
Pneumoderma	0	1
Lymphorrhea	0	1
Chylothorax	1	0
Infection	1†	2‡
Pancreatic juice leakage	0	1

†One patient developed cholecystitis after surgery. ‡One patient developed methicillin-resistant Staphylococcus aureus bacteremia and another developed mediastinal abscess after surgery. FP: Cisplatin plus 5-fluorouracil; DCF: docetaxel plus 5-fluorouracil and cisplatin.

patients in the DCF group and in 1/14 of patients in the FP group (p=0.0017). Despite antibiotic prophylaxis, the rate of febrile neutropenia was higher in the DCF group. The percentages of patients with grade 3 or 4 anemia and thrombocytopenia were similar in both groups. Although grade 3 oral mucositis occurred in two patients in the DCF group, there were no major differences in the incidence rates of severe nonhematologic toxicity during induction chemotherapy in the two groups. None of the patients developed treatment-related perforation of the esophageal wall, esophagobronchial fistula, mediastinal fistula, or aortic fistula. There were no treatment-related deaths in either group.

Postoperative complications. The in-hospital mortality rate after surgery was 0% in both of the treatment groups. The postoperative complication rate was 4/10 in the FP group and 6/12 in the DCF group. Details of the postoperative complications are listed in Table IV. Overall, there were no remarkable differences in the postoperative complications among the two study groups (Table IV). Notably, the incidence of overall infections, including pneumonia, wound infection, and other infections, was similar in the two groups.

Survival. PFS was analyzed for 22 patients who underwent induction chemotherapy followed by surgery. The median PFS for the DCF group was 15.7 months, which was longer than that for the FP group (8.4 months); however, the difference was not significant (p=0.740; Figure 1A). OS was analyzed for all patients who underwent induction chemotherapy regardless of surgery. The OS for the DCF group was also longer compared to that of the FP group (35.9 months *vs.* 19.0 months); however, the difference was not significant (p=0.285; Figure 1B). The 1-year survival rate in the DCF group was 90.0%, which was superior to 1-year survival in the FP group (58.3%, Figure 1B).

Patterns of postoperative recurrence. At the time of analysis, the recurrence rates after surgery were 7/10 in the FP group and 5/12 in the DCF group (p=0.1839). There were 7 patients with distant metastases in the FP group. The sites of distant metastases included the bone (N=1), lung (N=2), abdominal lymph node (N=2), and cervical lymph nodes (N=1); and one patient had recurrences in the bone, adrenal gland, and an abdominal lymph node. In another patient, recurrence in an abdominal lymph node was followed by liver metastasis. There were five patients in the DCF group with distant metastasis, and one patient with both locoregional and distant metastasis. The sites of distant metastases included abdominal lymph node (N=1), chest wall (N=1), and muscle (N=1); and, notably, bone metastases (N=5) were observed in all DCF patients who had recurrences.

Discussion

The prognosis of esophageal cancer patients with locally advanced SCC remains poor (15). Because of the high rate of postoperative complications, attention has shifted to neoadjuvant treatment. In the JCOG 9907 study, preoperative chemotherapy with FP was found to be superior to postoperative FP for OS in patients with resectable (non-T4), clinical stage II or III esophageal cancer (3). Based on this result, the standard treatment strategy for unequivocal T3

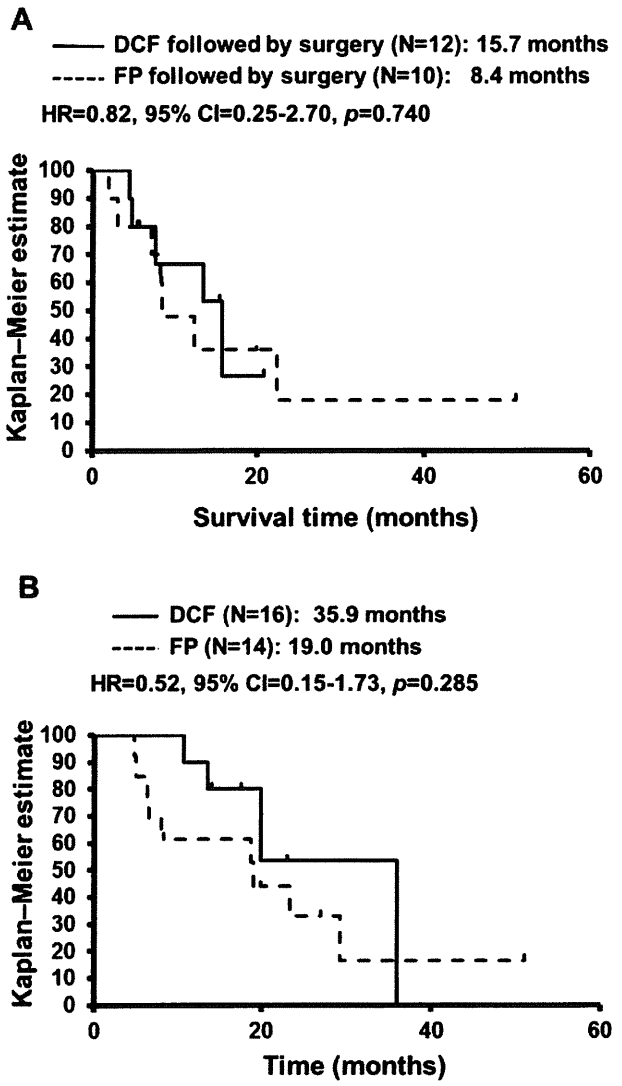

Figure 1. *Kaplan-Meier plot showing progression-free survival (A) and overall survival (B) in the docetaxel plus 5-fluorouracil and cisplatin (DCF) and cisplatin plus 5-fluorouracil (FP) induction chemotherapy groups.*

disease is preoperative chemotherapy with FP followed by radical surgery. However, local recurrence is commonly observed among the patterns of postoperative recurrence in patients receiving preoperative chemotherapy, even after three-field lymphadenectomy. In a meta-analysis of clinical trials of neoadjuvant chemotherapy, Gebski *et al.* demonstrated that there was no significant preoperative chemotherapy effect on all-cause mortality in patients with SCC (hazard ratio 0.88; p=0.12) (16). Furthermore, subgroup analysis of the JCOG 9907 study revealed that the survival benefit of neoadjuvant chemotherapy in stage III disease was less than the benefit in stage II disease. Although development of more intensive preoperative therapy is needed for local tumor control of advanced esophageal cancer in order to improve survival, there is no consensus on whether chemotherapy or chemoradiotherapy should be performed as preoperative treatment.

Preoperative chemoradiotherapy with FP is expected to be a promising, new standard preoperative therapy for esophageal cancer. Indeed, in Western countries, many patients with stage II or III SCC have received neoadjuvant chemoradiotherapy followed by surgery. Stahl *et al.* reported that chemoradiotherapy (40 Gy) followed by surgery improves local tumor control in patients with locally advanced esophageal SCC (17). However, treatment-related mortality was significantly increased in the group undergoing chemoradiotherapy followed by surgery compared to the group undergoing chemoradiotherapy alone (12.8% *vs.* 3.5%, respectively; p=0.03). Thus, there remains concern regarding the potential risks of surgery after chemoradiotherapy. Most randomized controlled studies of neoadjuvant chemoradiotherapy have included surgery alone as the control arm, and these studies failed to demonstrate significant improvement in survival, particularly among patients with histologic subtypes of SCC (18-22).

In this study, we retrospectively investigated if DCF was a more powerful preoperative chemotherapy agent than FP for the treatment of patients with locally advanced esophageal cancer, which were suspected of invading adjacent organs, but were not unequivocal T4 lesions (*i.e.*, borderline-resectable T4 disease). This is a patient subgroup for which we hypothesized that preoperative intensive chemotherapy could contribute to conversion of the lesion to curative resectability, which could lead to improved survival outcomes. Because patients with unequivocal T4 tumors have poor survival outcomes after surgical treatment and are usually treated in the palliative setting with FP or nedaplatin plus 5-fluorouracil with concurrent radiotherapy (4, 23, 24), we excluded unequivocal T4 patients from our analysis. Our results demonstrated that the overall response rate and R0 resection rate were better in patients receiving DCF than in patients receiving FP. One patient treated with DCF achieved complete response.

Histopathological findings in resected specimens revealed more favorable post-chemotherapeutic effects in DCF patients than in FP patients. These findings suggest that DCF induction chemotherapy for advanced esophageal cancer may be a promising preoperative option for local tumor control and may result in a high rate of curative resection. The Medical Research Council Oesophageal Cancer Working Group (MRC) found a 60% R0 resection rate among patients treated with neoadjuvant FP compared with a 54% rate in patients treated with surgery alone, which led to improved overall survival (p<0.0001) (25). Furthermore, it was reported that pathologic response after neoadjuvant therapy is associated with survival in patients with esophageal cancer (26). These findings suggest that pathologic response to neoadjuvant therapy and R0 resection are the major determinants of

survival. Our survival analysis indicated that the 1-year survival rate in the DCF group was 90.0%, which is superior to that seen in the FP group, and this DCF result is also superior to survival in patients with unequivocal T4 disease (4). The addition of docetaxel to cisplatin plus 5-fluorouracil may further improve pathologic response and subsequently improve survival in patients with advanced esophageal cancer.

As expected, the DCF regimen induced more leucopenia and neutropenia than FP, but did not lead to more frequent infectious complications. The myelotoxicity seen in the DCF group was consistent with that seen in other studies (7, 8), and was manageable probably because patients received prophylactic antibiotics. No significant differences in nonhematologic toxicity were observed during induction chemotherapy. Furthermore, the DCF regimen did not increase the risk of postoperative complications compared to the FP regimen. This result suggests that esophagectomy after DCF therapy is as safe as after FP therapy.

However, 5/12 patients receiving DCF followed by surgery experienced distant failure within 24 months after surgery. Therefore, we cannot conclude that preoperative DCF chemotherapy is able to provide local tumor control and also to prevent distant failure. Furthermore, the present analysis lacks the statistical power to demonstrate a significant survival benefit of the DCF regimen, because this is a single-institution retrospective study based on a small patient group and short observation period. To achieve better survival after DCF, it may be necessary to determine the predictive factors for tumor recurrence, in order to prevent the occurrence of distant metastasis, as well as to provide locoregional control.

In conclusion, induction chemotherapy using a DCF regimen may be an effective preoperative treatment that allows subsequent curative surgery for locally advanced borderline-resectable T4 esophageal cancer. However, it is still controversial whether preoperative chemotherapy or chemoradiotherapy should be performed. Our observations should be confirmed by longer follow-up and larger sample size. Therapeutic strategies for controlling distant metastasis, as well as locoregional lesions need additional consideration.

Conflict of Interest Statement

None declared.

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BRAF mutation is a powerful prognostic factor in advanced and recurrent colorectal cancer

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BACKGROUND: Activating mutation of *KRAS* and *BRAF* are focused on as potential prognostic and predictive biomarkers in patients with colorectal cancer (CRC) treated with anti-EGFR therapies. This study investigated the clinicopathological features and prognostic impact of *KRAS/BRAF* mutation in advanced and recurrent CRC patients.

METHOD: Patients with advanced and recurrent CRC treated with systemic chemotherapy ($n=229$) were analysed for *KRAS/BRAF* genotypes by cycleave PCR. Prognostic factors associated with survival were identified by univariate and multivariate analyses using the Cox proportional hazards model.

RESULTS: *KRAS* and *BRAF* mutations were present in 34.5% and 6.5% of patients, respectively. *BRAF* mutated tumours were more likely to develop on the right of the colon, and to be of the poorly differentiated adenocarcinoma or mucinous carcinoma, and peritoneal metastasis. The median overall survival (OS) for *BRAF* mutation-positive and *KRAS 13* mutation-positive patients was 11.0 and 27.7 months, respectively, which was significantly worse than that for patients with wild-type (wt) *KRAS* and *BRAF* (40.6 months) (*BRAF*; HR = 4.25, $P<0.001$, *KRAS13*; HR = 2.03, $P=0.024$). After adjustment for significant features by multivariate Cox regression analysis, *BRAF* mutation was associated with poor OS (HR = 4.23, $P=0.019$).

CONCLUSION: Presence of mutated *BRAF* is one of the most powerful prognostic factors for advanced and recurrent CRC. The *KRAS13* mutation showed a trend towards poor OS in patients with advanced and recurrent CRC.

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Keywords: *BRAF*; *KRAS*; prognostic marker; colorectal cancer; chemotherapy

Although the epidermal growth factor receptor (EGFR) has important roles in cell differentiation and proliferation in normal cells, activation of EGFR signalling is frequently observed in colorectal cancer (CRC) cells, resulting in cell proliferation, migration and metastasis, evasion of apoptosis, or angiogenesis (Fang and Richardson, 2005). Indeed, ~35% of CRC tissues carry a mutation in codons 12 or 13 of *KRAS* that leads to the constitutive activation of downstream pathways, including the Ras/Raf/MAP/MEK/ERK and/or PTEN/PI3K/Akt pathways (Kinzler and Vogelstein, 1999; Wan *et al*, 2004; Benvenuti *et al*, 2007; Di Nicolantonio *et al*, 2008; Souglakos *et al*, 2009). *BRAF* is a downstream molecule of *KRAS*. Although more than 40 somatic mutations in the *BRAF* kinase domain have been described, the most common mutation across various cancers is the classic GTG→GAG substitution at the position 1799 of exon 15, which results in the V600E amino acid change, and the subsequent constitutive activation of the EGFR signalling pathway. Recent studies from Western countries have suggested that *BRAF* mutations occur in 10–20% of patients with sporadic disease (Jass, 2007; Benvenuti *et al*, 2007; Di Nicolantonio *et al*, 2008; Souglakos *et al*, 2009; Fariña-Sarasqueta *et al*, 2010), whereas other reports have revealed that tumours harbouring *BRAF* mutations have different clinical and histopathological features compared with tumours that harbour *KRAS* mutations (Kim *et al*, 2006; Deng *et al*, 2008; Zlobec *et al*, 2010). However, the frequency and clinicopathological features of *KRAS/BRAF* mutation in Japanese CRC patients remain unknown.

Information on *KRAS/BRAF* genotype is extremely useful in systemic chemotherapy for advanced and recurrent CRC patients, not just for predicting the therapeutic efficiency of anti-EGFR therapy, but also for identifying patients with poor prognoses. Therefore, both *KRAS* and *BRAF* are currently being focused on as potential prognostic and predictive biomarkers in patients with metastatic disease treated with anti-EGFR therapies, such as panitumumab and cetuximab (Karapetis *et al*, 2008; Bokemeyer *et al*, 2009; Tol *et al*, 2009; Van Cutsem *et al*, 2009). A number of retrospective analyses have revealed that patients with *KRAS* mutations do not benefit from cetuximab treatment, suggesting that *KRAS* genotype is a useful predictive marker for cetuximab therapy in CRC (Karapetis *et al*, 2008; Bokemeyer *et al*, 2009; Van Cutsem *et al*, 2009). It has also been reported that wild-type (wt) *BRAF* is required for a successful response to panitumumab or cetuximab therapies in metastatic CRC (Di Nicolantonio *et al*, 2008; Laurent-Puig *et al*, 2009; Souglakos *et al*, 2009; De Roock

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et al, 2010). In contrast, the prognostic relevance of *KRAS* genotype in CRC has been controversial despite a number of multi-institutional investigations dating from the 1990s (Andreyev *et al*, 1998; French *et al*, 2008; Kakar *et al*, 2008; Ogino *et al*, 2009; Roth *et al*, 2010). Although few studies have investigated the impact of *KRAS12* and *KRAS13* mutations on CRC prognosis, a series of recent studies have highlighted the potential adverse prognostic impact of *BRAF* mutations, using both patients with stage II and III disease and patients across all disease stages (Ogino *et al*, 2009; Fariña-Sarasqueta *et al*, 2010). Although Tol *et al* (2009) analysed *BRAF* genotypes in 520 metastatic CRC patients, all the patients were treated with chemotherapy plus bevacizumab with or without cetuximab. Furthermore, *BRAF* genotypes were analysed in a large subgroup of 845 metastatic CRC treated with FOLFIRI and FOLFOX chemotherapy with or without cetuximab as the first-line treatment in the CRYSTAL and OPUS studies, respectively (Bokemeyer *et al*, 2010). Thus, although the prognostic value of *BRAF* has been analysed in CRC patients treated with specific chemotherapy regimens, it remains unclear what impact the *KRAS12, KRAS13,* and *BRAF* mutations have on clinical outcomes of all patients with advanced or recurrent CRC treated with systemic treatments.

We have previously introduced the cycleave PCR technique as applicable to the routine screening of *KRAS/BRAF* mutations in CRC from pathological specimens, such as surgical and biopsy specimens (Yokota *et al*, 2010). Cycleave PCR utilises chimeric DNA-RNA-DNA probes labelled with a fluorescent dye and quencher, and the accuracy of cycleave PCR in detecting *KRAS/BRAF* mutations has been confirmed by assessment of the concordance between cycleave PCR and reverse transcriptase PCR-coupled direct sequencing (Yatabe *et al*, 2006; Yokota *et al*, 2010).

The aim of this study was to evaluate the *KRAS/BRAF* genotypes of advanced and recurrent CRC patients and to assess the effects of these genotypes on clinical outcome. To this end, we analysed the frequencies of the *KRAS12, KRAS13* and *BRAF* mutations, and correlated these results with the clinicopathological features of 229 Japanese CRC patients.

PATIENTS AND METHODS

Patients and tissues

Analysis of the genes encoding *KRAS* and *BRAF* was performed on surgically resected or biopsied specimens from CRC patients at our institution from 2002 to 2010. Hematoxylin and eosin (H and E)-stained slides were retrospectively collected and histologic subtypes were reviewed by an experienced gastrointestinal pathologist. Clinicopathological and survival analyses were subsequently performed on all patients with advanced and recurrent CRC who underwent systemic chemotherapy. Clinical data, including patient age at diagnosis, tumour location, and metastatic sites, were retrieved from patient records. Right-sided cancers included tumours from the caecum to transverse colon, left-sided included tumours from the splenic flexure to the rectosigmoid junction. Specimens used for *KRAS/BRAF* genotyping were either frozen or paraffin embedded tissues. For the *KRAS/BRAF* genotyping, appropriate approvals were obtained from the institutional review committee and written informed consent was obtained from all patients.

DNA extraction

DNA was extracted from surgical or biopsy specimens. Briefly, tumour cell-rich areas in H and E-stained sections were marked under a microscope, and tissues scratched from the same areas were sequentially deparaffinised and unstained. Recovered tissues were incubated in 1X PCR buffer containing 100 $\mu g\,ml^{-1}$ proteinase K for 1 h at 54 °C. After heat inactivation at 95 °C for 3 min, samples were used directly as template DNA for PCR assay.

KRAS/BRAF genotyping by cycleave PCR

To detect point mutations at *KRAS* codons 12, 13 and 61, we used the cycleave PCR technique (Yatabe *et al*, 2006; Sakamoto *et al*, 2007; Yokota *et al*, 2010). Each chimeric DNA-RNA-DNA probe was labelled with a fluorescent dye and quencher at each end that targeted the G12D, G12V, G12R, G12C, G12S, or G12A mutations in codon 12, the G13D or G13C mutations in codon 13, or the G61H, G61L, G61E, or G61K mutations in codon 61 of *KRAS*. We also designed probes that targeted the V600E mutation in *BRAF*. The PCR reactions were performed using a cycleave PCR core kit (TAKARA, Co. Ltd, Ohtsu, Japan). Fluorescent signals were quantified using the Smart Cycler system (SC-100; Cepheid, Sunnyvale, CA, USA).

Statistical analysis

The χ^2, Fischer's exact tests and Student's *t*-tests were used to analyse the relationship between variables using SYSTAT software (SYSTAT Software Inc., Richmond, CA, USA). The *KRAS* wt/*BRAF* wt (wild/wild), *KRAS12* mutant (G12X), *KRAS13* mutant (G13X), and *BRAF* mutant (V600E) groups were analysed separately. Overall survival (OS) was calculated from the starting date of the first-line chemotherapy until death from any cause, or censored at last follow-up visit. Survival data were analysed using the Kaplan–Meier product-limit method. Comparison of survival curves was carried out using the log-rank test. We first performed a univariate comparison of survival functions for factors that could potentially affect the survival time using the log-rank test, and then a multivariate analysis using the Cox proportional hazards model. *P*-values <0.05 were considered statistically significant, and all *P*-values represent two-sided significance tests.

RESULTS

Frequency of *KRAS* and *BRAF* gene mutations in CRC patients

According to our previous investigation on the spectrum of *KRAS* genotypes in our database of CRC cases, the most frequent mutations at *KRAS* codon 12 were the G12D, G12V, G12R, G12C, G12S and G12A mutations, which accounted for more than 95% of the codon 12 mutations. Similarly, the G13D and G13C mutations at codon 13, and the G61H, G61L, G61E, and G61K mutations at codon 61 were also found to be the most common at each site (Yokota *et al*, 2010). All the *KRAS* mutations we located have been previously described as oncogenically active and were present in the COSMIC (catalogue of somatic mutations in cancer) database (Sanger Institute, Cambridge, UK). Therefore, a series of specific probes targeting the common mutations in *KRAS* codons 12, 13 and 61 were designed for subsequent analysis of *KRAS* mutation frequency in our population of CRC patients. Because the most common mutation in *BRAF* is a valine to glutamate transition at position 600 of the protein (V600E), we designed probes targeting the V600E mutation in *BRAF*.

We initially analysed the *KRAS* genotypes of 349 CRC patients at our institution for which pathological specimens were available by cycleave PCR. The *KRAS* mutations were present in 35.7% ($n=126$) of patients tested, including 24.4% ($n=86$) that exhibited codon 12 mutations and 11.3% ($n=40$) that exhibited codon 13 mutations. However, only 4.7% ($n=15$) of the patients tested were positive for the *BRAF* V600E mutation ($n=319$). None of the *KRAS*-mutated samples carried a concomitant *BRAF* mutation. Approximately 2–3% of the surgical specimens could

Table 1 Spectrum of *KRAS/BRAF* mutations in CRC

	KRAS			
BRAF	**Wild type**	**G12**	**G13**	**61**
Wild type	135	53	26	0
V600E	15	0	0	0

Abbreviation: CRC = colorectal cancer. $n = 229$.

not be evaluated by cycleave PCR, probably due to over-fixation by formalin, as we reported previously (Yokota *et al*, 2010).

For the subsequent clinicopathological and survival analysis, we picked out 229 patients with advanced and recurrent CRC for which we could access complete clinicopathological information. The *KRAS* mutations were present in 34.5% ($n = 79$) of advanced and recurrent CRC patients, including 23.1% ($n = 53$) with codon 12 mutations and 11.4% ($n = 26$) with codon 13 mutations. The *BRAF* mutation was found in 6.6% ($n = 15$) of this population (Table 1).

Association of *BRAF/KRAS* mutations with clinicopathological features

We then correlated the *KRAS* and *BRAF* genotypes with clinicopathological features of CRC, including primary tumour location, histological findings, and sites of metastases. We categorised the population into four subtypes; those with wt *KRAS* and *BRAF* (wild/wild), *KRAS12* mutations (G12X), *KRAS13* mutations (G13X), and *BRAF* mutations (V600E).

For disease status, recurrent disease was more frequent in the *KRAS12* and *KRAS13* mutant groups than in the wild/wild group. There was no association between *KRAS/BRAF* genotype and age, gender or PS. Primary tumours were located at the rectum in almost half of the wild/wild and G12X populations. However, right-side tumour location was more frequent (60%) in patients with *BRAF* mutation in all subtypes ($P = 0.0391$) (Table 2). Furthermore, 46.2% (12 out of 26) of the primary tumours with *KRAS13* mutations were located on the right side whereas the frequencies of right-side location were 20.7% (28 out of 135) and 26.4% (14 out of 53), for the wild/wild and G12X groups, respectively (Table 2). The *BRAF* and *KRAS13* mutations were present in 14.3% (9 out of 63) and 19.0% (17 out of 63) of right-sided CRC, respectively. These results suggested that the *BRAF* and *KRAS* codon 13 mutations were associated with a right-sided tumour location.

Analysis with respect to histology showed that the frequencies of poorly differentiated adenocarcinoma (por), mucinous carcinoma (muc) and signet-ring cell carcinoma (sig) were $<10.9\%$ in patients with wt *BRAF*, which supported previous reports that such histologies are rare in CRC (Ogino *et al*, 2006; Catalano *et al*, 2009). However, 60.0% (9 out of 15) of CRC cases with *BRAF* mutation were of the por or muc subtypes, although no signet-ring cell carcinomas were observed. The *BRAF* mutations were present in 36.0% (9 out of 25) of patients with por/muc histology. Furthermore, 60.0% (9 out of 15) of CRCs with *BRAF* mutation metastasised to the peritoneum, compared with $\sim 15\%$ of CRCs with other subtypes ($P = 0.0062$) (Table 2). However, Fisher's exact test indicated no statistically significant correlation between tumour histology and peritoneal metastasis in *BRAF* mutant patients. No other significant differences or trends in metastatic patterns with respect to *KRAS/BRAF* genotypes were observed.

Details of the first line chemotherapy regimens used are shown in Table 2. In all, 66.4% of patients were treated with oxaliplatin-based regimens, 14.4% with irinotecan-based regimens, and 19.2% with fluoropyrimidine-based chemotherapy without oxaliplatin or irinotecan. There were no significant differences in treatment regimens between *KRAS/BRAF* genotypes. A total of 86 (63.7%) patients with wild/wild tumours and five (33.3%) patients with *BRAF* mutation-positive tumours received anti-EGFR therapy, whereas few patients with *KRAS12* or *KRAS13* mutations received anti-EGFR therapy (1.9% and 3.8%, respectively).

Survival

The median OS for *BRAF* mutation-positive patients was 11.0 months, which was significantly worse than for patients with wt *KRAS* and *BRAF* (40.6 months) (HR = 4.25, 95% CI 2.08–8.67, $P < 0.001$; Figure 1). The median OS for all *KRAS* mutation-positive patients, including those with *KRAS12* or *KRAS13* mutations, was not statistically different to that of wt *KRAS* and *BRAF* patients (HR = 1.51, 95% CI 0.97–2.36, $P = 0.071$). However, if OS for *KRAS13* mutation-positive patients was analysed separately from *KRAS12* mutation-positive patients, then the median OS for *KRAS13* mutation-positive patients was significantly worse than that for wt *KRAS* and *BRAF* patients (27.7 months *vs* 40.6 months, HR = 2.03, 95% CI 1.10–3.74, $P = 0.024$; Figure 1). In contrast, the median OS for *KRAS12* mutation-positive patients was 38.8 months, similar to that for wt *KRAS* and *BRAF* patients (HR = 1.28, 95% CI 0.74–2.19, $P = 0.376$; Figure 1). Univariate analysis showed that two other variables were also significantly associated with poor survival, PS ECOG $\geqslant 2$ and gender (Table 3). *KRAS13* mutation was not statistically associated with poor survival by univariate analysis. This was because we compared OS for *KRAS13* mutation-positive patients with that for wt *KRAS13* patients, which included *KRAS12* and *BRAF* mutation-positive patients as well as wt *KRAS* and *BRAF* patients. The por/sig/muc histology and lung metastasis showed a trend towards poor OS ($P = 0.066$ and $P = 0.061$, respectively).

To correct for significant prognostic factors, a Cox proportional hazards model that included age, gender, PS, *KRAS* status, *BRAF* status, pathological finding, number of metastasis and metastatic sites, was used. As two variables, WBC and ALP, had missing data, they were not included in the multivariate analysis. *BRAF* mutation and PS ECOG $\geqslant 2$ were confirmed as poor prognostic factors. Specifically, the relative risk of death for patients with *BRAF* mutation was 4.23 (95% CI 1.76–10.2) compared with patients with wt *BRAF* tumours ($P = 0.001$) (Table 3). Multivariate analysis also found that por/sig/muc histology, age > 65, and liver metastasis were negative independent prognostic factors. However, *KRAS13* mutation was not found to be an independent prognostic factor.

DISCUSSION

In this study, we examined the incidence of *KRAS* and *BRAF* mutations in advanced and recurrent CRC patients, and clarified the relationship between *KRAS/BRAF* genotypes and clinicopathological features, including survival. Up to now, estimates of *KRAS* gene mutation frequency in metastatic CRCs have been based on selective clinical studies or drug admission trials with variable inclusion criteria. To our knowledge, the present report is the first to provide data on the frequency and type of *KRAS/BRAF* mutations from a large Japanese population of advanced and recurrent CRC patients tested in a routine setting.

Our results showed that *KRAS* mutation was observed in around 35% of CRC cases, which included 25% of patients with mutations at codon 12 and 10% of patients with mutations at codon 13. This observation agreed well with previous studies on selected cohorts that reported frequencies in the range of 30–42% (Table 1). The cycleave PCR technique was simultaneously applied to the detection of *BRAF* mutation, thought to be an adverse prognostic marker as well as a predictive marker for anti-EGFR therapy. Our analysis demonstrated that the *BRAF* V600E mutation was observed in $\sim 5\%$ of CRC patients, which appeared to be lower

Table 2 Association of *BRAF* and *KRAS* mutational status with clinicopathological features in colorectal cancer

KRAS/BRAF status	Wild/wild	KRAS mutant			BRAF mutant		
Clinicopathological features	$n=135$	G12X $n=53$	G13X $n=26$	Total (G12X+G13X) $n=79$	V600E $n=15$	*P-value	Overall $n=229$
Age at diagnosis (median)	62 (27–83)	62 (40–85)	68 (41–79)	63 (40–85)	62 (30–80)		
Gender							
Female	47 (34.8%)	27 (50.9%)	13 (50.0%)	40 (50.6%)	8 (53.3%)	0.1082	95
Male	88 (65.2%)	26 (49.1%)	13 (50.0%)	39 (49.4%)	7 (46.7%)		134
ECOG PS							
0–1	115 (85.2%)	46 (86.8%)	22 (84.6%)	68 (86.1%)	13 (86.7%)	0.7898	196
>2	9 (6.7%)	4 (7.5%)	3 (11.5%)	7 (8.9%)	2 (13.3%)		18
Unknown	11 (8.1%)	3 (5.7%)	1 (3.8%)	4 (5.1%)	0 (0.0%)		15
Tumour location							
Right sided	28 (20.7%)	14 (26.4%)	12 (46.2%)	26 (32.9%)	9 (60.0%)	0.0391	63
Left sided	41 (30.4%)	13 (24.5%)	3 (11.5%)	16 (20.3%)	3 (20.0%)		60
Rectum	64 (47.4%)	25 (47.2%)	11 (42.3%)	36 (45.6%)	3 (20.0%)		103
Other	2 (1.5%)	1 (1.9%)	0 (0.0%)	1 (1.3%)	0 (0.0%)		3
Disease status							
Advanced	82 (60.7%)	26 (49.1%)	11 (42.3%)	37 (46.8%)	9 (60.0%)	0.2269	128
Recurrence	53 (39.3%)	27 (50.9 %)	15 (57.7%)	42 (53.2%)	6 (40.0%)		101
Histological subtype							
Well	28 (20.7%)	8 (15.1%)	7 (26.9%)	15 (19.0%)	1 (6.7%)	<0.0001	44
Mod	91 (67.4%)	37 (69.8%)	18 (69.2%)	55 (69.6%)	5 (33.3%)		151
por/sig/muc	10 (7.4%)	5 (9.4%)	1 (3.8%)	6 (7.6%)	9 (60.0%)		25
Other	1 (0.7%)	0 (0.0%)	0 (0.0%)	0 (0%)	0 (0.0%)		1
Unknown	5 (3.7%)	3 (5.7%)	0 (0.0%)	3 (3.8%)	0 (0.0%)		8
Metastatic sites							
Liver	90 (66.7%)	31 (58.5%)	15 (57.7 %)	46 (58.2%)	10 (66.7%)	0.6595	146
Peritoneum	30 (22.2%)	11 (20.8%)	4 (15.4%)	15 (20.0%)	9 (60.0%)	0.0062	54
Lung	42 (31.1%)	21 (39.6%)	10 (38.5%)	31 (39.2%)	5 (33.3%)	0.6867	78
CNS	1 (0.7%)	0 (0.0%)	1 (3.8%)	1 (1.3%)	0 (0.0%)	0.3503	2
Bone	9 (6.7%)	3 (5.7%)	2 (7.7%)	5 (6.3%)	2 (13.3%)	0.7736	16
Number of metastatic sites							
>2	64 (47.4%)	23 (43.4%)	14 (53.8%)	37 (46.8%)	10 (66.7%)	0.4078	111
<1	71 (52.6%)	30 (56.6%)	12 (46.2%)	42 (53.2%)	5 (33.3%)		118
WBC							
WBC>10000	9 (6.7%)	4 (7.5%)	2 (7.7%)	6 (7.6%)	0 (0.0%)	0.7622	15
WNL	100 (74.1%)	38 (71.7%)	20 (76.9%)	58 (73.4%)	14 (93.3%)		172
Unknown	26 (19.3%)	11 (20.8%)	4 (15.4%)	15 (20.2%)	1 (6.7%)		42
ALP							
ALP>300	59 (43.7%)	18 (34.0%)	12 (46.2%)	30 (38.0%)	6 (40.0%)	0.6635	95
WNL	49 (36.3%)	24 (45.3%)	10 (38.5%)	34 (43.0%)	8 (53.3%)		91
Unknown	27 (20.0%)	11 (20.8%)	4 (15.4%)	15 (20.0%)	1 (6.7%)		43
First-line regimen							
IRI-based	24 (17.8%)	6 (11.3%)	2 (7.7%)	8 (10.1%)	1 (6.7%)	0.4062	33
OXA-based	85 (63.0%)	37 (69.8%)	17 (65.4%)	54 (68.4%)	13 (86.7%)		152
Others	26 (19.3%)	10 (18.9%)	7 (26.9%)	17 (21.5%)	1 (6.7%)		44
Anti-EGFR treatment							
Yes	86 (63.7%)	1 (1.9%)	1 (3.8%)	2 (2.5%)	5 (33.3%)	<0.0001	93
No	44 (32.6%)	52 (98.1%)	25 (96.2%)	77 (97.5%)	10 (66.7%)		131
Unknown	5 (3.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)		5

Abbreviations: CNS = central nervous system; ECOG = Eastern Cooperative Oncology Group; EGFR = epidermal growth factor receptor; PS = performance status; well = well-differentiated adenocarcinoma; mod = moderately differentiated adenocarcinoma; por = poorly differentiated adenocarcinoma; muc = mucinous carcinoma; sig = signet-ring cell carcinoma; CNS = central nervous system; IRI = irinotecan; OXA = oxaliplatin, ALP = alkaline phosphatase; WNL = within normal range; WBC = white blood cells. Patients with both wild-type *KRAS* and wild-type *BRAF* were designated as wild/wild. All patients with *KRAS* mutations ($n=79$) either in codon 12 (G12X) or in codon 13 (G13X) are shown as total (G12X+G13X). *P-values calculated between wild-type *KRAS* and *BRAF* (wild/wild), *KRAS12* mutant (G12X), *KRAS13* mutant (G13X), and *BRAF* mutant (V600E) groups.

than that previously reported from Western countries. None of the CRC patients in our study carried both *KRAS* and *BRAF* mutations, supporting the hypothesis that *KRAS* and *BRAF* mutations occur in a mutually exclusive manner (Rajagopalan *et al*, 2002; Frattini *et al*, 2004; Ahlquist *et al*, 2008). One possible explanation for the comparatively low frequency of *BRAF* mutation might be the

different ethnic group. Indeed, several studies have reported that the mutation rates of DNA mismatch repair (MMR) genes, such as *hMSH2* and *hMLH1*, in hereditary non-polyposis colorectal cancer, is variable between countries. Therefore, geographical variation may account for differences in the mutation spectrum of *BRAF*, as observed for MMR genes (Wei *et al*, 2003; Lee *et al*, 2005; Goldberg *et al*, 2008).

We also investigated the clinicopathological characteristics of CRC patients with respect to *KRAS12*, *KRAS13* and *BRAF* mutations. In accordance with previous reports (Kim *et al*, 2006; Deng *et al*, 2008; Zlobec *et al*, 2010), *BRAF* mutation occurred more frequently in right-sided tumour locations. We also found that 60.0% of the *BRAF* mutation-positive specimens were of the poorly differentiated adenocarcinoma or mucinous carcinoma subtypes. It was recently reported that mucinous histology predicts a poor response to oxaliplatin- and/or irinotecan-based chemotherapies and is correlated with poor OS (Catalano *et al*, 2009). As *BRAF* mutation was more frequent in mucinous groups than non-mucinous carcinoma, as demonstrated by the present study and others (Ogino *et al*, 2006), the poor prognosis associated with mucinous histology may be at least partially explained by *BRAF* gene mutation. These specific clinicopathological features support the hypothesis that the *BRAF* mutation-mediated carcinogenesis in CRC is initiated by altered *BRAF* function as an early step in the serrated pathway (Bennecke *et al*, 2010), leading to activation of RAF-MEK-ERK-MAP signalling.

In contrast to *BRAF* mutation, no significant differences in clinicopathological parameters were observed according to *KRAS* genotype. However, our analysis did suggest that *KRAS13* mutations were also associated with right-sided tumour location. This result raises the possibility that *KRAS13* may have a distinct phenotype from that of other *KRAS* genotypes.

Using a representative cohort of 229 sporadic CRCs, we identified the *BRAF* V600E mutation as an independent prognostic factor for survival in patients with advanced and recurrent CRC. The presence of the *BRAF* mutation is associated with a significantly higher risk of dying of cancer-related causes, independently of other factors such as age, gender, PS, *KRAS* status, pathological finding, number of metastasis and metastatic sites, in agreement with other recent studies (Ogino *et al*, 2009; Tol *et al*, 2009; Bokemeyer *et al*, 2010; Fariña-Sarasqueta *et al*, 2010). For example, analysis of stage II and stage III CRC patients (Fariña-Sarasqueta *et al*, 2010) was consistent with the finding that 44% of our population included recurrent disease. The *BRAF* mutation was correlated with survival in a heterogeneous group of CRC patients that included all disease stages (Ogino *et al*, 2009). Furthermore, a positive correlation between *BRAF* mutation and shorter survival was demonstrated in a homogeneous group of metastatic CRC patients treated with a specific chemotherapy regimen with or without cetuximab (Tol *et al*, 2009; Bokemeyer *et al*, 2010). However, our study focused on the advanced and recurrent group who received systemic chemotherapy, including fluoropyrimidines, in combination with oxaliplatin, irinotecan, bevacizumab and anti-EGFR antibody in several lines. Even though all of the patients in our study received systemic chemotherapy, a positive correlation between *BRAF* mutation and shorter survival was still demonstrated, independent of treatment arm.

The prognostic value of *KRAS* mutations in CRC remains controversial, even though *KRAS* mutations have been associated with a poor response to anti-EGFR antibody therapy in metastatic CRC (Karapetis *et al*, 2008; Bokemeyer *et al*, 2009; Van Cutsem *et al*, 2009). Despite a number of studies investigating a prognostic role for *KRAS* mutations, no definitive conclusions can be drawn (Castagnola and Giaretti, 2005). This may be due to differences

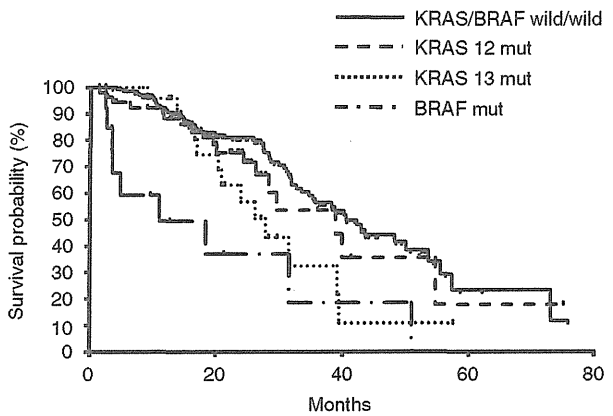

Figure 1 Kaplan–Meier plot showing overall survival in metastatic and recurrent colon cancer patients according to *KRAS* and *BRAF* V600E mutational status ($n = 229$). mut, mutated.

Table 3 Factors associated with overall survival in univariate and multivariate analyses

	Univariate analysis		Multivariate analysis	
Variable	**Hazard ratio (95% CI)**	***P*-value**	**Hazard ratio (95% CI)**	***P*-value**
Age >65	0.74 (0.48–1.13)	0.157	0.55 (0.34–0.90)	0.018
Female	1.59 (1.06–2.37)	0.025	1.35 (0.85–2.12)	0.201
PS (ECOG) ⩾2	6.14 (3.15–12.0)	<0.001	7.66 (3.68–16.0)	<0.001
BRAF mutant	3.78 (1.89–7.54)	<0.001	4.23 (1.76–10.2)	0.001
KRAS 12 mutant	1.03 (0.62–1.74)	0.897	1.57 (0.88–2.81)	0.128
KRAS 13 mutant	1.67 (0.93–3.02)	0.086	1.51 (0.76–2.98)	0.239
Pathology, por/sig/muc	1.74 (0.96–3.14)	0.066	2.38 (1.16–4.90)	0.018
Number of metastasis ⩾2	0.93 (0.63–1.40)	0.738	1.12 (0.61–2.05)	0.714
Liver metastasis	1.36 (0.88–2.11)	0.162	1.72 (1.02–2.90)	0.042
Lung metastasis	0.66 (0.42–1.02)	0.061	0.59 (0.32–1.11)	0.100
Peritoneal metastasis	1.21 (0.76–1.93)	0.417	1.56 (0.85–2.88)	0.154
WBC ⩾10000	1.27 (0.51–3.15)	0.605	—	—
ALP ⩾300	1.21 (0.78–1.88)	0.395	—	—
Anti-EGFR treatment	0.80 (0.53–1.20)	0.277	—	—

Abbreviations: ALP = alkaline phosphatase; PS = performance status; ECOG = Eastern Cooperative Oncology Group; EGFR = epidermal growth factor receptor; por = poorly differentiated adenocarcinoma; muc = mucinous carcinoma; sig = signet-ring cell carcinoma; CI = confidence interval; WBC = white blood cells.

between the studies in terms of study size, patient selection, tumour sampling, use of archival versus fresh/frozen material, or laboratory methods and data analyses. More importantly, few studies have differentiated *KRAS* mutations at codon 12 from those at codon 13 with respect to clinicopathological features and survival (Bazan *et al*, 2002). Our analysis revealed that mutation at *KRAS12* had no effect on patient OS. In contrast, our Kaplan–Meier curves clearly demonstrated that OS for patients with *KRAS13* mutations were significantly worse than for those who had wt *KRAS* and *BRAF*. It has been reported that stage III patients with *KRAS* mutations displayed significantly worse disease-free survival, as compared with those with wt *KRAS* (Fariña-Sarasqueta *et al*, 2010). This finding may be partially explained by the impact of *KRAS13* mutations on prognosis. As both univariate and multivariate analysis failed to confirm *KRAS13* mutation as an independent prognostic factor, the prognostic value of mutations at *KRAS13* remains unclear in advanced and recurrent CRC. In non-small-cell lung cancer there are differences in transforming potential and EGFR tyrosine kinase inhibitor sensitivity associated with EGFR somatic mutations L858R and deletion mutant Del (746-750) (Carey *et al*, 2006). Therefore, it remains a possibility that the different *KRAS* mutations at codons 12 and 13 may have different biological consequences that could influence the prognosis for CRC.

With respect to technical issue on *KRAS* and *BRAF* genotyping, we evaluated the prognostic value of the mutations frequently found in *KRAS* and *BRAF* using specific PCR probes. In contrast, direct sequencing is able to detect all possible *KRAS* and *BRAF* mutations including some more rare mutations. In fact, it is reported that *KRAS* codon 146 mutation, which was identified by direct sequencing, was associated with resistance to cetuximab plus irinotecan therapy although this is a minor oncogenic *KRAS* mutation (Loupakis *et al*, 2009). Therefore, direct sequencing may be able to obtain further insights into predictive and prognostic impact of these mutations.

Our study found that the median OS of patients with wt *BRAF* was generally longer than that observed in other reports. It could be argued that the selection of patients with good prognosis could bias the results in this study. Indeed, more than half of our study population was screened for *KRAS/BRAF* genotype to determine the use of anti-EGFR antibody, and 42% of the patients were treated with cetuximab combined therapy mostly as a second- or third-line chemotherapy. Although treatment selection may be a major reason for the longer survival observed in the present study as compared with previous studies involving metastatic CRC patients, univariate analysis revealed no significant differences in survival between patients with and without anti-EGFR therapy (38.8 months *vs* 32.6 months, $P=0.277$) (Table 3). Furthermore, almost all recurrent and advanced CRC patients are routinely screened for *KRAS/BRAF* genotype at the initiation of the first line chemotherapy in our institution since the use of cetuximab was approved for the treatment of CRC patients in Japan.

Another key point of discussion is the potential treatment bias in this retrospective analysis. The focus of the present study is the patient group with advanced and recurrent CRC who received systemic chemotherapy. However, we need to take the difference in the specific treatment regimen among four genotypes into consideration. In particular, 63.7% (86 out of 135) of wt *KRAS* and *BRAF* patients have received anti-EGFR therapy whereas 33.3% (6 out of 15) and 2.5% (2 out of 79) of patients with *BRAF* and *KRAS12/13* mutations have received anti-EGFR therapy, respectively. Therefore, the prognostic advantage of wt *KRAS* and *BRAF* patients over *BRAF* or *KRAS13* mutation might be partially explained by the presence of anti-EGFR therapy. Nevertheless, it is noteworthy that the prognosis of wt *KRAS* and *BRAF* patients was similar to that of the patients with *KRAS12* mutation despite the frequent use of anti-EGFR therapy.

In conclusion, our retrospective analysis demonstrated that *BRAF* mutation was an independent prognostic factor in advanced and recurrent CRC. Although the presence of *KRAS12* mutation had no apparent effect on OS in advanced and recurrent disease, the prognostic value of *KRAS13* mutation remains uncertain. Our results are useful not only for predicting the efficacy of anti-EGFR therapy, but also for identifying patients with shorter OS in response to systemic chemotherapy, regardless of the use of anti-EGFR therapy. The exact effects of *KRAS12* and *KRAS13* mutations on survival require further study. The application of novel strategies targeting *BRAF* kinase is warranted for the treatment of CRC patients with *BRAF* mutation.

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ORIGINAL ARTICLE

Meta-analysis of neutropenia or leukopenia as a prognostic factor in patients with malignant disease undergoing chemotherapy

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Abstract
Purpose We performed a systematic review and meta-analysis to determine the impact of neutropenia or leukopenia experienced during chemotherapy on survival.
Methods Eligible studies included prospective or retrospective analyses that evaluated neutropenia or leukopenia as a prognostic factor for overall survival or disease-free survival. Statistical analyses were conducted to calculate a summary hazard ratio and 95% confidence interval (CI) using random-effects or fixed-effects models based on the heterogeneity of the included studies.
Results Thirteen trials were selected for the meta-analysis, with a total of 9,528 patients. The hazard ratio of death was 0.69 (95% CI, 0.64–0.75) for patients with higher-grade neutropenia or leukopenia compared to patients with lower-grade or lack of cytopenia. Our analysis was also stratified by statistical method (any statistical method to decrease lead-time bias; time-varying analysis or landmark analysis), but no differences were observed.
Conclusions Our results indicate that neutropenia or leukopenia experienced during chemotherapy is associated with improved survival in patients with advanced cancer or hematological malignancies undergoing chemotherapy. Future prospective analyses designed to investigate the potential impact of chemotherapy dose adjustment coupled with monitoring of neutropenia or leukopenia on survival are warranted.

Keywords Chemotherapy · Neutropenia · Leukopenia · Prognostic factor · Meta-analysis

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Introduction

Neutropenia or leukopenia induced by cytotoxic chemotherapy is a common adverse event in patients with cancer. In general, the recommended doses of cytotoxic agents are determined in dose-finding phase I studies. However, sample sizes in phase I studies are not large enough to examine individual differences in drug metabolism; therefore, toxicity profiles are likely to be highly variable [1]. In other words, the determined standard dose may be conservatively low for some patients with faster drug elimination times [1]. In support of this hypothesis, toxicities such as neutropenia or leukopenia experienced during chemotherapy have been reported to be associated with favorable clinical outcomes in several cancer types. Recently, we analyzed the neutropenia that occurs during first-line FOLFOX (infusional 5-fluorouracil/leucovorin and oxaliplatin) chemotherapy in patients with advanced colorectal cancer [2] or during second-line chemotherapy with weekly paclitaxel in patients with advanced gastric cancer [3], using time-varying covariate (TVC) analysis. Since several studies, including ours, have primarily been retrospective analyses that lacked a statistically testable hypothesis, we conducted the present meta-analysis to evaluate the prognostic impact of neutropenia or leukopenia on patients with advanced cancer undergoing chemotherapy with a statistical power much higher than that of each individual trial.

Patients and methods

Selection of studies

This study was performed to assess whether neutropenia or leukopenia has an important effect upon survival in patients with cancer undergoing chemotherapy. A systematic review and meta-analysis of published articles were performed. Two authors (KS and KM) conducted a literature search for trials through computer-based searches of the Medline database (January 1966 and May 20, 2010) and of abstracts from conference proceedings of the American Society of Clinical Oncology (1995–2010) and European Society for Medical Oncology (1995–2009).

Search keywords included "neutropenia", "leukopenia", "prognostic", and "chemotherapy". The search was also guided by a thorough examination of reference lists of original and review articles. No limitation based on language was defined. We included abstracts or unpublished data if sufficient information on study design, characteristics of participants, interventions, and outcomes was available.

Procedures

Two investigators (KS and KM) abstracted data, according to Quality of Reporting of Meta-analyses (QUORUM) guidelines. Each study was assessed for quality and potential bias using a structured checklist based on the Method for Evaluating Research and Guideline Evidence criteria [4]. Studies that met the following criteria were analyzed: patients with malignant disease treated with chemotherapy; prospective and retrospective analyses in randomized study or cohort study that evaluated neutropenia or leukopenia as a prognostic factor; and attainment of hazard ratio (HR) with 95% confidence interval (CI). Adverse events were assessed and recorded according to the National Cancer Institute's Common Toxicity Criteria (NCI-CTC; version 2 or 3), which have been adopted widely in cancer clinical trials, in as many cases as possible. For each study, the following information was extracted: first author's name; year of publication; study design (prospective or retrospective); number of enrolled patients; underlying malignant disease; median age; treatment regimen(s); methods of analysis, including specific analysis to decrease lead-time bias (i.e., landmark analysis or TVC analysis); methods of comparison (i.e., grade 0 vs. grade 1–4, grade 0–2 vs. grade 3–4, or mild vs. moderate); and HR and 95% CI for clinical outcome (overall survival or disease-free survival).

Statistical methods

For each study, a HR (and 95% CI) was derived according to neutropenia or leukopenia. If HRs according to both univariate and multivariate analysis were reported, HR in multivariate analysis was used in this analysis. To estimate a summary HR for death for patients with neutropenia or leukopenia, patients with lower-grade (grade 0, grade 0–2, or lowest tertile) versus higher-grade neutropenia or leukopenia were compared, since the cut-off values used to divide neutropenia or leukopenia into low versus high grades differed between studies. Some trials used tertiles without using NCI-CTC grades. For meta-analyses, both the fixed-effects model (weighted with inverse variance) and the random-effects model were used. Statistical heterogeneity among studies with the Q statistic was assessed, and inconsistency was quantified with the I^2 statistic. The assumption of heterogeneity was judged as invalid if $P < 0.1$. To investigate possible reasons for heterogeneity, subgroup analyses were performed by disease type or specific methods such as landmark analysis or TVC analysis, and meta-regression analyses were performed to test for variation in risk estimates by those variables. A cumulative meta-analysis was also performed. Publication bias was assessed by a funnel plot. Statistical analyses were performed using STATA ver. 10 (StataCorp LP, College Station, TX, USA). All tests were 2-sided, and P values less than 0.05 were considered statistically significant.

Results

Selection of studies

A total of 753 potentially relevant reports were identified, of which 688 were initially excluded (Fig. 1). After a review of the remaining publications, 13 trials with sufficient data were identified for this meta-analysis, with a total of 9,528 patients [2, 3, 5–15]. Table 1 shows the baseline characteristics of patients from each trial. Malignant diseases included non-small cell lung cancer in three reports, breast cancer in three reports, gastric cancer in two reports, and colorectal cancer, uterine cervical cancer, ovarian cancer, esophageal cancer, and Hodgkin's lymphoma in one report each. Seven studies enrolled chemo-naive patients, one included pretreated patients, two evaluated chemotherapy in the adjuvant setting, and two assessed chemoradiotherapy for locally advanced disease. All studies used multivariate analysis to calculate HRs, and pretreatment neutrophil counts or leukocyte counts were included in five studies. Five studies used specific analysis methodology (landmark analysis in two and TVC analysis in three). Ten studies evaluated neutropenia, and three evaluated leukopenia. Six studies compared prognosis of patients without neutropenia or leukopenia to that of patients that experienced these cytopenias. Four studies compared patients

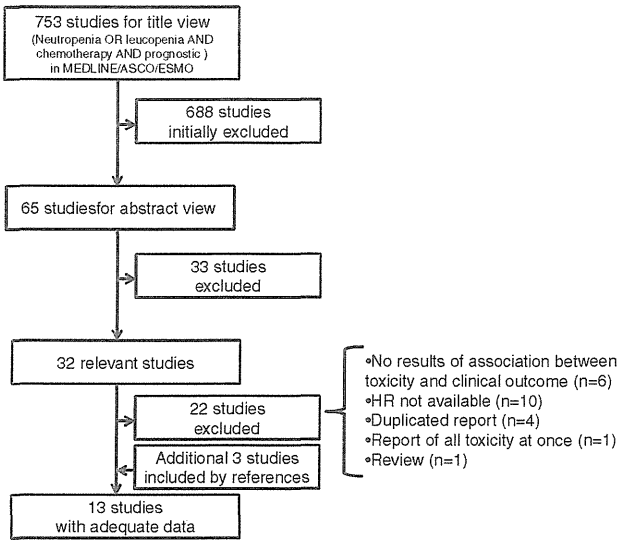

Fig. 1 Selection process for studies

with grade 0–2 versus grade 3–4 neutropenia. Two studies divided patients by tertile.

Survival analyses for neutropenia and leukopenia

The results of the meta-analysis revealed a combined estimate HR of 0.69 (95% CI, 0.64–0.75) (random-effects model) and 0.70 (95% CI, 0.65–0.75) (fixed-effects model). No apparent evidence for heterogeneity between these studies was detected ($P = 0.124$). A forest plot (Fig. 2) of the random-effects model analysis showed that eleven studies provided relatively similar HRs favoring higher-grade neutropenia or leukopenia, whereas the Kim et al. [11] and Miyoshi et al. [13] studies did not. The present analysis was also stratified by underlying disease (solid tumor in metastatic setting or solid tumor in adjuvant setting or hematologic malignancy; $P = 0.52$, Fig. 3), variable (neutropenia or leukopenia; $P = 0.55$), statistical method (landmark analysis or TVC analysis vs. without these methods; $P = 0.39$), and quality of report (low vs. high; $P = 0.46$); however, no differences were observed. Funnel plots showed that the possibility of bias is low (Fig. 4).

Discussion

We conducted the first meta-analysis to answer the question of whether patients with a higher grade of neutropenia or leukopenia during chemotherapy experienced superior survival compared to patients with lower-grade neutropenia or leukopenia. We found an approximately 30% risk reduction in mortality for patients with higher-grade cytopenias. Patients cannot be randomized to experience cytopenia or not, and so the only practical method of assessing the effect is by observational studies. These have a higher risk of bias than randomized trials, and so their results must be interpreted with caution, but well-conducted meta-analysis may reduce this risk. A lack of an obvious source of heterogeneity may support the consistency of our findings across heterogeneous methods of analysis, sites of malignancy, and clinical settings.

Based on our observation that patients who experience higher-grade neutropenia or leukopenia during chemotherapy have a better prognosis, we speculate that neutropenia, an indication of bone marrow suppression caused by a particular dose of a chemotherapeutic agent, may also be a surrogate marker that indicates that the same dose is adequate to provide an antitumor effect. Thus, lack of neutropenia or leukopenia may indicate a weak or absent biological effect by chemotherapy, which could possibly be caused by underdosing in an individual patient. Such underdosing may at least partly be the consequence of the methodology of phase I clinical trials in which the maximum tolerated dose (MTD) is selected according to body surface area (BSA) [7, 16]. Several studies have indicated that the pharmacokinetics of several cytotoxic drugs is poorly correlated with BSA due to inter-patient variability in metabolism (e.g., variability in enzymatic activity, genetic polymorphisms) [17–19]. If this inter-patient variability in pharmacokinetics is indeed a cause of underdosing, dose adjustment (increased or reduced) based on observed toxicity may be a possible solution. For example, dose increases of the epidermal growth factor receptor (EGFR) monoclonal antibody cetuximab in the absence of skin toxicity have been shown to result in an improved objective response in patients with colorectal cancer [20].

Several other possible explanations in addition to chemotherapy dose may support the present findings. The first is the potential relationship between pretreatment neutrophil or leukocyte count and vulnerability to cytopenia during chemotherapy. Several reports have indicated that patients with high neutrophil or leukocyte counts prior to treatment might have a poor prognosis and be less likely to experience cytopenia during treatment [16, 21, 22]. However, our previous two studies [2, 3] and three other studies [8, 9, 12] included pretreatment neutrophil counts or leukocyte counts as adjusted factors, and these studies demonstrated that neutropenia or leukopenia experienced during chemotherapy was independently associated with prognosis. Therefore, this explanation is less likely to account for the findings of this meta-analysis.

Another possible explanation is that the association between cytopenia and prognosis is the result of bias introduced by the different analytical methods used in different studies. Since neutropenia does not exist prior to the initiation of chemotherapy, a false association between neutropenia and patient outcome might have been observed due to a

Table 1 Baseline characteristics of patients of the 13 included trials

Primary author	Year	Study type	Analysis	Disease	*n*	Setting	Treatment	Variable	Endpoint
Saarto [5]	1997	Prospective	MA	Breast	193	Adjuvant	AC, 5-FU	Leukopenia	DFS
Poikonen [6]	1999	Retrospective	MA	Breast	368	Adjuvant	CMF	Leukopenia	DFS
Di Maio [7]	2005	Prospective	MA with landmark[b]	NSCLC	1,265	Metastatic (1st-line)	GEM or VNR combinations	Neutropenia	OS
Klimm [8]	2005	Prospective	MA[a]	Hodgkin's lymphoma	4,626	1st-line	COPP/ABVD, BEACOPP ± RT	Leukopenia	FFTF
Yamanaka [9]	2007	Retrospective	MA[a] with TVC	Gastric	1,055	Metastatic (1st-line)	S-1	Neutropenia	OS
Pallis [10]	2008	Prospective	MA	NSCLC	858	Metastatic (1st-line)	GEM + DOC	Neutropenia	OS
Kim [11]	2009	Retrospective	MA	Cervical	107	Adjuvant	PTX + CBDCA + RT	Neutropenia	DFS
Kishida [12]	2009	Prospective	MA[a] with landmark	NSCLC	337	Metastatic (1st-line)	VNR + GEM followed by DOC vs. PTX + CBDCA	Neutropenia	OS
Miyoshi [13]	2009	Retrospective	MA	Esophageal	42	Preoperative	FP/FAP + RT	Leukopenia	OS
Shitara [2]	2009	Retrospective	MA[a] with TVC	Colorectal	153	Metastatic (1st-line)	FOLFOX ± BV	Neutropenia	OS
Kim [14]	2010	Retrospective	MA	Ovarian	179	Metastatic (1st-line)	PTX + CBDCA	Neutropenia	OS
Ishitobi [15]	2010	Retrospective	MA	Breast	103	Neoadjuvant	Epirubicin combination	Neutropenia	DFS
Shitara [3]	2010	Retrospective	MA[a] with TVC[c]	Gastric	242	Metastatic (2nd-line)	Weekly PTX	Neutropenia	OS

MA multivariate analysis, *TVC* time-varying covariate analysis, *NSCLC* non-small cell lung cancer, *AC* doxorubicin + cyclophosphamide, *5-FU* 5-fluorouracil, *CMF* cyclophosphamide + methotrexate + 5-fluorouracil, *C-MOPP* cyclophosphamide + vincristine + procarbazine + prednisone, *ABVD* adriamycin + bleomycin + vinblastine + dacarbazine, *BEACOPP* bleomycin + etoposide + doxorubicin + cyclophosphamide + vincristine + procarbazine + prednisolone, *RT* radiotherapy, *VNR* vinorelbine, *GEM* gemcitabine, *DOC* docetaxel, *PTX* paclitaxel, *CBDCA* carboplatin, *DFS* disease-free survival, *OS* overall survival, *FFTF* freedom from treatment failure

[a] Pretreatment neutrophil counts or leukocyte counts were included

[b] Landmark analysis in 436 patients and out of landmark analysis in 829 patients

[c] TVC with landmark analysis in 202 patients

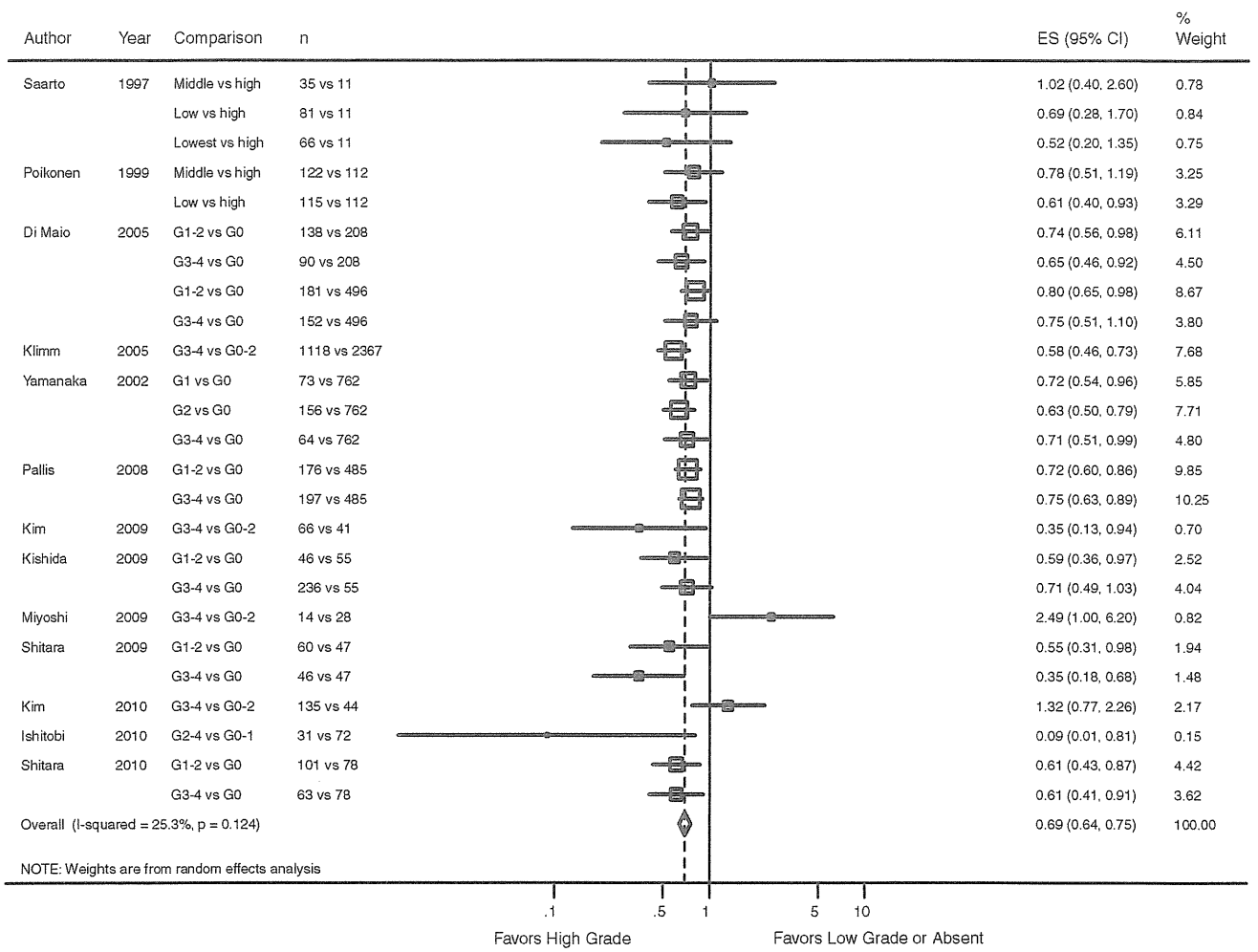

Fig. 2 Forest plots of hazard ratios. The size of the *gray markers* (*squares*) corresponds to the weight of the study in the meta-analysis. Combined hazard ratio was calculated using the random-effects model

higher incidence of neutropenia with increasing cycles of chemotherapy in patients with a better prognosis (lead-time bias). Therefore, some studies, including our previous studies, used landmark analysis and/or TVC analysis to decrease lead-time bias as much as possible. However, the present meta-analysis revealed the limited impact of survival analysis methods as shown by lack of significant heterogeneity. In our two previous studies in colorectal cancer [2] and gastric cancer [3], the majority of patients with neutropenia experienced their highest grade within 4 weeks of initiating treatment, and those who did not experience neutropenia during the first 4 weeks rarely experienced severe late-onset neutropenia. These observations support the possibility that false-positive association by lead-time bias is low and indicate that the impact of landmark analysis and/or TVC analysis is not high, as shown in this meta-analysis. The impact of neutropenia was shown in this study despite the treatment bias by severe neutropenia, which might reduce the effect of treatment by dose reduction or delay. Although the use of G-CSF was not evaluated in detail in each study, the possibility that G-CSF itself prolonged the survival of patients with neutropenia might be low.

This study has several methodological issues. Although the sample size was considered to be sufficient, the disease types and study settings were variable. Therefore, it is difficult to completely rule out potential heterogeneity across disease types. Second, the evaluation of neutropenia or leukopenia was performed differently in different studies; however, a lack of obvious heterogeneity among the results of different studies suggests this had little, if any, impact. Third, although most studies calculated HR using multivariate analysis, the variables used in multivariate analysis could have been insufficient. Fourth, although the funnel plot of our study suggested publication bias was low, there might be we did comprehensive literature search, the studies that failed to show an association between lack of neutropenia and outcome are less likely to have been published; therefore, this might have led to an exaggeration

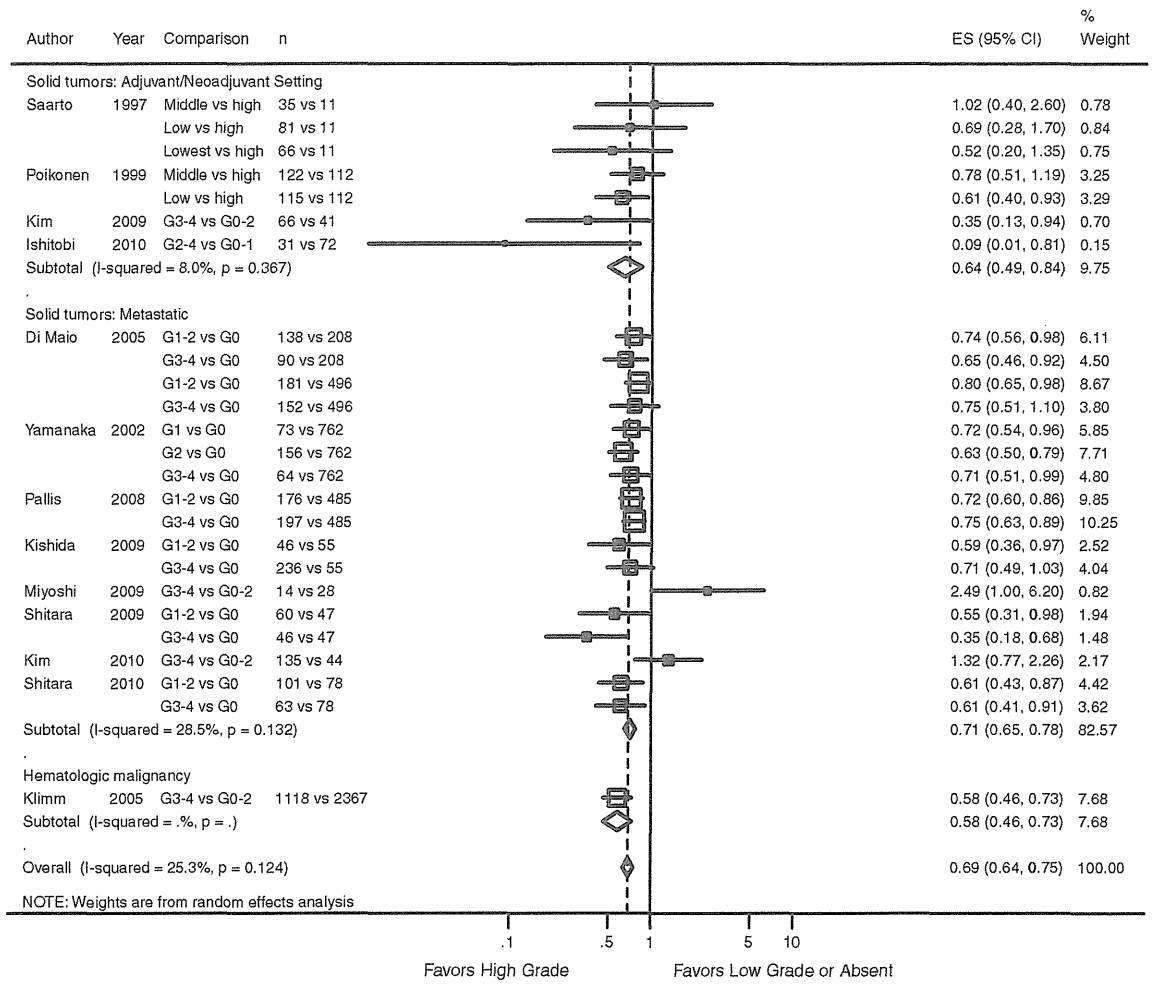

Fig. 3 Subset-analysis according to disease type

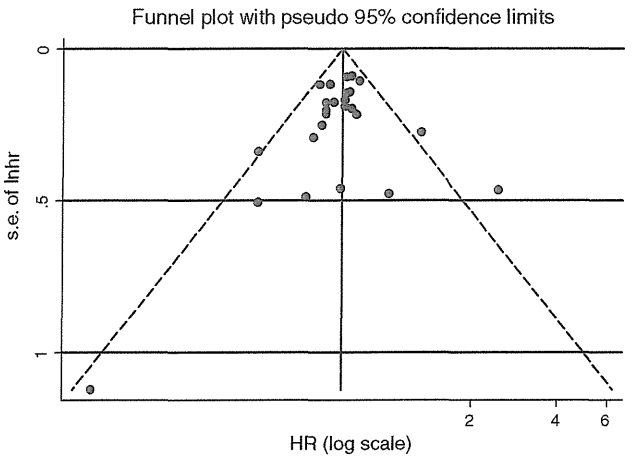

Fig. 4 Funnel plot of included studies

of the purported benefit in this meta-analysis. Ideally, an individual data-based meta-analysis might clarify this issue. Further study is warranted.

In conclusion, this meta-analysis indicated that neutropenia or leukopenia occurring during chemotherapy in patients with solid tumors or hematological malignancies is strongly associated with better prognosis. This suggests that neutropenia or leukopenia could be utilized as a surrogate marker to determine adequate antitumor doses of chemotherapeutic agents. An additional well-defined prospective trial designed to evaluate dose escalation in patients without neutropenia or leukopenia during the early course of treatment is warranted. We are currently planning a dose-escalation study of weekly paclitaxel in patients with advanced gastric cancer based on incidence of neutropenia.

Conflict of interest None of the authors have financial or personal conflicts of interest to disclose.

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ORIGINAL ARTICLE

Fluoropyrimidine plus cisplatin for patients with advanced or recurrent gastric cancer with peritoneal metastasis

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Abstract
Background There are few data on the efficacy of combination chemotherapy with a fluoropyrimidine plus cisplatin for patients with advanced or recurrent gastric cancer (AGC) complicated by peritoneal metastasis, especially massive ascites.
Methods We retrospectively evaluated the efficacy and safety of a fluoropyrimidine (S-1 or capecitabine) plus cisplatin as first-line chemotherapy in 120 patients with AGC and peritoneal metastasis.
Results Ascites was detected in 50 patients, with 11 patients having massive ascites. Median progression-free survival (PFS) and overall survival (OS) of all patients was 6.1 and 15.9 months, respectively. The PFS and OS were shorter in patients with massive ascites ($n = 11$; 3.7 and 9.5 months) compared with patients with small or moderate ascites ($n = 39$; 5.8 and 13.5 months) or patients without ascites ($n = 70$; 6.9 and 18.1 months). The objective response in terms of ascites was similar whether ascites was massive (4 of 11 patients; 36.4%) or small or moderate (16 of 39 patients; 41%). The frequencies of grade 3 or higher toxicity or treatment discontinuation due to toxicity are relatively similar across ascites groups.
Conclusions Fluoropyrimidine plus cisplatin appears to be tolerated in selected patients with peritoneal metastasis.

Keywords Chemotherapy · Cisplatin · Fluoropyrimidine · Gastric cancer · Peritoneal metastasis

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Introduction

Gastric cancer is the fourth most common malignancy in the world (988,602 cases in 2008, 7.8% of all malignancies) and the second leading cause of cancer death (737,419 deaths, 9.7% of all cancer deaths) [1]. The prognosis for patients with advanced or recurrent gastric cancer (AGC) remains poor; chemotherapy confers only a minimal survival advantage, with a median overall survival (OS) of approximately 1 year. In a pivotal phase III trial (SPIRITS trial) in Japan that compared S-1 alone with S-1 plus cisplatin (combination = SP), patients treated with SP showed a significantly higher response rate (54 vs. 31%), longer progression-free survival (PFS; 6.0 vs. 4.0 months), and longer OS (13 vs. 11 months) than patients receiving S-1 alone [2]. Therefore, SP is now considered to be one of the standard regimens for AGC in Japan. Capecitabine, another oral fluoropyrimidine, when combined with cisplatin (combination = XP), is also reported to have an effectiveness that is statistically indistinguishable from that of 5-fluorouracil (5-FU) plus cisplatin (ML17032 trial [3]), which was used as a reference regimen in recent global studies, including those in Japan [4, 5]. Thus, the most commonly used treatments for AGC are combination

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chemotherapy regimens consisting of a fluoropyrimidine (5-FU or an oral fluoropyrimidine) plus a platinum agent, although docetaxel or anthracyclines are sometimes combined in Western countries [6, 7].

Peritoneal metastasis, a common type of metastasis in AGC, causes several complications such as ascites, bowel obstruction, and hydronephrosis—all leading to a deterioration of the patient's general condition. Several reports have suggested that the presence of peritoneal metastasis or ascites is associated with poor survival in patients with AGC [8–11]. To improve the prognosis for patients with AGC and peritoneal metastasis, several clinical trials have been conducted [12–18]. However, there are few data on the efficacy of a fluoropyrimidine plus cisplatin for peritoneal metastasis as the current standard treatment for patients with AGC. Moreover, since patients with massive ascites have usually been excluded in previous pivotal randomized studies, the efficacy and feasibility in this patient population is also unclear. Therefore, we retrospectively evaluated the efficacy and safety of a fluoropyrimidine plus cisplatin regimen in patients with AGC and peritoneal metastasis.

Patients and methods

Patients

This retrospective study was designed to evaluate the efficacy and safety of first-line chemotherapy with a fluoropyrimidine plus cisplatin (SP and XP) in patients with AGC from January 2005 to March 2011. Since capecitabine was not available in Japan until February 2011, most patients had been treated by SP, although we included patients who had been treated with XP in the context of two global studies [3, 4]. Patients who had received XP plus experimental agents (i.e., trastuzumab or bevacizumab) were excluded from our analysis.

Eligibility criteria were as follows: (1) presence of histologically proven, inoperable AGC; (2) Eastern Cooperative Oncology Group performance status (ECOG PS) 0–2; (3) sufficient oral intake to take oral agents; (4) adequate bone marrow, hepatic, and renal function; (5) diagnosis of peritoneal metastasis, which could be confirmed either by macroscopic evaluation (upon laparotomy or laparoscopy) with cytology or by imaging data [computed tomography (CT) scan or barium enema] with relevant signs such as ascites, hydronephrosis, and intestinal stenosis; (6) no previous chemotherapy other than adjuvant chemotherapy, which was required to have been finished more than 6 months before enrollment. Written informed consent for chemotherapy was obtained from each patient prior to treatment initiation.

Treatment plan

Patients were treated with either: (1) a standard regimen of SP [S-1 (80 mg/m^2) for 21 consecutive days followed by a 14-day rest; cisplatin (60 mg/m^2) intravenous infusion on day 8] with repetition of the 35-day cycle [2]; or (2) XP [capecitabine (1,000 mg/m^2) for 14 days followed by a 7-day rest; cisplatin (80 mg/m^2) intravenous infusion on day 1] with repetition of the 21-day cycle [4, 5]. Intravenous hydration (1,500 mL) was performed on the day of cisplatin administration and on the next 2 days. Dose modification and scheduling of the two regimens were performed as reported in the literature [2, 4, 5]. Patients could continue with the fluoropyrimidine alone if they experienced severe toxicity with cisplatin. Treatment was discontinued if the tumor progressed, severe toxicity occurred, or at the patient's request.

Evaluation of treatment and statistical analysis

In patients with measurable lesions, the tumor response was assessed objectively according to the guidelines of the Response Evaluation Criteria In Solid Tumors (RECIST, ver. 1.0), and the best overall response was recorded as the antitumor effect for that patient. The objective response rate in these patients was presented as the percentage of patients with a complete response (CR) or partial response (PR). According to the Japanese Classification of Gastric Carcinoma [19], the amount of ascites was assessed by a radiologist using CT. Response rate for ascites represented the percentage of patients with complete disappearance (CR) or a dramatic decrease in ascites (PR). Time to treatment failure (TTF) was measured from the date of initiation of chemotherapy to the date of the last administration of fluoropyrimidine or cisplatin. The PFS was measured from the date of chemotherapy to the date of progressive disease or death from any cause. The OS was estimated from the date of initiation of chemotherapy to the date of death or last follow-up visit. Median PFS and median OS were estimated by the Kaplan–Meier method. Toxicities were graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events, version 4.0.

Our primary interest was in comparing the clinical outcomes among patient groups that had different amounts of ascites. The amount of ascites was defined as follows: small (limited to pelvic cavity or around liver); moderate (not small or massive); or massive (continuous ascites from surface of liver to pelvic cavity). This definition of massive ascites was the same as that used in the JCOG 0106 study [13]. The volume of ascites was also estimated by the five-point method, as previously reported [16, 20]. We divided patients into the following three groups: (1) patients