LMC, other metastatic disease was also observed in 9 patients, including peritoneal dissemination ($n = 8$), lymph node metastasis ($n = 6$), brain metastasis ($n = 2$), bone metastasis ($n = 2$), and liver metastasis ($n = 1$); the remaining three patients showed no evidence of metastasis other than LMC.

Of the 12 patients, 9 had received chemotherapy for gastric cancer prior to the diagnosis of the LMC. At the onset of LMC, the efficacy of the previous chemotherapy was rated as partial response or stable in 7 (78%) patients, progressive disease in one patient, and unevaluated in one patient. Of the remaining 3 patients who had no history of previous chemotherapy, one developed LMC immediately after the gastric cancer diagnosis, and in the other two patients LMC was detected simultaneously with other recurrence(s) after curative surgery. The median duration from the diagnosis of gastric cancer to the diagnosis of LMC was 15.6 months (range 1.0–91.1 months).

Table 2 Patients' characteristics

Characteristics	
Categories	Number of patients
Sex	
Male/female	9/3
Age, years, median (range)	63 (30–73)
RTOG-NFC	
2/3/4	2/7/3
Primary tumor	
Yes/no	5/7
Histological type	
Intestinal/diffuse/small cell	1/10/1
Metastatis sites	
Peritoneum/lymph nodes/brain/bone/liver/lung	8/6/2/2/1/1
Number of metastatic sites (except for LMC)	
0/1/2/3/4	3/2/2/4/1
Prior chemotherapy	
Yes/no	9/3
Number of prior chemotherapy regimen	
1/2/3/4	2/2/4/1
Response to prior chemotherapy at LMC diagnosis	
Response or stable/progression/not evaluated	7/1/1

Clinical symptoms and LMC diagnosis

The most frequent symptom of LMC was headache, and various clinical neurological signs were noted, including consciousness disturbance, cataplexy, vomiting, convulsion, and cerebellar ataxia.

The initial LMC diagnosis was made by gadolinium-enhanced MRI in 8 patients and by CSF cytology in the remaining 4 patients. Finally, leptomeningeal enhancement (Fig. 1) was detected in 10 patients, and the CSF cytology was class IV or V in 9 of the 10 patients in whom the examination was performed.

Treatment

Among the 12 patients, best supportive care alone was selected for treatment in 2 patients, including one with a past history of WBI for brain metastasis and another who was comatose and developed disseminated intravascular coagulation immediately after being diagnosed with LMC.

The therapeutic modalities applied for the remaining 10 patients were as follows: ITC alone in 3 patients, ITC plus WBI in 4 patients, and ITC plus WBI plus VP shunt in 3 patients. For the intrathecal administration of methotrexate, which was undertaken in all the 10 patients, a subcutaneous (Ommaya) reservoir was implanted in 8 of the patients.

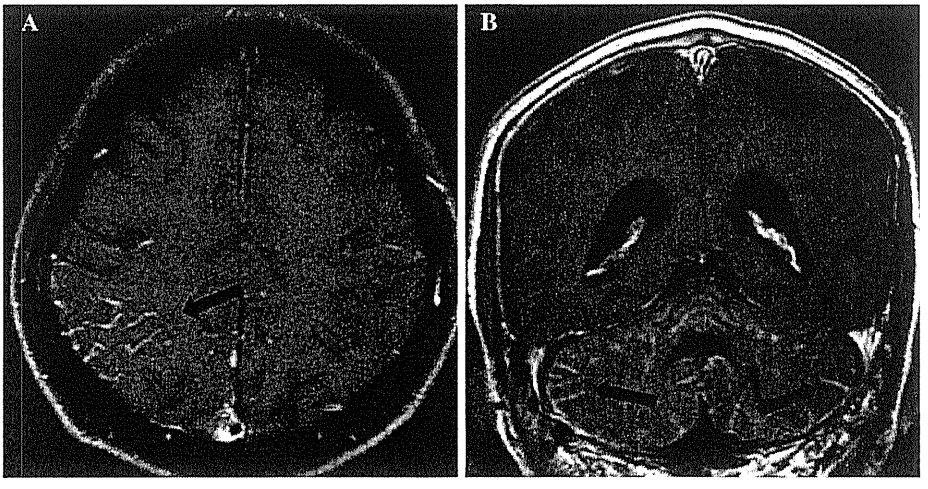

Fig. 1 Magnetic resonance imaging (MRI) scan finding in LMC. MRI scan of the brain shows peripheral contrast enhancement of the cerebellar (*arrows* in **a**) and cerebral (*arrows* in b) sulci

Table 3 Toxicity profiles ($n = 10$)

Toxicity	Grade (NCI-CTC, version 3.0; %)					
	Intrathecal chemotherapy alone ($n = 3$)			Intrathecal chemotherapy + WBI ($n = 7$)		
	3	4	% grade 3 or lower	3	4	% grade 3 or lower
Luekopenia	1	0	33	1	1	29
Neutropenia	0	1	33	0	1	14
Hemoglobin	1	0	33	3	0	43
Thrombocytopenia	2	0	67	0	1	14
AST	0	0	10	1	0	14
ALT	1	0	33	2	0	29
Febrile neutropenia	0	0	0	0	1	14
Headache	0	0	0	1[a]	0	14
Nausea	0	0	0	1[a]	0	14
Appetite loss	0	0	0	1[a]	0	14

Five patients (50%) died within 30 days of the last administration of intrathecal methotrexate

NCI-CTC National Cancer Institute common toxicity criteria

[a] All the grade 3 or lower headache, nausea, and appetite loss were observed at the beginning of the treatment

Table 4 Leptomeningeal carcinomatosis with gastric cancer: treatment and outcome

Case	Age (years)	Sex	RTOG-NFC at diagnosis of LMC	Treatment of LMC	RTOG-NFC after treatment[a]	Transition to home care	Survival after LMC diagnosis (days)
1	73	M	3	IT MTX	4	No	14
2	63	M	3	IT MTX	2	Yes	92
3	41	F	3	IT MTX	2	Yes	60
4	70	M	3	IT MTX,WBI	4	No	13
5	30	F	3	IT MTX,WBI	2	Yes	89
6	63	M	3	IT MTX,WBI	3	No	61
7	59	M	3	IT MTX,WBI	2	Yes	47
8	59	M	2	IT MTX,WBI, VP shunt	1	Yes	532
9	66	F	4	IT MTX,WBI, VP shunt	3	No	114
10	59	M	2	IT MTX,WBI, VP shunt	2	Yes	104

[a] At the time intrathecal methotrexate was finished (intrathecal methotrexate alone cases) or WBI was finished

IT MTX intrathecal methotrexate

Toxicities

The worst grades of toxicity in each patient during the intrathecal methotrexate therapy and WBI are summarized in Table 3. Grade 3 or 4 neutropenia was observed in two patients and grade 3 or 4 leukopenia in three patients. All of the grade 3 adverse events, namely headache, nausea and appetite loss, were observed at the beginning of the treatment. The main non-hematological adverse events that appeared anew after the beginning of the treatment were headache (2 cases), nausea (2 cases), vomiting (2 cases), and general fatigue (1 cases). All of these adverse events were grade 1 in severity, except for headache, which was grade 2 in severity. Early death within 30 days of the last administration of intrathecal methotrexate occurred in five patients, of whom one patient who developed grade 4 neutropenia, leukopenia and febrile neutropenia died on the day following the last intrathecal methotrexate administration. The major complications caused by VP shunt were not observed.

Efficacy

The treatment and clinical outcomes of the 10 patients who received treatment are summarized in Table 4. RTOG-NFC improvement was obtained in 6 (60%) patients, 5 of whom and another patient could leave the hospital temporarily (Table 4). All the patients had died by the time of this analysis. One patient was considered to have died a treatment-related death, 1 died from progression of peritoneal dissemination, and all of the remaining 10 patients died from the progression of the LMC. The median overall survival time from the diagnosis of LMC was 60 days in the 12 patients (Fig. 2), but 90 days in those who had received any treatment for LMC. The RTOG-NFC class did not become worse with VP shunt in any of the 3

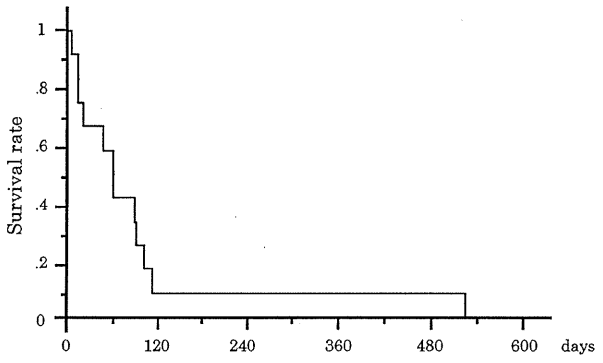

Fig. 2 Overall survival from the diagnosis of LMC

patients in whom VP shunt was performed, and 1 patient in whom ITC, WBI, and VP shunt were administered survived for a considerably long period of 532 days.

Discussion

Although cancer cells may seed the leptomeninges in patients with any type of solid tumors, the highest incidence of LMC has been reported in breast and lung cancer patients (12–34 and 10–26%, respectively); the incidence of LMC complicating gastrointestinal tract cancer is comparatively low (4–14%) [11]. Recently, the advances in systemic chemotherapy brought about by the development of new agents, including molecular-targeted agents, has contributed to a longer survival of cancer patients than that in the twentieth century. The median survival time of advanced gastric cancer patients treated by systemic chemotherapy has exceeded one year. In this retrospective study, the median interval between the initial diagnosis of gastric cancer and development of LMC was 15.6 months. Thus, it is anticipated that the incidence of LMC complicating gastric cancer will increase along with the prolonged survival brought by the advances in systemic chemotherapy.

In this study, the histological type of the tumor in 10 of the 12 patients (83%) was poorly differentiated adenocarcinoma or signet-ring cell carcinoma. This result is consistent with that suggested by previous reports. The majority of gastric cancer patients with LMC have poorly differentiated or signet-ring cell cancer [3, 4, 12]. In the diffuse type of gastric cancer, peritoneal dissemination and lymph node metastasis appear to be the major metastatic sites. Thus, the most frequent sites of concurrent metastasis associated with LMC in patients with gastric cancer were lymph node metastasis and peritoneal dissemination, as corroborated by both this study and previous reports [13].

Once LMC develops, irrespective of the primary cancer site, depressed neurological functions and neurological deficits cause reduction in the activities of daily living and extreme deterioration of the quality of life, and the prognosis is very poor. Because systemic chemotherapy is not effective against LMC, intraventricular chemotherapy and/or radiation have been most commonly employed for its treatment. Although no novel administration method of intrathecal methotrexate has been established, use of a low dose and daily intraventricular administration of methotrexate have been reported to be associated with reduced neurotoxicity [14]. Notwithstanding this treatment, the overall median survival of LMC patients was as low as 0.7–5.8 months [15–17]. In the largest case series of 90 patients with LMC complicating various kinds of cancers at the Memorial Sloan-Kettering Cancer Center who received focal irradiation and intraventricular methotrexate from 1975 to 1980, the overall median survival was reported to be 5.8 months [17]. However, breast cancer (46 patients) was the most commonly documented primary tumor in that study, and no gastric cancer patients were included in the report. As for the breast cancer patients in the case series, 28 patients (61%) showed symptomatic improvement or stabilization, and their overall median survival was 7.2 months. Consistent with the prognosis of LMC complicating gastric cancer being much worse, with an overall median survival ranging from 4 to 6–7 weeks [3, 4], the median overall survival of the 12 patients was about nine weeks (60 days) in our present study. Thus, establishment of an effective treatment strategy for LMC complicating gastric cancer is warranted.

Omuro et al. [9] reported that VP shunt resulted in improvement of the symptoms of intracranial hypertension in 27 (77%) of the 37 patients with LMC. In our study, the treatment strategy changed with the passage of time and accumulation of experience, and VP shunt was applied to the last three patients, one of whom survived for a considerably long period of 532 days. Retrospectively, among the five patients who were actually treated with the two modalities of intraventricular methotrexate plus WBI, two patients would have fulfilled the eligibility criteria for VP shunt; the survival times of these two patients were as short as 61 and 47 days. This study had its limitations, e.g., the small sample size of the study population and the retrospective study design; nonetheless, the results suggest that VP shunt may have potential clinical benefit for selected gastric cancer patients with complicating LMC. Ideally, the survival benefit of VP shunt should be evaluated in future clinical trials, although most patients with LMC are generally poor candidates for clinical trials.

The goals of treatment of LMC include not only prolongation of survival, but also improvement of the neurological symptoms. ITC and/or WBI have been tried in the palliative setting for selected patients. Lee et al. [3] reported that incomplete resolution of neurological symptoms was observed in only one among the 10 patients who

received ITC, and five patients showed no significant changes in clinical symptoms. Once LMC is diagnosed in gastric cancer patients, immediate hospitalization is indicated, because of the severe neurological symptoms. In this study, improvement of RTOG-NFC was seen in 2 of 3 patients by ITC and 4 of 7 by ITC followed by WBI. It is considered a very significant result that 6 of the 10 patients who received multidisciplinary treatment could temporarily leave the hospital.

In conclusion, this study suggests that ITC might be effective for obtaining improvement of neurological functions, and further accumulation of clinical experience is necessary to evaluate the efficacy of multidisciplinary treatment, including WBI and VP shunt.

Conflict of interest No author has any conflict of interest.

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Cancer Research

Priority Report

HERC2 Interacts with Claspin and Regulates DNA Origin Firing and Replication Fork Progression

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Abstract

DNA replication, recombination, and repair are highly interconnected processes the disruption of which must be coordinated in cancer. HERC2, a large HECT protein required for homologous recombination repair, is an E3 ubiquitin ligase that targets breast cancer suppressor BRCA1 for degradation. Here, we show that HERC2 is a component of the DNA replication fork complex that plays a critical role in DNA elongation and origin firing. In the presence of BRCA1, endogenous HERC2 interacts with Claspin, a protein essential for G_2–M checkpoint activation and replication fork stability. Claspin depletion slowed S-phase progression and additional HERC2 depletion reduced the effect of Claspin depletion. In addition, HERC2 interacts with replication fork complex proteins. Depletion of HERC2 alleviated the slow replication fork progression in Claspin-deficient cells, suppressed enhanced origin firing, and led to a decrease in MCM2 phosphorylation. In a HERC2-dependent manner, treatment of cells with replication inhibitor aphidicolin enhanced MCM2 phosphorylation. Taken together, our results suggest that HERC2 regulates DNA replication progression and origin firing by facilitating MCM2 phosphorylation. These findings establish HERC2 as a critical function in DNA repair, checkpoint activation, and DNA replication. *Cancer Res; 71(17); 5621–5. ©2011 AACR.*

Introduction

DNA replication, recombination, and repair coordinately maintain genome stability, and their defect is a hallmark of cancer cells. The DNA replication and damage response share many critical proteins. Among them are the ATR-Chk1 pathway, which is activated in response to stalled replication forks and prevents inappropriate entry into mitosis, while it also regulates normal DNA replication by stabilizing replication forks and inhibiting excess origin firing (1, 2). Claspin is a checkpoint mediator that facilitates the phosphorylation and activation of Chk1 by ATR (3). In addition, Claspin in combination with TIPIN-TIM1-AND1 complex physically links the DNA polymerase and helicase activities, preventing fork collapse, and is required for a normal rate of fork progression (2, 4, 5).

HERC2 is a large HECT and RCC-like domain-containing protein comprising 4,834 amino acids, and has recently been implicated in homologous recombination repair of DNA double-strand breaks (DSB; ref. 6). HERC2 is recruited to sites of DSBs and facilitates assembly of the RNF8-Ubc13 complex, and is thereby essential for ubiquitin-dependent retention of repair factors (6). HERC2 is also implicated in nucleotide excision repair by ubiquitinating and degrading XPA (7, 8). In addition, we showed that HERC2 is an E3 ubiquitin ligase that targets BARD1-uncoupled BRCA1 for degradation (9). Depletion of HERC2 does not produce G_2–M checkpoint failure (9) in spite of the fact that HERC2 is essential for recruiting the repair factors including BRCA1 that mediate G_2–M checkpoint activation, to sites of DNA damage (6). Because depletion of HERC2 compensated for BRCA1 instability in BARD1-deficient cells and restored G_2–M checkpoint function, we propose that HERC2 inhibits G_2–M checkpoint function by destabilizing BRCA1. HERC2 interacts with BRCA1 and may inhibit its G_2–M checkpoint function during normal S-phase or during recovery from the checkpoint. However, role of HERC2 in normal S-phase is unknown.

Because BRCA1 interacts with Claspin and acts as a second regulator of Chk1 activation (10), HERC2 may interact with Claspin and regulate DNA replication. Here, we show that HERC2 is a component of replication fork complex and regulates the fork progression and origin firing in conjunction with Claspin.

Materials and Methods

Cell culture and transfection

HCT116, Hela, HEK293T, U2OS, and either BRCA1-negative or -positive UWB1.289 cells were purchased from American

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic agent. Cell lines were not further tested or authenticated. siRNA oligonucleotides targeting HERC2 (#1: 5′-GGAAAGCACUGGAUUCGUU-3′ and #2: 5′-GAAGGUGGCUGUUCACUCA-3′, sense strand), Claspin (#1: 5′-GGACGUAAUUGAUGAAGUA-3′ and #2: 5′-GGAAAUAC-CUGGAGGAUGA-3′, sense strand), and nontargeting control (D-001206-14) were purchased from Dharmacon. Myc-tagged *N*-terminus (1-2329, NT) and *C*-terminus (2292-4834, CT) of HERC2 fragments were subcloned in pcDNA3 by fusing smaller fragments of HERC2 as described previously (9). Transfection was carried out as previously described (11). Aphidicolin was purchased from Sigma-Aldrich.

Cell-cycle analysis

Seventy-two hours after siRNA transfection, HCT116 cells were treated with 100 ng/mL of nocodazole for 12 hours to trap cells in mitosis and to analyze a single cycle of S-phase. Bromodeoxyuridine (BrdUrd) was added for the last hour. Cells were then harvested and DNA was stained with anti-BrdUrd monocolonal antibody (mAb; Santa Cruz) and propidium iodide, and analyzed by flow cytometry as described elsewhere (12).

Immunoprecipitation, immunoblotting, and immunocytochemistry

Rabbit polyclonal antibodies to HERC2 (Bethyl Laboratories), Claspin (Bethyl Laboratories), ATRIP (Bethyl Laboratories), phospho-MCM2-S108 (Bethyl Laboratories), goat polyclonal antibodies to MCM2 (Santa Cruz) and mouse mAbs to HERC2 (BD Bioscience), MCM7 (Santa Cruz), PCNA (Neomarkers), TopBP1 (BD Bioscience), α- and β-tubulin (DMIA+BMIB, Neomarkers), Actin (Sigma), and Myc (9E10, BabCo) were purchased commercially. Immunoprecipitation and immunoblotting were done as described (11) with 0.5% NP-40–based lysis buffer in the presence or absence of benzonase nuclease (Novagen) or with RIPA buffer for whole-cell lysates (11, 13). For indirect immunocytochemistry, cells were fixed with cold methanol for 60 minutes and permeablized with cold acetone for 5 seconds. Cells were then stained as previously described (13) with the modification that blocking buffer contained 0.1% Triton X-100.

DNA fiber experiments

Forty-eight hours after siRNA transfection, cells were pulse labeled with 25 μmol/L BrdUrd for indicated time length, followed by 250 μmol/L iododeoxyuridine (IdUrd) for 20 minutes. DNA combing was carried out as described elsewhere (14, 15) with modifications. Briefly, 3,000 labeled cells spread on a glass slide were overlaid with 10 μL of buffer containing 0.5% sodium dodecyl sulfate, 200 mmol/L Tris-HCl (pH 7.4), and 50 mmol/L EDTA. After 10 minutes, the slide was tilted at 30 degrees and the resulting DNA spreads were air-dried, and fixed in 3:1 methanol/acetic acid for 5 minutes. The slides were treated with 2.5 mol/L HCl for 60 minutes, washed in PBS, and blocked in 2% bovine serum albumin in PBS for 30 minutes. The DNA fibers were then immunostained with rat anti-BrdUrd mAb and mouse anti-BrdUrd/IdUrd mAb (BD Biosciences) followed by AlexaFluor 488-conjugated chicken anti-rat IgG (Invitrogen) and AlexaFluor 555-conjugated goat anti-mouse IgG (Millipore). Fluorescent-labeled fibers were then examined with an LSM 510 confocal microscope (Carl Zeiss). The lengths of BrdUrd- (green) and IdUrd (red)-labeled patches were measured using LSM software (Carl Zeiss).

Results and Discussion

HERC2 interacts with Claspin and affects S-phase progression

To examine whether HERC2 cooperates with Claspin, we first analyzed the interaction between endogenous HERC2 and Claspin by coupled immunoprecipitation and Western blotting. HERC2 was readily detected in Claspin immunocomplexes precipitated from HCT116 or HeLa cell lysate (Fig. 1A). The interaction was diminished in BRCA1-defective UWB 1.289 cells (Fig. 1B), suggesting that BRCA1 facilitates the interaction. We next examined whether HERC2 has some role in the effect of Claspin on S-phase progression. HCT116 cells

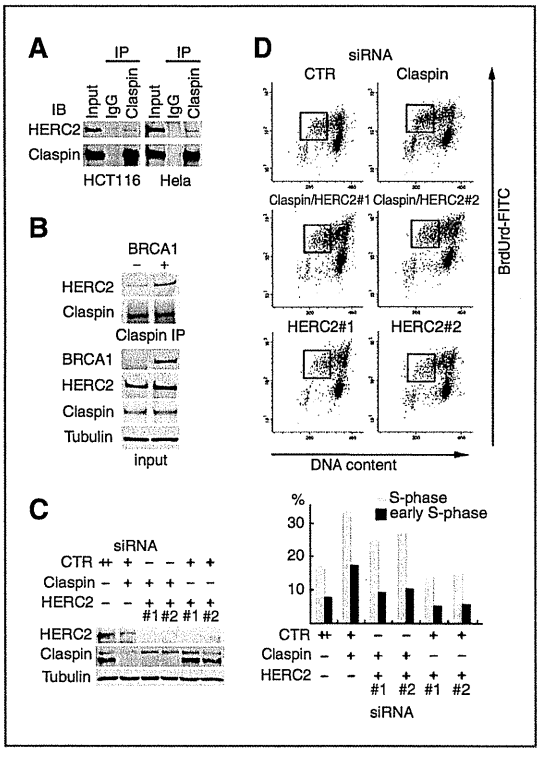

Figure 1. HERC2 interacts with Claspin and affects S-phase progression. A and B, lysates prepared from HeLa, HCT116 (A), and BRCA1-negative and positive UWB 1.289 (B) cells were immunoprecipitated (IP) and immunoblotted (IB) with indicated antibodies. Inputs (1.5%) were also loaded. C, HCT116 cells transfected with indicated siRNA were IB with indicated antibodies. Tubulin was the loading control. D, cells from (C) were analyzed for cell cycle. The percentages of early S- (indicated with square boxes) and S-phase cells are shown in bottom. CTR, control.

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were transfected with siRNA for Claspin and/or HERC2, and the S-phase progression was analyzed with BrdUrd. The siRNA treatment successfully inhibited expression of Claspin and HERC2 (Fig. 1C; Supplementary Fig. S1). Depletion of Claspin expression increased cells in S-phase, including that in early S-phase (Fig. 1D), indicating slowed S-phase progression as previously reported (4). Importantly, additional HERC2 depletion with 2 different siRNAs both reduced the effect of Claspin depletion and decreased cells in S-phase to the level of control cells. This suggests that HERC2 suppresses S-phase progression in the absence of Claspin. Single HERC2 knockdown also slightly decreased cells in S-phase when compared with the control cells.

HERC2 localizes at DNA replication fork

We previously showed that HERC2 and BRCA1 colocalize at S-phase nuclear foci (9). To analyze whether HERC2 localizes at DNA replication foci, exponentially proliferating HeLa cells were immunostained with anti-HERC2 antibody in combination with antibodies to proteins in the replication fork complex. Interphase cells exhibited nuclear HERC2 foci as we showed previously (9). Importantly, clear colocalization of HERC2 with nuclear PCNA foci was visualized (Fig. 2A). The colocalization was especially remarkable in cells with larger PCNA foci (light upper cell in top panels), an indication of cells in late S-phase (16). To further show the interaction between HERC2 and PCNA, HEK-293T cells were transfected with HERC2 fragments. As shown in Fig. 2B, PCNA was detected in the Myc-HERC2-CT immunocomplexes. Reciprocally, Myc-HERC2-CT was detected in PCNA immunocomplexes. The detection of the interaction was dependent on endonuclease treatment of cell lysates, suggesting that HERC2 physically interacts with PCNA complex on chromatin. HERC2 also colocalized at nuclear foci with TopBP1 and MCM7 (Fig. 2A). Together the results indicate that HERC2 is a component of the DNA replication complex.

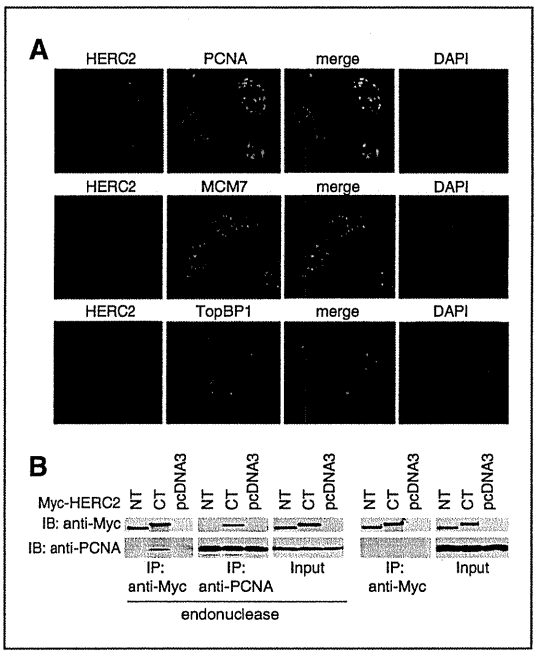

Figure 2. HERC2 localizes at DNA replication foci. A, HeLa cells were immunostained with anti-HERC2 antibody in combination with indicated antibodies. The nucleus was counterstained with DAPI. B, HEK-293T cells were transfected with Myc-HERC2-NT or CT fragment, or parental pcDNA3 vector, and lysed with or without endonuclease. Interaction between Myc-HERC2 fragments and PCNA were assessed by IP followed by IB with indicated antibodies.

HERC2 suppresses DNA replication progression in the absence of Claspin

S-phase progression is regulated by 2 main mechanisms: replication origin firing and elongation. Claspin and Chk1 stabilize replication fork and maintain elongation while they suppress excess origin firing (5, 17). Therefore the observed effect on S-phase progression in cells depleted of Claspin and/or HERC2 (Fig. 1) could result from the 2 contradictory factors. To analyze this mechanism more precisely, we used DNA combing experiments. The nascent DNAs were labeled with BrdUrd followed by IdUrd and the DNA lengths were analyzed with immunofluorescent detection. The reliability of the procedure was first verified by proportionate increase of the DNA length and labeling time length (Supplementary Fig. S2). HCT116 cells were then transfected with Claspin- or HERC2-specific siRNAs. The inhibition of HERC2 and/or Claspin expression was verified by Western blot (Fig. 3A, Supplementary Fig. S1). The lengths of the nascent DNAs in each sample were determined (Fig. 3B–D). Consistent with the previous report (4, 18), inhibition of Claspin shortened the DNA by approximately one third of that in control cells (41.4 vs. 14.0 or 14.7 kbp). Importantly, depletion of HERC2 can alleviate the slow replication fork progression in the Claspin-deficient cells (14.0 vs. 19.1 or 17.9 kbp). Interestingly, however, single HERC2 knockdown slightly shortened, rather than lengthened, the DNA lengths (41.4 vs. 35.6 or 36.5 kbp). The observed effect of HERC2 likely did not rely on its E3 activity for protein degradation, because proteasome inhibitor MG132, instead of HERC2 deletion, did not affect the nascent DNA length shortened by Claspin inhibition (Supplementary Fig. S3).

HERC2 enhances origin firing in the absence of Claspin and facilitates MCM2 phosphorylation in response to replication stress

Inhibition of Claspin leads to uncoupling of MCM helicase complex and polymerase complexes, resulting in enhanced single-strand DNA (SSD) during replication elongation (5). Therefore, the observed complement effect of HERC2 depletion on the slow replication fork progression in the Claspin-deficient cells could be explained by a direct role of HERC2 on the fork stabilization. However, HERC2 depletion in the Claspin-deficient cells did not show an obvious complementary effect on the foci formation of a SSD-binding protein RPA70 (Supplementary Fig. S4), suggesting that HERC2 is not directly involved in the fork stabilization.

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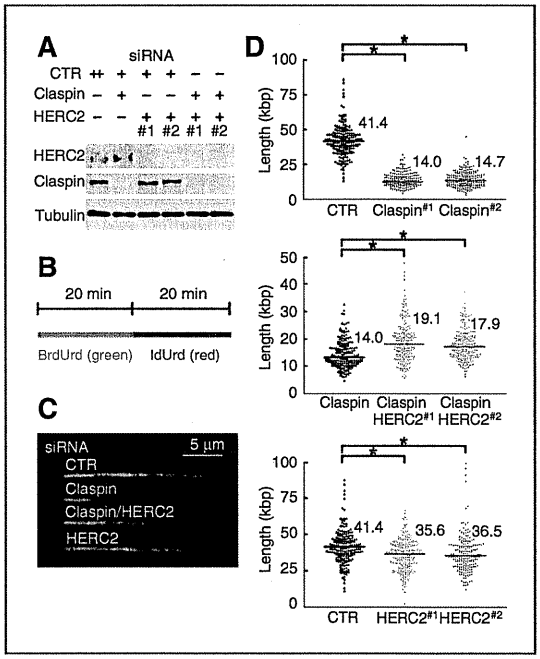

Figure 3. HERC2 suppresses DNA strand elongation in the absence of Claspin. A and B, HCT116 cells transfected with the indicated siRNA were subjected to immunoblot (A) or sequentially treated with BrdUrd and IdUrd for 20 minutes each to label the nascent DNAs (B). C, representative images of labeled DNA fibers from cells with indicated siRNA measured with confocal microscopy. D, distributions of replication fork length during the entire labeling period in cells with the indicated siRNA are shown with the mean percentages (bars). Significance was analyzed by Student *t* test. *, $P < 0.0001$.

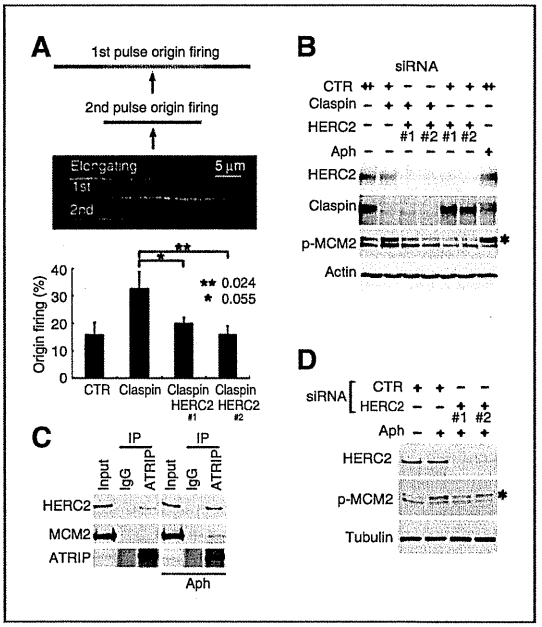

Figure 4. HERC2 enhances origin firing and MCM2 phosphorylation in replication stress. A, the nascent DNAs were labeled as in Fig. 3. Representative images of the labeled DNA fibers of each origin firing or elongation pattern from control cells are shown. Arrows indicate the position of replication initiation. Bottom, quantification of origin firing in cells with the indicated siRNA. Summation of number of first label origins (red-green-red), second label origins (red only), and interspersed patterns (repeated red-green-red) are shown as percentage of all labeled tracks. Data represent the mean of triplicate experimental samples and error bars indicate SD. *, $P = 0.055$; **, $P = 0.024$ by Student *t* test. B, HCT116 cells were transfected with the indicated siRNA and whole-cell lysates were IB with indicated antibodies. Aphidicolin (Aph, 4 μmol/L) was added for 3 hours as a positive control for MCM2 phosphorylation. C, Hela cells untreated or treated with 4 μmol/L aphidicolin for 3 hours were IP in the presence of endonuclease, and IB with indicated antibodies. D, HCT116 cells were transfected with the indicated siRNA, untreated or treated with 1 μmol/L aphidicolin for 3 hours, and whole-cell lysates were IB with indicated antibodies. *, phosphorylated MCM2.

We next analyzed the effect of HERC2 on origin firing. Origin firing is regulated by 2 contradictory mechanisms. Claspin and Chk1 inhibits excess origin firing while promoting elongation (17, 19). In contrast, ATR-mediated phosphorylation of MCM2 recruits Plk1, which upregulates origin firing as a compensatory mechanism for survival of replication blocks (5, 20). Supporting this mechanism, Claspin-deficient cells showed enhanced origin firing (Fig. 4A). Significantly, additional depletion of HERC2 resuppressed the origin firing to the level of that in control cells. In addition, depletion of Claspin enhanced the MCM2 phosphorylation and additional inhibition of HERC2 dramatically suppressed the MCM2 phosphorylation in the Claspin-deficient cells (Fig. 4B). To test the effect of HERC2 on replication stress in physiologic condition, we treated the cells with aphidicolin and ATRIP was immunoprecipitated. In addition to MCM2, HERC2 was coprecipitated from endonuclease-treated cell lysates (Fig. 4C; Supplementary Fig. S5). Importantly, the aphidicolin treatment increased the amount of HERC2 in the ATRIP immunocomplex (Fig. 4C). In addition, HERC2 depletion suppressed the MCM2 phosphorylation in the aphidicolin-treated cells (Fig. 4D), in similar fashion to that in the Claspin-deficient cells (Fig. 4B).

In summary, our results show that HERC2, an E3 ligase critical for DNA damage repair pathways, also regulates DNA replication progression and origin firing by facilitating MCM2 phosphrylation (Supplementary Fig. S6). Because HERC2 targets BRCA1 for degradation, it is possible that HERC2, Claspin, and BRCA1 cooperate on activation of Chk1 and Plk1. HERC2 in maintenance of DNA stability warrants further study into its potential roles in cancer development and therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Phase II Study of Bolus 5-Fluorouracil and Leucovorin Combined with Weekly Paclitaxel as First-Line Therapy for Advanced Gastric Cancer

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Key Words

Gastric cancer · Chemotherapy · Weekly paclitaxel · Bolus 5-fluorouracil · Leucovorin

Abstract

Objective: We evaluated the efficacy and safety of bolus 5-fluorouracil (5-FU) and leucovorin combined with weekly paclitaxel (FLTAX) in advanced gastric cancer (GC) patients. ***Methods:*** Patients with untreated stage IV GC received paclitaxel 80 mg/m^2 as a 1-hour infusion, followed by 5-FU 600 mg/m^2 as a bolus infusion and L-leucovorin 250 mg/m^2 as a 2-hour infusion on days 1, 8 and 15. Treatment cycles were repeated every 28 days. The primary endpoint was response rate. ***Results:*** Thirty-five patients were enrolled. The median age was 62 years (range 34–75). Twenty-one patients (60%) had diffuse-type cancer and 11 had peritoneal metastasis. The confirmed response rate was 43% (95% CI 26–61) with 15 partial responses. Stable disease was observed in 16 (46%) patients. Median progression-free survival and overall survival were 6.8 months (95% CI 5.8–7.4) and 16.2 months (95% CI 10.0–22.8), respectively. Grade 3–4 adverse events were: neutropenia (54%), febrile neutropenia (3%), diarrhea (6%) and sensory neuropathy (11%). ***Conclusion:*** FLTAX showed a desirable safety profile, and the efficacy against advanced GC was encouraging. FLTAX may be a good option for GC patients with deteriorated general condition, and a randomized clinical trial in such patients is currently underway.

Introduction

Gastric cancer (GC) is the second leading cause of cancer death both worldwide and in Japan [1, 2]. Even though the incidence of GC is declining, approximately 930,000 cases are newly diagnosed each year worldwide [1]. Because of the vague and nonspecific symptoms associated with GC, the disease is often advanced on diagnosis, after which chemotherapy is the main treatment option. Oral fluoropyrimidines plus cisplatin-containing therapy is now considered to be the standard for advanced GC patients in most countries [3–5]. However, GC patients who are in poor condition or who have peritoneal dissemination cannot tolerate aggressive hydration and severe toxicity. Therefore, treatment with cisplatin is not indicated

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in a considerable portion of GC patients. Furthermore, patients with advanced GC often have gastrointestinal symptoms, negatively affecting their oral intake and sometimes precluding the administration of oral drugs. Intravenous administration-based therapies with mild toxicity and sufficient efficacy are needed.

Despite optimal treatment, advanced GC remains an aggressive malignancy, with a median survival of 9–13 months [3, 6, 7]. Recently, trastuzumab was shown to be the first molecularly targeted agent to provide survival benefits in HER2-positive GC patients [8]. However, HER2, the target of trastuzumab, is reportedly overexpressed only in approximately 20% of GC tumors, and the majority of these are of intestinal type [9, 10], which means patients with diffuse-type GC are not treated with trastuzumab. Previous reports have indicated that patients with diffuse-type GC have a significantly shorter survival than those with intestinal-type tumors, mainly due to the higher malignancy grade, with a high incidence of peritoneal metastases and deteriorated general condition [9, 11, 12]. Therefore, new chemotherapy regimens to improve the outcome in GC patients, particularly in diffuse-type cancer, are required.

Paclitaxel is pharmacologically and clinically considered to be effective against diffuse-type GC, as well as for intestinal types [13–15]. Because a weekly regimen of paclitaxel is less toxic than paclitaxel given once every 3 weeks [16], weekly regimens have become common in Japan, producing good results in GC patients [17]. 5-Fluorouracil (5-FU) is generally accepted as a key drug in the treatment of GC patients. Based on a sequence-dependent, synergistic cytotoxic effect of paclitaxel followed by 5-FU without overlapping toxicity [18, 19], we developed a chemotherapy regimen consisting of leucovorin-modulated weekly bolus 5-FU combined with weekly paclitaxel (FLTAX) on an outpatient basis. We subsequently conducted a phase I study to determine the recommended dose for the phase II study [20]. Preliminary safety data for the FLTAX regimen showed only mild toxicity.

This multi-institutional phase II study was designed to evaluate the efficacy and safety of the new non-platinum regimen of FLTAX as a first-line treatment for patients with metastatic or recurrent GC. The primary objective was to determine the overall response rate.

Patients and Methods

Patient Eligibility

This was a prospective, multi-institutional phase II clinical trial performed at the National Cancer Center Hospital, Kochi Health Sciences Center, Kanagawa Cancer Center, and Mitsubishi Kyoto Hospital. To be eligible, patients had to meet the following criteria: histologically proven unresectable or recurrent GC; age of 20–75 years; performance status of ≤2 according to the Eastern Cooperative Oncology Group scale; estimated life expectancy of >8 weeks after study entry, no prior chemotherapy for metastatic disease; adequate hematological function (white blood cell count between 3,000 and 12,000/mm^3, platelet count of ≥100,000/mm^3); adequate hepatic function (serum total bilirubin level of ≤2.0 mg/dl, AST and ALT levels of ≤100 IU/l); adequate renal function (serum creatinine level of ≤1.5 mg/dl); serum C-reactive protein level of ≤10 mg/dl; and written informed consent. Patients also had to have radiographically measurable disease according to the Response Evaluation Criteria in Solid Tumors guidelines [21]. Adjuvant chemotherapy with an oral fluoropyrimidine alone, not exceeding 1-year duration, completed more than 6 months before entry, was allowed.

Exclusion criteria were watery diarrhea; marked pleural effusion or ascites; active infection; severe comorbidity such as heart disease or renal disease; metastasis to the central nervous system; mental disorder; history of alcoholic hypersensitivity; active concomitant malignancy; pregnant or nursing women, and women of childbearing age, unless they were practicing effective contraception. This study was approved by the Institutional Review Boards of all participating institutes.

Treatment Plan

Paclitaxel (Taxol; Bristol-Myers K.K., Tokyo, Japan) at a dose of 80 mg/m^2 was administered as a 1-hour intravenous infusion followed by 5-FU (5-FU; Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) 600 mg/m^2 as a bolus intravenous infusion on days 1, 8 and 15 of a 28-day cycle. A 2-hour intravenous infusion of L-leucovorin (Isovorin; Wyeth K.K., Tokyo, Japan) 250 mg/m^2 in normal saline solution was started at the same time as the paclitaxel infusion on the same days. This treatment was repeated until disease progression or unacceptable toxicity occurred. Short-term premedication was given to prevent paclitaxel-associated hypersensitivity reactions 30 min before the infusion of paclitaxel: dexamethasone 8 mg, ranitidine 50 mg and chlorpheniramine 10 mg.

Dose Attenuation

If patients had leukocytes <2,500/mm^3, platelets <100,000/mm^3, total bilirubin >2.0 mg/dl, AST and ALT >100 IU/l, or serum creatinine >1.5 mg/dl, both 5-FU/leucovorin and paclitaxel were withheld until recovery. To receive a subsequent cycle of chemotherapy, patients had to have a leukocyte count of ≥3,000/mm^3 and the recovery of any treatment-related nonhematological toxicity to grade ≤1 (except alopecia and neuropathy). Treatment was delayed for no more than 3 weeks to allow patients to recover from toxicities.

If a patient developed one of the following toxicities, the dose of 5-FU was reduced to 500 mg/m^2 for the subsequent cycle of treatment: grade 4 neutropenia lasting for 4 or more days; grade 3–4 thrombocytopenia; grade 3–4 febrile neutropenia; grade 3–4 diarrhea despite adequate antidiarrheal medication; any grade 3–4 nonhematological toxicity (excluding anorexia, nausea, vomiting, electrolyte abnormalities, and alopecia); treatment interruption for ≥2 weeks, and a delay of the start of the second cycle by ≥8 days because of toxicity. Any patient who required more

dose reduction of the FLTAX regimen was withdrawn from the treatment protocol.

Toxicity and Response Evaluation

Treatment-related toxic effects were assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0. During treatment, patient histories were obtained, and physical examination, complete blood counts with differential counts, serum chemical analyses, and urinalyses were carried out at least once a week. Tumor response was evaluated according to Response Evaluation Criteria in Solid Tumors guidelines [21] every 8 weeks until tumor progression.

Statistical Analysis

The primary endpoint of this study was overall response rate. In a design with an unacceptable response rate of 20%, an acceptable response rate of 40%, a statistical power of 80% and an α-error of 0.05, 35 patients needed to be enrolled and treated. Our secondary endpoints were progression-free survival time (PFS), time to treatment failure (TTF), overall survival time (OS), and toxicity. PFS was defined as the time from the date of starting treatment to the date of first documentation of disease progression or death. PFS in patients with protocol treatment cessation for toxicity was calculated as the time to the date of first documentation of disease progression in subsequent therapies. TTF was measured from the date of starting treatment to the date of treatment cessation for any reason. OS represented the duration from the date of starting treatment to the date of death from any cause. PFS, TTF and OS were calculated by the Kaplan-Meier method. All statistical analyses were performed using JMP software (version 9.0.1; SAS Institute Inc., Cary, N.C., USA), with the final analysis conducted in January 2011. This study was registered with UMIN-CTR (ID number: UMIN000000502).

Results

Patient Characteristics and Drug Delivery

Thirty-five patients were enrolled in this study between September 2006 and October 2009. Response and toxicity were assessable in all patients. The clinical characteristics of the patients are shown in table 1. The majority of patients were males (86%), with a median age of 62 years (range 34–75). More than half of patients had diffuse-type GC (60%), and 77% had unresected primary tumor.

The median number of treatment cycles per patient was 5 (range 1–9; table 2). At the time of analysis, all patients had completed protocol treatment. The median relative dose intensity during the protocol treatment was 82% (range 55–100) for 5-FU, 88% (range 58–100) for L-leucovorin and 88% (range 60–100) for paclitaxel. After a 1-week observation period in the hospital, all patients were able to receive treatment on an outpatient basis.

Table 1. Patient characteristics

	Patients	
	n	%
Total patients	35	100
Sex		
Male	30	86
Female	5	14
Age, years		
Median	62	
Range	34–75	
ECOG performance status		
0	16	46
1	19	54
2	0	0
Histological type		
Intestinal	14	40
Diffuse	21	60
Prior surgery		
None	27	77
Gastrectomy	8	23
Adjuvant chemotherapy	0	0
Site of metastasis		
Lymph nodes	35	100
Liver	21	60
Peritoneum	11	31
Lung	4	11
Adrenal gland	1	3
Subsequent lines of chemotherapy		
2nd line	32	91
3rd line	14	40
4th line and more	6	17
Unknown	2	6

ECOG = Eastern Cooperative Oncology Group.

Efficacy

All 35 patients were included in the evaluation of response. Confirmed tumor response was partial response in 15, stable disease in 16, and progressive disease in 4 subjects (table 2). The confirmed overall response rate was 43% [95% confidence interval (CI) 26–61], and the best overall response rate including the unconfirmed partial response was 63% (95% CI 45–79). Four patients (11%) underwent secondary curative resection after major response to protocol treatment, and 2 patients underwent surgery before confirmation of partial response. No patients had a complete response.

At a median follow-up time of 17.8 months, 23 patients (66%) had died. The median PFS and TTF were 6.8 months (95% CI 5.8–7.4) and 5.6 months (95% CI 3.1–6.2), respectively (fig. 1a). The median OS was 16.2

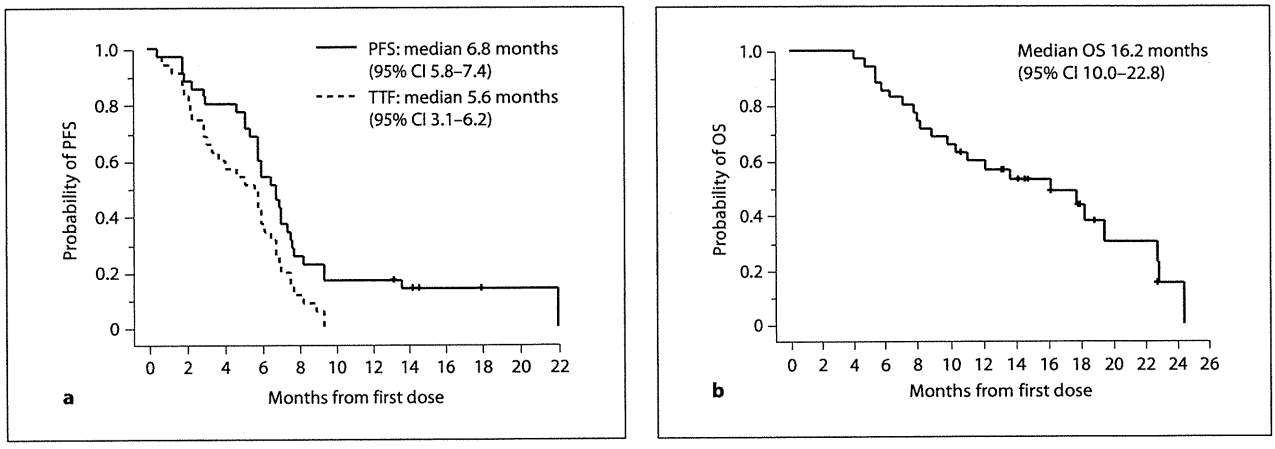

Fig. 1. Kaplan-Meier curves for PFS and TTF (**a**) and OS (**b**) in all patients (n = 35).

months (95% CI 10.0–22.8). Thirty-two (91%) patients received second-line chemotherapy (table 1). Among these, 15 patients were given cisplatin-based therapy, 8 patients S-1 alone, 4 patients 5-FU plus leucovorin, 3 patients irinotecan alone, and 2 patients paclitaxel-based therapy.

In an exploratory subgroup analysis, the best response rate and PFS in patients with diffuse-type GC were 62% (95% CI 38–82) and 6.8 months (95% CI 5.1–8.2), respectively. On the other hand, these results in patients with intestinal-type cancer were 64% (95% CI 35–87) and 7.0 months (95% CI 1.9–7.7), respectively. The median OS was 17.8 months (95% CI 8.2–19.5) in diffuse-type GC and 16.2 months (95% CI 7.2 to infinite) in intestinal-type GC. There were no significant differences in response rate, PFS or OS among the histological types.

Table 2. Drug delivery and treatment response

	Median	Range
Drug delivery		
Chemotherapy cycles	5	1–9
Relative dose intensity		
5-FU	0.82	0.55–1.00
L-Leucovorin	0.88	0.58–1.00
Paclitaxel	0.88	0.60–1.00
Confirmed tumor response[1]		
Total	35 (100)	
CR	0	
PR	15 (42.9)	
SD	16 (45.7)	
PD	4 (11.4)	
Overall response rate, %	42.9	
Overall response rate, 95% CI	26.3–60.7	
Best overall response rate, %	62.9	
Best overall response rate, 95% CI	44.9–78.5	

Figures in parentheses are percentages. CR = Complete response; PR = partial response; SD = stable disease; PD = progressive disease.

[1] According to Response Evaluation Criteria in Solid Tumors.

Toxicity

Major toxic effects occurring during the protocol treatment are summarized in table 3. The most common grade 3–4 toxicity was neutropenia (54%). One patient developed neutropenic fever and recovered with appropriate therapy. No patients developed grade 3–4 thrombocytopenia. Nonhematologic toxicities observed in the present study were generally mild. Only a small portion of patients experienced grade 3 nonhematologic toxicities: diarrhea in 2 patients (6%) and sensory neuropathy in 4 patients (11%; table 3). Although grade ≥1 diarrhea developed in 21 patients (60%), it was mild and promptly resolved after appropriate medical treatment such as with antidiarrheal agents. There was no grade 4 nonhematologic toxicity or treatment-related death in the present study. As for cumulative toxicity, 12 patients (34%) had grade ≥2 sensory neuropathy. In 6 of these patients, the treatment protocol was discontinued because of prolonged sensory neuropathy, and the median number of cycles delivered before discontinuation was 4.5 (range 2–7). All grade ≥2 neuropathy patients recovered within 2 months after treatment discontinuation. No other cumulative toxicity was observed.

The reasons for cessation of protocol treatment were progressive disease in 23 patients (66%), sensory neuropathy in 6 (17%), radical gastrectomy after tumor regression in 4 (11%), and infection in 2 (6%). One patient with a history of mild emphysema discontinued the protocol treatment and switched to another chemotherapy regimen because of recurrent pneumonia. In another patient, the protocol treatment had to be withdrawn at the end of the second cycle because of grade 3 diarrhea and infectious colitis.

Table 3. Number of patients with toxicity

Toxicity	By patient (n = 35)		By cycle (n = 154)	
	all grades	grade 3/4	all grades	grade 3/4
Hematologic toxicity				
Leukopenia	32 (91)	9 (26)	114 (74)	12 (8)
Neutropenia	31 (89)	19 (54)	103 (67)	38 (25)
Anemia	35 (100)	7 (20)	150 (97)	15 (10)
Thrombocytopenia	6 (17)	0	9 (6)	0
Febrile neutropenia	–	1 (3)	–	1 (1)
Nonhematologic toxicity				
Nausea	23 (66)	0	57 (37)	0
Vomiting	9 (26)	0	18 (12)	0
Diarrhea	21 (60)	2 (6)	49 (32)	2 (1)
Stomatitis	12 (34)	0	19 (12)	0
Skin rash	7 (20)	0	12 (8)	0
Sensory neuropathy	30 (86)	4 (11)	94 (61)	4 (3)
Hand-foot syndrome	11 (31)	0	32 (21)	0
Alopecia	31 (89)	–	112 (73)	–
AST elevation	19 (54)	0	34 (22)	0
ALT elevation	13 (37)	0	24 (16)	0
ALP elevation	17 (49)	0	34 (22)	0
T-bil elevation	0	0	0	0

Figures in parentheses are percentages. T-bil = Total bilirubin.

Discussion

Most patients with advanced GC receive chemotherapy as a part of palliative therapy. Although oral fluoropyrimidine plus cisplatin-containing regimens are considered to be the standard for advanced GC, such regimens are toxic and cannot be tolerated by many patients with advanced GC because of poor performance status at initial diagnosis. Therefore, a chemotherapy regimen with the least toxicity and sufficient efficacy is desirable, and treatment on an outpatient basis is ideal for patient quality of life. Before conducting clinical trials of FLTAX in patients with deteriorated general condition, we needed to confirm its antitumor activity in advanced GC patients with relatively good performance status [20]. Our current multi-institutional phase II study of the FLTAX regimen showed that the best response rate was 63% and median PFS and OS were 6.8 and 16.2 months, respectively. The toxicity profile of FLTAX was similar to those in previous studies using different administration schedules for protracted 5-FU infusion and paclitaxel [22–25]. The incidence of grade 3 sensory neuropathy (11%) in our study was also identical to previous reports on weekly paclitaxel [26, 27]. Accordingly, FLTAX for advanced GC patients would be a good option and has the advantage of avoiding the use of indwelling venous access devices or ambulatory pumps for outpatient treatment. Furthermore, as the toxicity profile of FLTAX was found to be favorably mild, the FLTAX regimen is also a promising substitute therapy for GC patients who cannot tolerate cisplatin-containing therapy.

Oral fluoropyrimidines such as S-1 and capecitabine have been evaluated in numerous large-scale clinical trials [4–6, 28]. However, they cannot be used in a considerable portion of patients with advanced GC. For example, tumors associated with obstruction of the pylorus or cardia or with peritoneal dissemination as a common characteristic of diffuse-type GC cause dysphagia, nausea, vomiting and intestinal obstruction, often precluding the administration of oral anticancer drugs. Recently, the non-inferiority of leucovorin-modulated weekly bolus 5-FU regimen in comparison with S-1 [29] and good anti-tumor activity of S-1 plus docetaxel [30] in advanced GC patients have been reported in phase III trials. Unfortunately, there are no data directly comparing the antitumor activity of 5-FU plus taxane (paclitaxel or docetaxel) with that of S-1 plus taxane. Although there are limitations in comparing the results of different studies, the efficacy of S-1 plus paclitaxel has been reported in phase II trials with a response rate of 40–55% and a median survival of 9–15 months, and this is comparable to the present data for FLTAX [31–33]. Accordingly, the FLTAX regimen may be a good option for GC patients who are not suitable for treatment with platinum agents.

The median OS in our study was slightly longer than those in previous phase II/III studies for 5-FU or S-1 combined with taxane, although the median PFS was comparable [25, 30–32]. The longer OS in the present study may be related to the better performance status of patients enrolled as compared with that in other studies. Furthermore, most patients in our study received subsequent lines of chemotherapy (table 1). As second-line chemotherapy, 15 patients were given cisplatin-based therapy (cisplatin plus irinotecan for 10, cisplatin plus S-1 for

4, and cisplatin plus 5-FU for 1 patient). Both better performance status and the additional lines of chemotherapy administered in the present study may have contributed to the better OS outcome, as reported previously [34, 35].

The survival outcome of patients with diffuse-type GC is reportedly worse than that of subjects with the intestinal type [9, 36]. Diffuse-type GC has higher malignancy grades, is more resistant to chemotherapy, is more likely to metastasize to the peritoneum causing intestinal obstruction and ascites, and consequently, is more likely to exhibit a poor performance status [11, 12, 37–39]. Therefore, a new effective therapy for diffuse-type GC patients with deteriorated condition is needed. In the subgroup analysis of the present study, the response rate, PFS and OS of FLTAX in diffuse-type GC (21 patients) were favorable and similar to those in intestinal-type cancer (14 patients). The FLTAX regimen would be an effective regimen for GC, irrespective of histological type. The favorable toxicity profile of FLTAX may make it a viable alternative treatment for patients who cannot receive intensive standard platinum regimens. The good toxicity profile might also permit the concurrent use of new targeted agents in such patients.

Subsequent phases of the present study for FLTAX are currently underway. First, a multi-institutional feasibility study of FLTAX for GC patients with massive ascites or inadequate oral intake was conducted [40]. Based on the dose-finding part of the feasibility study (n = 13), the recommended dose was set at 5-FU/L-leucovorin/paclitaxel = 500/250/60 mg/m^2. A subsequent part of the study (n = 25) showed that the objective response rate of ascites was 44%, the ascites control rate was 96%, and the toxicity profile of FLTAX was acceptable in these patients [40]. A randomized clinical trial is currently being planned to examine whether the FLTAX regimen can be a standard first-line treatment for GC patients with massive ascites or inadequate oral intake.

In conclusion, the present multi-institutional phase II study demonstrated that FLTAX would be an active chemotherapy regimen with satisfactory efficacy and safety in advanced GC patients. The desirable toxicity profile of this encouraging non-platinum regimen will serve as the basis for establishing FLTAX as a standard therapy for GC patients in deteriorated general condition, and further clinical trials are warranted.

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Disclosure Statement

We declare no conflicts of interest.

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ORIGINAL ARTICLE

Association of fluoropyrimidines, platinum agents, taxanes, and irinotecan in any line of chemotherapy with survival in patients with advanced gastric cancer

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Abstract
Background Although fluoropyrimidines, platinum agents, taxanes, and irinotecan are used in the treatment of advanced gastric cancer (AGC), it remains unclear whether these agents in any line of chemotherapy are associated with overall survival (OS) in these patients.
Methods We retrospectively analyzed 704 patients with AGC. To avoid possible lead-time bias, we applied time-varying covariate analysis for chemotherapy with four agents in any line.
Results Median OS was 12.3 months. The frequency of exposure to each agent class during all lines of treatment was 92.6% for FU (5-fluorouracil or oral fluoropyrimidine), 48.2% for platinum agents, 65.1% for taxanes, and 39.1% for irinotecan. According to a multivariate Cox model with exposure to each agent class as a time-varying covariate, the hazard ratios (HRs) of death were 0.41 (95% confidence interval [CI], 0.27–0.57; $p < 0.001$) for FU, 0.71 (95% CI, 0.58–0.84; $p < 0.001$) for platinum agents, 0.51 (95% CI, 0.41–0.63; $p < 0.001$) for taxanes, and 0.53 (95% CI, 0.43–0.65; $p < 0.001$) for irinotecan. Although other agents were used in 18.6% of the patients, they did not affect survival.
Conclusions Each of the four agent classes (FU, platinum agents, taxanes, and irinotecan) appears to be independently associated with improved OS in patients with AGC regardless of timing. This result suggests the importance of developing strategies which make these active agents available to all patients with AGC to prolong OS.

Keywords Gastric cancer · Chemotherapy · Fluorouracil · Platinum agent · Paclitaxel · Irinotecan

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Introduction

Gastric cancer is the fourth most common malignancy in the world (989,000 cases in 2008, 7.8% of the total number of cases of malignancy) and the second leading cause of cancer death (737,000 deaths, 9.7% of the total number of cancer deaths) [1]. The prognosis of patients with advanced or recurrent gastric cancer (AGC) remains poor; chemotherapy confers only a minimal survival advantage, with a median survival of approximately 1 year. The most commonly used regimens are combination chemotherapy consisting of a fluoropyrimidine [FU; 5-fluorouracil (5-FU) or oral fluoropyrimidine] plus a platinum agent [2–7]. While docetaxel and anthracyclines are also used in first-line combination regimens in western countries [2, 3], docetaxel or paclitaxel is commonly used as second- or third-line chemotherapy in Japan [4, 8, 9]. Although irinotecan has been evaluated as a first-line agent [10–12], results have shown no survival benefit, partially reflecting its use in post-disease progression in trial control arms [10, 11].

In contrast, a recent small randomized study has suggested its efficacy as second-line chemotherapy [13].

Because these agents are variously used in any of several lines of chemotherapy, in both clinical studies and clinical practice, their individual impact on overall survival (OS) in AGC patients in any line of treatment has not yet been clarified. Here, we used a novel time-varying covariate (TVC) analysis to retrospectively evaluate the impact of exposure to different classes of agents on OS in AGC patients who had undergone chemotherapy.

Patients and methods

Patients

This was a retrospective cohort study of AGC patients who received chemotherapy. Other principal inclusion criteria were the presence of histologically proven, inoperable gastric cancer; Eastern Cooperative Oncology Group performance status (ECOG PS) 0–2; and sufficient bone marrow, liver, and renal function.

Between March 2001 and April 2009, 758 consecutive patients with AGC were treated with chemotherapy in our institution, of whom 704 met the inclusion criteria. FU (5-FU or S-1), cisplatin, docetaxel, and paclitaxel were approved for use and consistently available throughout this period. Written informed consent for treatment was obtained from all patients. Chemotherapeutic regimens were selected individually by the attending physician or within the context of a clinical trial. Dosing and scheduling of most regimens were performed as reported in the literature [4, 8–11, 14].

Statistical methods

The primary objective of this study was to evaluate the association between exposure to each class of chemotherapeutic agent; namely, FU (5-FU or oral fluoropyrimidine), platinum agents (cisplatin or oxaliplatin), taxanes (docetaxel or paclitaxel), irinotecan, or others, and OS.

OS was defined as the number of days from the date of initial chemotherapy to the date of death or last follow-up visit. Disease progression associated with each line of chemotherapy was also measured from the beginning of treatment to the date of disease progression, as evaluated by the attending physician. Vital status and disease status were confirmed by checking medical records at the date of the last follow-up visit. In cases lost to follow-up, vital status was confirmed by census registration, which is conducted annually in Japan.

The impact of exposure to each agent class on OS was evaluated by univariate and multivariate analyses, using a Cox proportional hazards model and presented as hazard ratio (HRs) and 95% confidence intervals (95% CIs). As the length of exposure to each agent class varied over time (i.e., between first-, second-, and third-line treatment), analyses might have been compromised by possible lead-time bias, which would have resulted in a false-positive association between a larger number of chemotherapeutic lines and longer survival. To minimize this potential bias, exposure to each agent class was analyzed as a simple prognostic factor or as a TVC. In addition, because disease progression was the primary reason for proceeding to the next line of chemotherapy, tumor progression during each line of chemotherapy was also included in the TVCs. Each TVC was constructed as a step function initially set at 0 and increased by 1 unit each time the corresponding event was observed (Fig. 1).

Models were constructed using forward and backward stepwise progression, with threshold p values for inclusion or exclusion in the model defined as 0.10 and 0.20, respectively. Other confounding variables considered in the univariate and multivariate analyses were age (<65 vs. ≥65 years), gender (male vs. female), ECOG PS (0–1 vs. 2), histological subtype (diffuse vs. intestinal), disease status (advanced vs. recurrent), prior gastrectomy (no vs. yes), prior adjuvant chemotherapy (no vs. yes), presence of peritoneal metastasis (no vs. yes), presence of liver metastasis (no vs. yes), number of metastatic sites (1 vs. ≥2), first-line chemotherapy (monotherapy vs. FU + platinum agent vs. other combinations), and date of initial chemotherapy (2001–2004 vs. 2005–2009). We applied the Kaplan–Meier product limit method to estimate survival probability at certain time points. Distribution of subject characteristics was assessed by the χ^2 test or Fisher's exact test, as appropriate. Statistical analyses were performed using STATA ver. 10 (Stata Corp LP, College Station, TX,

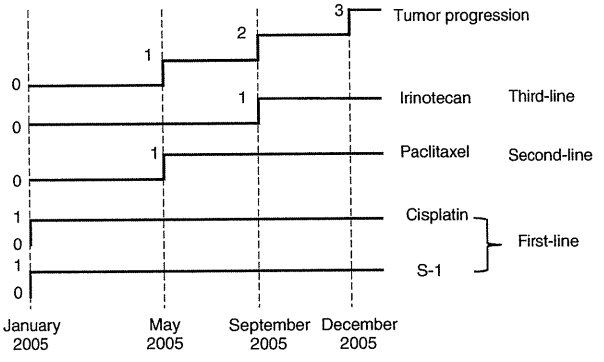

Fig. 1 Time-dependent variables describing exposure to each agent and tumor progression in an individual patient. S1 plus cisplatin was initiated in a patient as first-line chemotherapy in January 2005. Second-line paclitaxel and third-line irinotecan were administered, due to disease progression, beginning in May 2005 and September 2005, respectively

USA). All tests were two-sided, with p values less than 0.05 considered statistically significant.

Results

Patient characteristics

Patient characteristics are summarized in Table 1. Median follow-up at the time of analysis was 47 months (range, 10–108 months). Median OS of all patients was 12.3 months (95% CI; 11.5–13.1 months), with 576 patients (81.8%) already dead. Treatment regimens are shown in Fig. 2. A total of 198 (28.1%) patients received FU plus a platinum agent as first-line chemotherapy. Other patients received other combinations (n = 131; 18.6%) or monotherapy (n = 375; 53.3%). Second-line chemotherapy was used in 513 patients (72.9%), third-line in 264 patients (37.5%), and fourth-line in 117 patients (16.6%). Exposure frequencies to each agent class in all courses of treatment were as follows: FU, 92.6%; platinum agents, 48.2%; taxanes, 65.1%; irinotecan, 39.1%; and other agents, 18.6%. The median OS of patients exposed to one (n = 145; 20.6%), two (n = 233; 33.1%), three (n = 191; 27.1%), or four agents (n = 135; 19.2%) was 6.5 (95% CI, 5.0–8.1), 10.2 (95% CI, 9.1–12.1), 14.8 (95% CI, 12.9–16.5), and 20.4 months (95% CI, 16.2–21.7), respectively.

Table 1 Patient characteristics

Characteristic	All (n = 704)	
	n	%
Median age, years	62	–
Gender		
Male	470	67
Female	234	33
ECOG PS		
0–1	595	85
2	109	15
Histological type		
Diffuse	500	71
Intestinal	204	29
Disease status		
Advanced	472	67
Recurrent	232	33
Prior gastrectomy		
No	349	49
Yes	355	51
Adjuvant chemotherapy		
No	605	86
Yes	99	14
Peritoneal metastasis		
No	334	47
Yes	370	53
Liver metastasis		
No	504	72
Yes	200	28
No. of metastatic sites		
1	358	51
≥2	346	49
First-line chemotherapy		
Monotherapy	381	49
FU + platinum agent	198	28
Other combinations	125	18
Initiation of chemotherapy		
2001–2004	325	46
2005–2009	379	54

ECOG PS Eastern Cooperative Oncology Group performance status, *FU* 5-fluorouracil or fluoropyrimidine

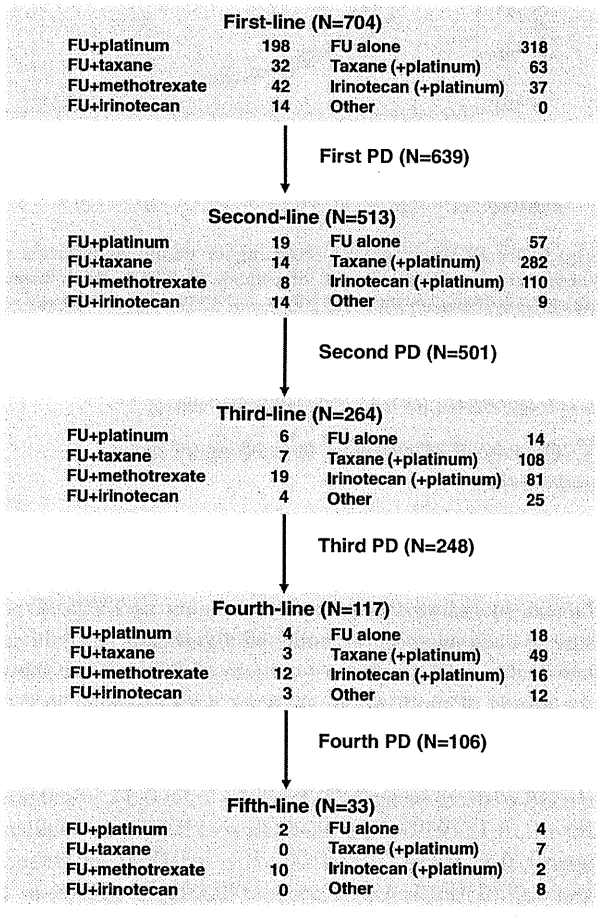

Fig. 2 Treatment regimens and lines of chemotherapy. *FU* 5-Fluorouracil or fluoropyrimidine, *PD* progressive disease

Table 2 Univariate and multivariate analyses with or without TVCs

Baseline and clinical features	Univariate analysis without TVC			Multivariate analysis without TVC			Multivariate analysis with TVC[a]		
	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
Exposure to agents									
FU	0.48	0.35–0.66	*<0.001*	0.37	0.26–0.51	*<0.001*	0.41	0.27–0.57	*<0.001*
Platinum agent	0.67	0.57–0.79	*<0.001*	0.73	0.60–0.88	*0.001*	0.71	0.58–0.84	*<0.001*
Taxane	0.67	0.56–0.79	*<0.001*	0.68	0.56–0.82	*<0.001*	0.51	0.41–0.63	*<0.001*
Irinotecan	0.66	0.56–0.78	*0.006*	0.79	0.65–0.97	*0.022*	0.53	0.43–0.65	*<0.001*
Others	0.92	0.75–1.11	0.47	1.00	0.80–1.26	0.94	0.87	0.69–1.10	0.25

Values in italics denote significant differences

TVC time-varying covariate, *HR* hazard ratio, *CI* confidence interval, *FU* 5-fluorouracil or fluoropyrimidine, *ECOG PS* Eastern Cooperative Oncology Group performance status

[a] Adjusted by age, gender, ECOG PS, histological type, disease status, prior gastrectomy, adjuvant chemotherapy, presence of peritoneal metastasis, presence of liver metastasis, number of metastatic sites, first-line chemotherapy, time of initiation of chemotherapy, and tumor progression during each line of chemotherapy

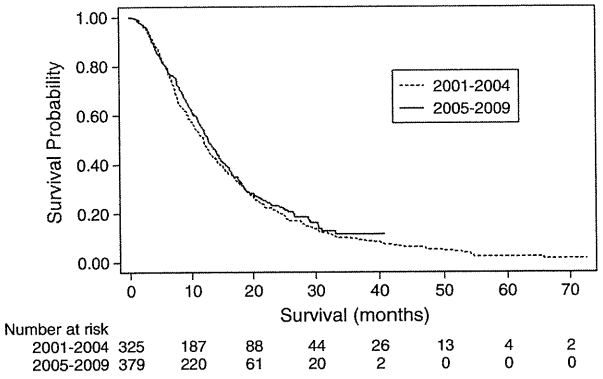

Fig. 3 Overall survival (OS) according to period of initiation of chemotherapy. The median OS of patients in whom chemotherapy was initiated between 2001 and 2004 ($n = 325$) was 12.0 months and that of patients in whom chemotherapy was initiated between 2005 and 2009 ($n = 379$) was 12.5 months, with no statistically significant improvement on multivariate analysis (HR 0.93: 95% CI, 0.78–1.12; $p = 0.44$). *HR* hazard ratio, *CI* confidence interval

TVC analyses of exposure to each agent class and survival

Table 2 shows the results of univariate and multivariate analyses of exposure to each agent class as prognostic factors, including exposure to each agent as TVCs. Exposure to each agent class remained significant on multivariate analyses. The rightmost column of Table 2 also shows the results of multivariate analyses with exposure to each agent class as a TVC. Exposure to each agent class remained significantly associated with better survival, with the HR of death being 0.41 (95% CI, 0.27–0.57; $p < 0.001$) for FU, 0.71 (95% CI, 0.58–0.84; $p < 0.001$) for platinum agents, 0.51 (95% CI, 0.41–0.63; $p < 0.001$) for taxanes, and 0.53 (95% CI, 0.43–0.65; $p < 0.001$) for irinotecan. In contrast, other agents had no impact on survival (HR, 0.87; 95% CI, 0.69–1.10; $p = 0.25$).

Table 3 Exposure to each agent between 2001–2004 and 2005–2009

Agent	Exposure to agents	
	2001–2004 (%)	2005–2009 (%)
FU	94	91
Platinum	40	54*
Taxane	65	62
Irinotecan	36	36
Any three drugs	43	49
Four drugs	18	20

* $p < 0.05$

Comparison of the periods 2001–2004 and 2005–2009

The median OS of patients for whom chemotherapy was initiated between 2001 and 2004 ($n = 325$) was 12.0 months, while that of patients who started on chemotherapy between 2005 and 2009 ($n = 379$) was 12.5 months, showing no statistically significant improvement on multivariate analysis (HR 0.93: 95% CI, 0.78–1.12; $p = 0.44$, Fig. 3). The proportion of patients who received each drug and any three drugs was not significantly different between the two periods, although exposure to platinum was slightly higher in 2005–2009 (Table 3).

Discussion

In this study, we found that each of the four classes of agents examined—FU, platinum agents, taxanes, and irinotecan—was independently associated with improved OS in patients with AGC according to TVC analysis. Although several pivotal studies [2–6, 8, 10–15] and one meta-