gastric cancer who were found to be positive for *MET* amplification by FISH [16]. Further investigation of the efficacy of MET-TKIs in patients with advanced gastric cancer positive for *MET* amplification is thus warranted.

Given the potential of MET-targeted therapy for gastric cancer with *MET* amplification, it is important to determine the prevalence of such gene amplification in patients with unresectable advanced gastric cancer, most of whom are currently treated with systemic chemotherapy. Our present study was limited to gastric cancer patients who underwent gastrectomy, and so further studies will be needed for patients with unresectable advanced tumors. Given the apparent low prevalence of *MET* amplification in gastric cancer, implementation of a sequential approach including screening with a PCR-based copy number assay followed by confirmatory FISH analysis should facilitate the identification of *MET* amplification in a large cohort of patients with unresectable advanced gastric cancer.

MATERIALS AND METHODS

Cell culture

The human gastric cancer cell lines SNU1, SNU5 and Hs746T were obtained from American Type Culture Collection (Manassas, VA); MKN1, MKN7, MKN45, and NUGC3 were from the Health Science Research Resources Bank (Japan Health Sciences Foundation, Tokyo, Japan); KATO III, MKN28, and HSC39 were from Immuno-Biological Laboratories (Gunma, Japan); and SNU216 was from the Korean Cell Line Bank (Seoul National University, Seoul, Korea). All of the cell lines were maintained under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated FBS (Gibco BRL, Grand Island, NY), penicillin, and streptomycin.

Patients

A total of 267 patients with histologically confirmed gastric cancer who had undergone surgery at the National Cancer Center Hospital (Tokyo, Japan) between 1996 and 2006 were included in the study. All the patients had an Eastern Cooperative Oncology Group performance status of 0 to 2. One patient was subsequently excluded as a result of an insufficient quantity of DNA extracted from the corresponding tissue specimen. The specimens from the remaining 266 patients were thus analyzed. The present study was approved by the Institutional Review Board of the National Cancer Center Hospital, and informed consent was obtained from all subjects.

Isolation of genomic DNA

Macrodissection of the surgical specimens preserved as FFPE tissue was performed after removal of paraffin in order to select a region of cancer tissue. Genomic DNA was extracted from the cancer tissue with the use of a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). The DNA concentration of the extracts was determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA).

PCR-based determination of MET copy number

The copy number of *MET* was determined with the use of a TaqMan Copy Number Assay [32] and the Hs05005660_cn (intron 16) primer (Applied Biosystems, Foster City, CA). The *TERT* locus was used as the internal reference, and DNA from noncancerous FFPE tissue was used as a normal control. Real-time PCR was performed in a total volume of 20 μL per well containing 10 μL of TaqMan genotyping master mix, 20 ng of genomic DNA, and each primer. The amplification protocol included an initial incubation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The resulting products were detected with the use of ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Data were analyzed with SDS 2.2 software and Copy Caller software (Applied Biosystems).

FISH

MET copy number per cell was determined by FISH with the use of the c-met / CEN7p Dual Color FISH Probe (GSP Laboratory, Kawasaki, Japan) [22], where CEN7p is the centromeric region of chromosome 7p. The signals were detected by fluorescence microscopy and were evaluated by independent observers (H.K. and I.O.). After screening all entire sections, images of tumor cells were captured and recorded and the signals for 60 random nuclei were counted for an area where individual cells were recognized in at least 10 representative images. Nuclei with a disrupted boundary were excluded from the analysis. Gene amplification was strictly defined by a mean *MET*/CEN7p copy number ratio of >2.2, corresponding to a previous definition of *MET* amplification [16]. The presence of polysomy or an equivocal *MET*/CEN7p ratio (1.8 to 2.2) were thus scored as negative for amplification.

Immunoblot analysis

Immunoblot analysis was performed as described previously [22]. Rabbit polyclonal antibodies to phosphorylated human MET (pY1234/pY1235), to total AKT, to phosphorylated AKT, to phosphorylated

extracellular signal–regulated kinase (ERK), to phosphorylated or total forms of STAT3 were obtained from Cell Signaling Technology (Danvers, MA); those to total ERK were from Santa Cruz Biotechnology (Santa Cruz, CA); those to total MET were from Zymed/Invitrogen (Carlsbad, CA); and those to β-actin were from Sigma. All antibodies were used at a 1:1000 dilution, with the exception of those to β-actin (1:200).

Cell growth inhibition assay

Cells were transferred to 96-well flat-bottomed plates and cultured for 24 h before exposure to various concentrations of JNJ38877605 (Janssen Pharmaceutica NV, Beerse, Belgium) or SGX523 (SGX Pharmaceuticals, San Diego, CA) for 72 h. Tetra Color One (5 mmol/L tetrazolium monosodium salt and 0.2 mmol/L 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku Kogyo, Tokyo, Japan) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems, Boston, MA). Absorbance values were expressed as a percentage of that for nontreated cells, and the IC_{50} values of JNJ38877605 and SGX523 for inhibition of cell growth were determined.

Annexin V binding assay

The binding of annexin V to cells was measured with the use of an Annexin-V-FLUOS Staining Kit (Roche, Basel, Switzerland). Cells were harvested by exposure to trypsin-EDTA, washed with PBS, and centrifuged at 200 × *g* for 5 min. The cell pellets were resuspended in 100 μL of Annexin-V-FLUOS labeling solution, incubated for 10 to 15 min at 15° to 25°C, and then analyzed for fluorescence with a flow cytometer (FACSCalibur) and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ).

Statistical analysis

Overall survival (OS) curves were estimated with the Kaplan-Meier method and compared with the log-rank test. Other statistical analysis was performed with Student's two-tailed *t* test or the chi-square test. A *P* value of <0.05 was considered statistically significant.

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Conflict of interest statement

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Phase II study of bi-weekly irinotecan for patients with previously treated HER2-negative metastatic breast cancer: KMBOG0610B

Hidetoshi Hayashi · Junji Tsurutani · Taro Satoh · Norikazu Masuda · Wataru Okamoto · Ryotaro Morinaga · Masaaki Terashima · Masaki Miyazaki · Isamu Okamoto · Yukihiro Nishida · Shusei Tominaga · Yukihiko Tokunaga · Masahide Yamaguchi · Junichi Sakamoto · Takahiro Nakayama · Kazuhiko Nakagawa

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Abstract

Background A trial was conducted to evaluate the feasibility, efficacy, and safety of biweekly administration of irinotecan, a novel topoisomerase I inhibitor, for patients with metastatic breast cancer (MBC) previously treated with either anthracycline-based or taxane-based chemotherapy.

Methods Eligible patients were HER2-negative, had a performance status of 0 to 2, and had been treated previously with either anthracyclines or taxanes for MBC. Patients received irinotecan intravenously at 150 mg/m^2 on days 1 and 15 every 4 weeks. The primary end-point was feasibility, and the treatment was considered feasible if a patient was able to receive three administrations of irinotecan within the first 8 weeks, as pre-specified in the protocol.

Results Eighteen patients (median age 60 years) were enrolled. Fifteen patients received irinotecan more than 3 times within the first 8 weeks, with resulting feasibility of 83.3%. The median number of treatment cycles was 2 (range 1–16) during this period, and the relative dose intensity was 91.2%. Partial response was observed for one patient, so overall response rate was 5.6%. Nine patients (50.0%) had stable disease, and overall disease control was 50.0%. Median progression-free survival and overall survival periods were 3.2 and 9.6 months, respectively. The only grade 3/4 hematological toxicity was neutropenia (22.2%). Grade 3/4 non-hematological toxicities were anorexia (11.2%), diarrhea (11.2%), and fatigue (5.6%). No treatment-related death occurred.

Conclusions This study demonstrated that biweekly administration of 150 mg/m^2 irinotecan was feasible for

H. Hayashi · J. Tsurutani (✉) · T. Satoh · W. Okamoto · R. Morinaga · M. Terashima · M. Miyazaki · I. Okamoto · K. Nakagawa
Department of Medical Oncology, Kinki University Faculty of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-8511, Japan
e-mail: tsurutani_j@dotd.med.kindai.ac.jp

N. Masuda
Department of Surgery, Breast Oncology, National Hospital Organization Osaka National Hospital, 2-1-14 Hoenzaka, Chuo-ku, Osaka, Osaka 540-0006, Japan

Y. Nishida · S. Tominaga
Department of Breast Surgery, Higashiosaka City General Hospital, 3-4-5 Nishi-Iwata, Higashiosaka, Osaka 578-8588, Japan

Y. Tokunaga
Department of Surgery, Osaka Kita Posts and Telecommunications Hospital, 1-1-6 Nakazaki, Kita-ku, Osaka, Osaka 530-8798, Japan

M. Yamaguchi
Department of Surgery, Matsushita Memorial Hospital, 5-55 Sotojima-cho, Moriguchi, Osaka 570-8540, Japan

J. Sakamoto
Young Leaders Program of Health Care Administration, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan

T. Nakayama
Department of Breast and Endocrine Surgery, Graduate School of Medicine, Osaka University, 2-15 Yamadaoka, Suita, Osaka 565-0871, Japan

patients with MBC treated previously with anthracyclines or taxanes.

Keywords Metastatic breast cancer · Irinotecan · Phase II trial · Topoisomerase I inhibitor · HER2-negative

Introduction

Metastatic breast cancer (MBC) remains an incurable disease, and the main objectives of treatment are prolongation of survival time and palliation. The standard first-line chemotherapy for MBC has been based on anthracyclines or taxanes [1, 2]. For patients with HER2-negative disease, there is limited consensus regarding the use of subsequent chemotherapy if treatment with anthracyclines and taxanes fails, whereas introduction of anti-HER2 therapy has resulted in improved survival of patients with HER2-positive MBC [3]. Anthracyclines and taxanes are the mainstays of adjuvant therapy for breast cancer, but after such therapy tumor cells often develop resistance to the drugs when the cancer recurs. To overcome the problem of refractory disease, drugs with different mechanisms of action would be of great value.

Irinotecan is an inhibitor of topoisomerase I, an enzyme necessary for DNA replication. The irinotecan metabolite, SN-38, binds to the topoisomerase I-DNA complex, preventing the enzyme from resealing the DNA during replication and transcription, and thereby producing DNA breaks, leading to apoptosis.

Irinotecan-based chemotherapy has been shown to be active against metastatic colorectal cancer and small-cell lung cancer. Similarly, this agent had clinical activity against MBC in phase II studies [4–6]. Previous trials involving patients with MBC have used two different irinotecan regimens (every 3 weeks or weekly on day 1 of each of 4 consecutive weeks, followed by a 2-week rest period), and moderate toxicity and promising efficacy have been observed. However, with regard to bi-weekly administration of irinotecan, no systematic studies have been reported in which the safety and efficacy of the regimen for patients with MBC were evaluated [5].

In this study, we investigated the feasibility and efficacy of irinotecan administered bi-weekly for patients with HER-2-negative MBC that had been treated previously with either anthracycline-based or taxane-based chemotherapy.

Patients and methods

Study design and patient selection

This was a multicenter single-arm phase II trial designed to evaluate the feasibility of a bi-weekly schedule of irinotecan for women in whom MBC had recurred or progressed after anthracycline-based or taxane-based chemotherapy, or both. The trial was conducted by the Kinki Multidisciplinary Breast Oncology Group (KMBOG).

Eligible patients were female, aged ≥20 and ≤75 years, and had cytologically or histologically confirmed metastatic breast cancer that was HER2-negative (IHC 0–1 or IHC 2+ confirmed as FISH-negative). Other eligibility criteria included: prior treatment with at least one regimen containing an anthracycline or a taxane for advanced disease, or relapse during or within 6 months for adjuvant chemotherapy, an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of ≤2, and a life expectancy of more than 3 months. Within 14 days of study registration, patients had to have an absolute neutrophil count of ≥1,500/mm^3 or a white blood cell count of ≥3,000, platelets ≥100,000/mm^3, and hemoglobin ≥8.0 g/dL. Also at baseline, creatinine had to be less than the upper limit of normal (ULN), total bilirubin had to be <1.5 mg/dL, and both AST and ALT had to be 2.5 times the ULN.

Patients who had undergone chemotherapy, immunotherapy, biological systemic anticancer therapy, and radiation therapy within ≤2 weeks, and hormonal therapy within 4 weeks of study registration were excluded. Previous administration of irinotecan was not permitted. Also excluded from participation were patients with other serious illnesses and medical conditions, for example uncontrolled infection, intestinal obstruction, persistent diarrhea, interstitial pneumonia, other malignancies, or CNS metastases that were still symptomatic. In addition, women who were pregnant, lactating, or unwilling to use adequate contraception were excluded.

The study was performed according to the precepts established by the Helsinki Declaration. The protocol was approved by the institutional review boards of all the participating centers, and each patient provided written informed consent to participate.

Treatment schedule

Irinotecan was administered at a dose of 150 mg/m^2 in normal saline or 5% dextrose as a 90-min infusion on days 1 and 15 every 4 weeks. Use of hematopoietic growth factors (G-CSF) was allowed for patients with febrile neutropenia. At the investigator's discretion, treatment with analgesics and antiemetics was permitted. Loperamide was also allowed for treatment of diarrhea during and between irinotecan administrations.

Administration of irinotecan on day 1 was delayed by 1 or 2 weeks for patients with diarrhea, >grade 2 non-hematological toxicity, leucopenia, neutropenia, or a platelet count <75,000. On day 15, administration of irinotecan was skipped if the same toxicity occurred. If grade 4

hematological or grade 3 non-hematological toxicity was observed, the dose was reduced to 120 mg/m^2 from the next cycle onwards. If, after dose reduction to 120 mg/m^2, the same toxicity recurred, the treatment was discontinued. No dose re-escalation was allowed after dose reduction.

Treatment was continued until the appearance of grade 4 non-hematological toxicity, patient refusal, disease progression, patient removal for clinical reasons at the investigator's discretion, or death.

Patient evaluation

Pretreatment evaluations included complete medical history, physical examination, tumor evaluation, hematology, and biochemistry, and ECG. Patients were evaluated for toxicity every 2 weeks, in accordance with the National Cancer Institute Common Toxicity Criteria, version 3.0 (NCI-CTC v3.0). Radiological tumor assessments were performed every 2 months to confirm response until progression. The response rate (RR) was defined as the percentage of evaluable patients whose best overall response was classified as either complete response (CR) or partial response (PR) according to the Response Evaluation Criteria in Solid Tumors (RECIST).

Data analysis

Intention-to-treat analysis considering all patients was performed. The primary endpoint was feasibility, and the treatment was regarded as feasible if a patient was able to receive three infusions of CPT-11 within the first 8 weeks, as pre-specified in the protocol. Assuming expected feasibility of 80%, accuracy of 20%, and a threshold completion rate of 60%, a minimum of 16 evaluable patients were required. In consideration of this number, and possible ineligible patients and/or dropouts, the target number of patients for this study was set at 18.

Secondary endpoints included the objective response (CR + PR) rate, median progression-free survival, median overall survival, relative dose intensity, and toxicity. Progression-free survival was calculated as the time from the first day of treatment to the first day of documented progression or death. Survival time was defined as the time from the day of registration to the final date of confirmed survival or the date of death. Analysis was performed with SPSS version 16.0 for Windows (SPSS, Chicago, IL, USA).

Results

Patient characteristics

Eighteen patients were enrolled in the trial between April 2006 and December 2009 (Table 1). All patients were evaluable for toxicity and response. Two-thirds of the patients (12/18) had previously received two or more chemotherapy regimens for advanced disease. In addition, more than three quarters of the patients (14/18) had received both anthracycline-based and taxane-based treatment, including an adjuvant setting. There were measurable lesions in all patients (involving the liver in 5 patients, lymph nodes in 8 patients, and lungs in 12 patients). Six patients had triple-negative breast cancer (TNBC).

Treatment

Eighteen patients received a total of 78 cycles of the therapy (1–18 cycles per patient). The relative dose intensity of irinotecan was 91.5%. Fifteen patients were administered irinotecan more than 3 times within the first 8 weeks, and the feasibility was 88.9%. The median number of treatment cycles was 2 (range 1–16) during this period. Over the course of the trial, 2 patients (11.1%) required dose reduction on day 1 of the second course because of toxicity (grade 3 diarrhea and anorexia, respectively), and treatment interruption was necessary for 7 patients.

Table 1 Patient characteristics (n = 18)

	N (%)
Median age (range)	59 (40–75) years
ECOG performance status	
0	3 (16.7)
1	14 (77.8)
2	1 (5.6)
Receptor status	
ER and/or PR positive	11 (61.1)
ER/PR negative	7 (38.9)
Previous treatment	
Anthracyclines	15 (83.3)
Taxanes	17 (94.4)
Both anthracyclines and taxanes	14 (77.8)
Number of chemotherapy regimens for metastases	
1	1 (5.6)
2	4 (22.2)
3 or more	13 (72.2)
Site of metastasis	
Lung	12 (66.7)
Bone	7 (38.9)
Liver	8 (44.4)
Lymph nodes	5 (27.8)
Chest wall	5 (27.8)

ER estrogen receptor, *PR* progesterone receptor, *ECOG* Eastern Cooperative Oncology Group

Table 2 Frequency of adverse events seen in >10% of patients, attributable to treatment

Adverse event	Highest grade/number of patients (%)		
	Grade 1–2	Grade 3	Grade 4
Fatigue	4 (22.2%)	1 (5.5%)	0 (0.0%)
Nausea	4 (22.2%)	1 (5.5%)	0 (0.0%)
Vomiting	2 (11.1%)	2 (11.1%)	0 (0.0%)
Anorexia	2 (11.1%)	2 (11.1%)	0 (0.0%)
Diarrhea	3 (16.5%)	2 (11.1%)	0 (0.0%)
Neutropenia	4 (22.2%)	3 (16.5%)	1 (5.5%)

Toxicity

Treatment-related toxicity was generally modest and manageable (Table 2). The most common grade 3 and 4 adverse events were neutropenia in 4 patients (22.2%), and febrile neutropenia occurred in one patient only. The other grade 3 adverse events were diarrhea ($n = 2$, 11.1%), anorexia ($n = 2$, 11.1%), and vomiting ($n = 2$, 11.1%). Only one patient experienced a grade 4 adverse event (neutropenia) during the course of the study. No instance of irinotecan-related death was observed.

The primary reason for trial drug discontinuation was disease progression ($n = 15$, 83.3%), followed by toxicity, patient refusal, and deterioration of PS ($n = 1$, 5.6%, respectively). None of the patients had continued receiving irinotecan by March 2011.

Efficacy

The objective response rate was 5.6% (95% CI 0.1–27.3%). An additional 8 patients had SD for ≥4 months, giving a clinical benefit rate (CR + PR + SD ≥ 4 months) of 50.0% (95% CI 26.0–80.0%). The median progression-free survival time was 2.9 months (95% CI 0.7–5.2 months) and the median overall survival period was 10.0 months (95% CI 8.9–11.1 months) (Fig. 1).

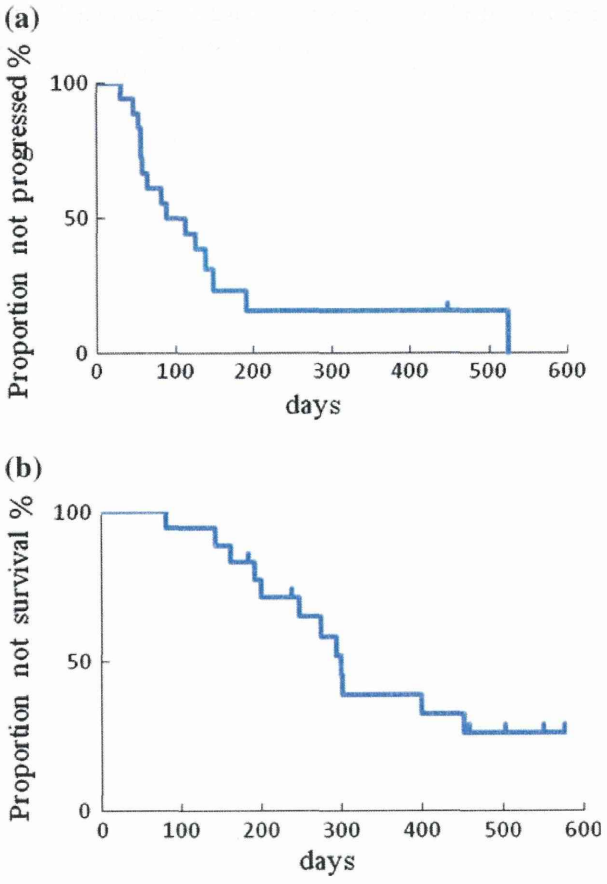

Fig. 1 Progression-free (**a**) and overall (**b**) survival ($N = 18$)

Discussion

In this trial we demonstrated that biweekly administration of 150 mg/m^2 irinotecan was feasible for patients with MBC treated previously with anthracyclines or taxanes. Even in the trial for pre-treated patients in MBC, feasibility was 88.9% and toxicity associated with biweekly administration of irinotecan was predictable and manageable. The results also suggested efficacy was promising, with disease control of 50.0% (95% CI 26.0–80.0%) and median progression-free survival of 2.9 months (95% CI 0.7–5.2 months).

Mono-chemotherapy with irinotecan for advanced MBC has also been investigated by another group, who used two different schedules: every 3 weeks or weekly on day 1 of each of 4 consecutive weeks, followed by a 2-week rest period [6]. Although the dose of irinotecan in that trial was equivalent to that in ours, both grade 3 and 4 hematological and non-hematological toxicity were more manageable for our bi-weekly schedule. In the other trial, weekly and tri-weekly regimens of irinotecan achieved response rates of 14 and 23%, respectively; the median response duration was 4.2 months, and the median OS was 8.6 months (95% CI 7.0–12.3 months). Although response to bi-weekly irinotecan was lower than that in the weekly or tri-weekly schedules, the PFS and OS were similar in both trials. Good tolerability of bi-weekly irinotecan may have contributed to this result, despite the lower response rate.

Recently, eribulin, a tubulin-targeting agent, has been shown to significantly improve overall survival in women with heavily treated MBC in comparison with other available cytotoxic therapy; the response rate was 12% (95% CI 9.4–15.5%), the median PFS 3.7 months (95% CI 3.3–3.9 months), and the median OS 13.1 months (95% CI

11.8–14.3 months) [7]. In several phase II trials, capecitabine, an orally available fluoropyrimidine, has also been shown to achieve a response rate of 15–28%, a median time to progression of 7.5–8.3 months, and a median OS of 10.1–15.2 months [8–11]. Although we are unable to reach any firm conclusion, because of the small size of this feasibility trial, the safety profiles are comparable with those of the other agents described above that had modest anti-tumor effects with good tolerability and survival.

Although a few phase II trials investigating bi-weekly irinotecan for other types of solid tumor were reported more than a decade ago [12], evaluation of feasibility was adopted as primary end point in this study. Recently, numerous patients with MBC received several lines of chemotherapy during their lifetimes, because of the increasing number of active compounds and prolongation of survival time. This study included many patients who had been intensively treated by use of other regimens, and we suppose that heavily-treated patient's profiles with advanced MBC are different from those of patients with other solid tumors. Moreover, several supportive medicines for management of toxicity from chemotherapy have been developed in the last decade, and assessment of the feasibility of bi-weekly irinotecan for this group of patients is meaningful. Given the different situation in the previous trials for other types of cancer, we reasonably evaluated the feasibility of bi-weekly irinotecan in pre-treated MBC and demonstrated that this regimen was quite feasible in this population.

Several studies have linked the *UGT1A1*28* allele to the severe toxicity of irinotecan. Patients homozygous for *UGT1A1*28* are at significantly high risk of severe adverse events, for example diarrhea and myelosuppression because of reduction of UGT1A1 protein expression and SN-38 glucuronidation capacity [13, 14]. In addition, a single nucleotide polymorphism, *UGT1A1*6*, has been proved to reduce the catalytic activity of UGT1A1, and to be associated with drug-induced severe toxicity in Asian populations [15, 16]. In this study, although *UGT1A1* genotypes were not analyzed, grade 3 diarrhea developed in only 2 patients and was manageable, suggesting that bi-weekly administration of 150 mg/m^2 irinotecan is feasible in unselected patients.

In conclusion, this study demonstrated that biweekly administration of 150 mg/m^2 irinotecan is feasible for patients with MBC who have already been heavily treated with anthracyclines and taxanes. Further trials to evaluate the efficacy of this promising regimen for MBC in this setting are warranted. Additionally, we are now conducting another phase II trial which investigates the efficacy and toxicity of irinotecan combined with trastuzumab for extensively pretreated patients with HER2-positive MBC.

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