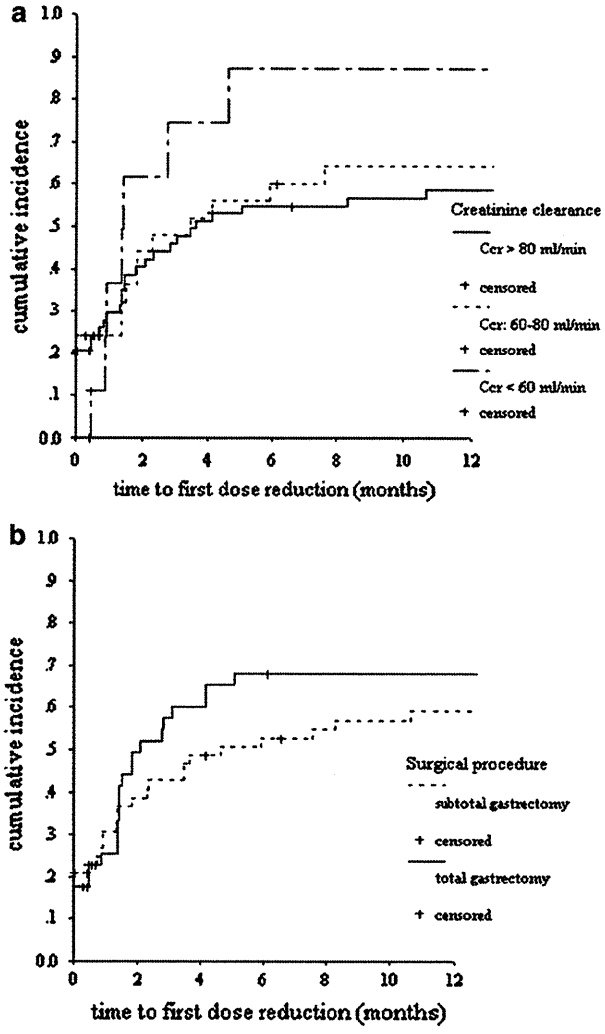

Fig. 2 Cumulative incidence of the first dose reduction. **a** By pretreatment creatinine clearance (*Ccr*) level; Ccr > 80 ml/min ($n = 58$), Ccr 60–80 ml/min ($n = 29$), Ccr < 60 ml/min ($n = 10$). Patients with a lower Ccr level had a greater tendency to require dose reduction ($P = 0.20$). **b** By surgical procedures; subtotal gastrectomy group ($n = 57$), total gastrectomy group ($n = 40$). Patients with total gastrectomy had a greater tendency to require dose reduction ($P = 0.36$)

of patients who continued treatment for the scheduled 12 months was 73%. Twenty-six patients (27%) discontinued treatment (Table 4). The reasons for treatment discontinuation were adverse events in 20 patients, recurrent disease in 2, and other reasons in 4. The median duration until treatment discontinuation was 4.0 months.

We performed univariate analyses using pretreatment patient profiles (sex, age, performance status, surgical procedure, creatinine clearance, and time interval between surgery and first S-1 administration) as the basis of dose reduction during the planned 1-year treatment. Although the univariate analyses revealed no significant predictive factors for dose reduction, the incidence of dose reduction tended to be higher in patients with a low creatinine clearance level and in those who underwent total gastrectomy (Fig. 2). Moreover, no significant difference in recurrence was found depending on the S-1 starting time (within 6 weeks vs. more than 6 weeks; data not shown).

Discussion

The present study demonstrated that in most patients, the planned 1-year adjuvant S-1 therapy for stage II or III gastric cancer could be completed by modifying the dose reduction and treatment schedule. Patients with a low creatinine clearance level at pretreatment and those starting the treatment after total gastrectomy may require careful observation for adverse events, particularly during the early period after treatment.

In the present study, patients with a low creatinine clearance level showed a trend to require dose reduction. Because CDHP, a biochemical modulator of 5-FU, is excreted mainly in the urine, renal function is critical for plasma CDHP clearance. Lower CDHP clearance leads to a prolonged high plasma CDHP concentration, which causes a sustained high plasma 5-FU concentration. This may lead to severe adverse events with an S-1 chemotherapeutic regimen for patients with a low creatinine clearance level. Post-marketing surveillance of S-1 in patients with advanced gastric cancer has demonstrated a close relationship between the incidence of grade 3 or worse hematological toxicities and renal function [11].

With regard to S-1 adverse events, gastrointestinal toxicity has become well recognized, particularly diarrhea, which was the identified dose-limiting toxicity of S-1 in phase I studies in Western countries [12–15]. In the adjuvant setting, these adverse events must be successfully managed to achieve the planned 1-year treatment with S-1. Of the 26 patients who discontinued treatment in the present study, 20 (77%) discontinued due to S-1 adverse events. Persistent gastrointestinal toxicities, even if the grade of adverse events was mild, were the major reasons for patients' refusal to undergo continuous treatment. Therefore, appropriate guidelines must be established for the proper management of adjuvant S-1 therapy in order that the planned 1-year treatment is completed. We recommended to the patients that they skip S-1 administration if they complained of uncomfortable gastrointestinal toxicities. Likewise, we explained to the patients to start taking S-1 again after the relief of symptoms.

Recently, S-1 pharmacokinetic data have demonstrated that the plasma Cmax and AUC of 5-FU after total gastrectomy were significantly higher with S-1 treatment than

these parameters were before surgery [16–18]. Moreover, statistically significant relationships were observed between the grade of S-1-induced diarrhea and AUC, as well as between the plasma Cmax and 5-FU concentration [12, 15]. Therefore, it is possible that S-1 toxicities might be enhanced by gastrectomy. In fact, the present study demonstrated that patients who had undergone total gastrectomy had a higher incidence of dose reduction than patients who had subtotal gastrectomy.

Post-hoc analyses of the ACTS-GC trial showed that patients who completed the planned 1-year S-1 treatment had a longer survival than patients who discontinued treatment [19]. Therefore, it is important to complete the planned 1-year treatment, if necessary by modifying the dose or treatment schedule. An appropriate strategy must be developed to achieve completion of treatment and appropriate management of adverse events. As an example, in our patients, when they could take the full 2-week S-1 treatment from the initial treatment but experienced uncomfortable gastrointestinal toxicities that prevented continuation of S-1 administration, we usually changed the treatment schedule from the original 4-week administration followed by a 2-week rest to 2-week administration followed by a 1-week rest. This change in the treatment schedule allows patients to have an earlier rest, which enables them to recover from the prolonged gastrointestinal toxicities or to discontinue treatment before symptoms develop. On the other hand, when patients had not taken the planned S-1 dosage at the time of examination 2 weeks from the initial treatment and had uncomfortable gastrointestinal toxicities that prevented continuation of S-1 administration, one dose-reduction level could be applied as follows: from 120 to 100 mg/day, from 100 to 80 mg/day, or from 80 to 60 mg/day. Thus, the greatest possible efforts to maintain dose intensity must be made.

As for the patients with dose reduction, there was a high probability of requiring dose reduction during the initial 3 months of the planned 1-year treatment. It is therefore deemed necessary that accurate examination and careful treatment must be carried out in light of the adverse events of S-1, particularly until 3 months after the initial S-1 treatment. Significant predictive factors of dose reduction were sought in the present study and in past studies of adjuvant S-1 treatment. We believe that clarification of the predictive factors of S-1 adverse events remains a major issue, because S-1 has been approved for the treatment of several cancers. In further large prospective trials, the exploration of predictive factors of adverse events, such as gene polymorphisms, is expected.

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ORIGINAL ARTICLE

Neuroendocrine tumors of the stomach: chemotherapy with cisplatin plus irinotecan is effective for gastric poorly-differentiated neuroendocrine carcinoma

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Abstract
Background Neuroendocrine tumors (NETs) occur in various primary sites, but rarely in the stomach. NETs are classified into three types, carcinoids, malignant carcinoids and poorly differentiated neuroendocrine carcinomas (PNECs), whose clinical behavior is different. Currently, clinical outcomes and standard chemotherapy for NETs of the stomach remain unclear.
Methods We conducted a retrospective review of histopathologically confirmed NETs of the stomach at our hospital between January 2000 and August 2006.
Results Thirty-seven NETs were identified. Fifteen patients had carcinoids while 22 had PNECs. Among the carcinoid patients, 7 underwent endoscopic mucosal resection and 5 had gastrectomy as first-line treatment. Three patients were observed without intervention. All patients were alive after an average follow-up period of 27 months. Among the 22 PNEC patients, 3 had no metastasis, 11 had regional lymph node metastasis, and 8 had distant metastasis. Eight of 14 patients relapsed at a median of 177 days (range 120–1459 days) after curative surgery. Twelve patients with metastatic or recurrent disease received palliative cisplatin plus irinotecan chemotherapy. The response rate was 75%, the median progression-free survival time was 212 days, and median survival time was 679 days.
Conclusion Gastric PNEC patients with distant metastasis had poor outcomes. Regimens containing cisplatin plus irinotecan produced a good response in gastric PNEC.

Keywords Carcinoid tumor · Poorly differentiated neuroendocrine carcinoma · Cisplatin · Irinotecan · Stomach

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Introduction

Neuroendocrine tumors (NETs) occur in various primary sites, but rarely in the stomach. Gastric carcinoids account for only 3% of all carcinoid tumors [1]. The World Health Organization classifies endocrine tumors into the following categories: well-differentiated (neuro)endocrine tumors, well-differentiated (neuro)endocrine carcinomas, poorly differentiated (neuro)endocrine carcinomas (PNECs), and mixed exocrine-endocrine tumors [2]. Regarding NETs of the stomach, well-differentiated endocrine tumors and well-differentiated endocrine carcinomas are regarded as carcinoids and malignant carcinoids, respectively. PNEC is mainly regarded as small cell carcinoma.

Well-differentiated NETs and PNECs show different biological behaviors. Carcinoids demonstrate slow growth, whereas PNECs grow rapidly and carry a poor prognosis [3, 4]. Carcinoids are treated by endoscopic mucosal resection (EMR) or surgery, and most patients with carcinoids have localized tumor and a good prognosis, with the 5-year survival for localized gastric carcinoids reported to be 93% [1]. Gastric PNECs without distant metastasis are mainly treated by surgical intervention, whereas PNECs

with distant metastasis or recurrence are treated by chemotherapy. Various types of chemotherapy have been used to treat PNECs or extrapulmonary small cell carcinomas [4–7]; however, there is no standard regimen for metastatic or recurrent gastric PNECs.

Small cell lung cancer (SCLC) is a far more common disease than PNEC, but the two diseases share many clinicopathological features. In SCLC with extended disease, cisplatin plus irinotecan has been associated with better survival than cisplatin plus etoposide [8]. Here we report the clinical outcomes of primary NETs of the stomach and evaluate the efficacy of cisplatin plus irinotecan for gastric PNECs.

Patients and methods

This study was a retrospective analysis of histopathologically confirmed NETs primarily arising in the stomach in patients treated between January 2000 and August 2006 at the National Cancer Center Hospital in Tokyo, Japan. We extracted information on age, sex, disease stage, laboratory findings, radiological findings, pathological findings, therapy, effectiveness of therapy, and outcomes.

The cisplatin plus irinotecan regimen was administered as follows: on days 1 and 15 irinotecan (70 mg/m^2) was given as a 90-min intravenous infusion. Cisplatin (80 mg/m^2) was given via 120-min intravenous infusion on day 1. This regimen was repeated every 4 weeks until the occurrence of tumor progression or severe adverse reactions. The chemotherapy response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) guideline version 1.0. Toxicity was graded according to the Common Terminology Criteria for Adverse Events version 3.0.

Statistical analysis was performed using SPSS version 11 software (SPSS Japan., Tokyo, Japan). Overall survival and progression-free survival curves were constructed by the Kaplan–Meier method.

Results

Thirty-seven patients [male, $n = 30$; female, $n = 7$; median age, 67 years (range 27–82 years)] had NETs arising in the stomach. Of these 37 patients, 15 had carcinoids and 22 had PNECs (16 small cell carcinomas, 4 small cell carcinomas with adenocarcinoma, and 2 large cell carcinomas). Among the carcinoid patients, EMR was performed in 7 patients, including 3 who later underwent surgery; surgery was performed in 5 patients, and 3 patients were observed, due to complication (other cancer) or tumor disappearance after biopsy. No patient had metastasis or carcinoid syndrome. All the patients with carcinoids were alive after an average follow-up period of 27 months (Fig. 1).

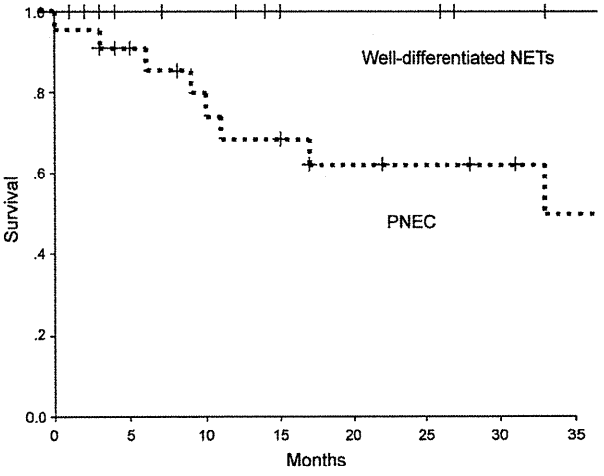

Fig. 1 Survival curves of patients with neuroendocrine tumors (*NETs*) primarily arising from the stomach. All patients with well-differentiated type ($n = 15$) were alive, and the median survival time of patients with poorly differentiated neuroendocrine carcinoma (*PNEC*) was 33 months ($n = 22$)

The clinical data regarding PNEC patients are summarized in Table 1. Among the 22 PNEC patients, 14 had local disease and 8 had distant metastasis. Serum carcinoembryonic antigen (CEA), neuron-specific enolase (NSE), and pro-gastrin-releasing peptide (GRP) were elevated in 23% (5/22), 38% (5/13), and 29% (4/14) of patients, respectively. Surgery was performed in 15 patients (14 patients with localized disease and 1 patient in whom peritoneal dissemination was detected by cytologic diagnosis during surgery). Among the 14 patients with localized disease, 2 patients received adjuvant S-1 chemotherapy after curative surgery. Of those who underwent surgery, only 4 (27%) were diagnosed with neuroendocrine carcinoma or small cell carcinoma from biopsy specimens prior to surgery.

Eight of 14 patients relapsed at a median of 177 days (range 120–1459 days) after curative surgery. A total of 14 patients (7 with unresectable disease at diagnosis, 7 with recurrent disease after surgery) received chemotherapy, and 1 relapsed patient received best supportive care.

The median survival time in the 22 PNEC patients was 33 months (Fig. 1), whereas in those with PNEC with distant metastasis the median survival time was 10.4 months. Twelve patients (5 with recurrent disease, 7 with unresectable disease) received chemotherapy with cisplatin plus irinotecan. The median number of treatment cycles was four. Six patients discontinued treatment because of disease progression, and 3 discontinued therapy because of adverse

Table 1 Clinical data of patients with poorly differentiated neuroendocrine carcinomas

Patient no.	Sex	Age (years)	Stage	CEA[a]	NSE[b]	GRP[c]	Treatment
1	M	61	1	3.7	NE	NE	Surgery, RF
2	M	82	2	3.8	NE	NE	Surgery, RC (liver), Palliation
3	M	71	2	1.1	8.5	13.9	Surgery, RF
4	M	74	2	2.1	14	19.5	Surgery, RC (liver), CX (IP)
5	M	72	2	2.5	33.6	44.6	Surgery, RC (LN), CX (IP)
6	M	61	2	1.5	12.8	42.5	Surgery, RC (liver), CX (IP)
7	F	67	3	4	NE	NE	Surgery, RF
8	M	75	3	2.7	NE	NE	Surgery, RF
9	F	70	3	8	NE	NE	Surgery, RC (LN), CX (S-1)
10	M	59	3	2	NE	NE	Surgery, AD (S-1), RF
11	M	69	3	6.7	NE	NE	Surgery, RC (LN), CX (S-1)
12	M	60	3	51.6	12.7	1039	Surgery, RC (liver), CX (IP)
13	M	75	3	4.5	9.7	21.2	Surgery, AD (S-1), RF
14	M	62	3	3	8.4	21.2	Surgery, RC (LN), CX (IP)
15	M	35	4	1.4	5.2	5.1	Surgery, CX (IP)
16	M	68	4	1.4	197	18	CX (IP)
17	M	62	4	7	89.6	5850	CX (IP)
18	M	27	4	3.2	NE	138	CX (IP)
19	F	61	4	2	3.5	43.5	CX (IP)
20	M	67	4	4.1	62.4	67.8	CX (IP)
21	M	73	4	2.1	181.2	23.6	CX (IP)
22	M	74	4	22.3	NE	NE	Palliation

NE not examined, *RF* relapse-free, *RC* recurrence (region), *CX* chemotherapy, *IP* irinotecan plus cisplatin, *AD* adjuvant chemotherapy, *LN* lymph node

[a] *CEA* carcinoembryonic antigen: normal range <5 ng/ml

[b] *NSE* neuron-specific enolase: normal range <15 ng/ml

[c] *GRP* pro-gastrin-releasing peptide: normal range <46 pg/ml

effects (neurotoxicity, febrile neutropenia, and diarrhea) at a median follow-up period of 29 months. The overall response rate was 75% (8/12) and 2 patients had stable disease. Rates of grade 3/4 neutropenia and diarrhea were 58% (7/12) and 17%, respectively, but there were no treatment-related deaths. The median progression-free survival (PFS) time was 212 days (95% confidence interval [CI] 121–302) and the median survival time was 679 days (95% CI 39–1319). No patient had brain metastasis during the time of observation.

Discussion

NETs arise from the widely distributed neuroendocrine cell system. Histologically, NET cells are argentophilic and express endocrine markers such as chromogranin or NSE. NETs of the stomach are classified into the following three categories; carcinoids, malignant carcinoids, and PNECs or small cell carcinomas, on the basis of clinical and pathological features [2, 9]. However, some confusion remains with regard to the histological classification.

Gastric carcinoids account for about 0.3% of all gastric tumors. Gastrointestinal small cell carcinomas account for about 0.1–1.0% of all gastrointestinal tumors, and gastric small cell carcinomas account for approximately 11% of gastrointestinal small cell carcinomas [4]. As for NETs of the stomach, Rindi et al. [10] reviewed the histology of 55 gastric endocrine tumors and reported 46 cases of well-differentiated tumors and 9 of neuroendocrine carcinomas. They further investigated 205 gastric endocrine tumors and reported 193 cases of well-differentiated and 12 of poorly differentiated tumors [11].

Well-differentiated NETs (carcinoids and malignant carcinoids) and PNECs have different biological behaviors. The 5-year survival rate of carcinoids without metastasis was reported to be 93–98% [1, 3]. In carcinoids with metastasis, the 5-year survival rate was reported to be 0–75%. The cumulative crude survival rate of PNEC was only 33.3% at 5 years after diagnosis. Carcinoid patients

have better survival than patients with PNEC. In our study, the difference in survival between carcinoid and PNEC was similar to that in previous studies.

Standard treatment of patients with localized gastric carcinoids involves endoscopic resection or surgical excision [9]. For functioning tumors (carcinoid syndrome) as well, surgery is the primary treatment option. For patients with metastasis, management is not well defined and thus they are treated using several strategies, including surgery, biotherapy, and chemotherapy. Rinke et al. [12] reported that a somatostatin analog retarded tumor progression in patients with functionally active and inactive metastatic midgut NETs. We had only patients with small and/or localized carcinoids; thus, resection or observation was performed and they showed a good prognosis.

There are no standard chemotherapy regimens for gastric PNEC. Surgery is performed to treat localized disease in patients with gastric PNEC. Matsui et al. [13] reported 17 patients with gastric small cell carcinoma who underwent surgery; 3 patients without metastasis survived for 6–20 months after the surgery and 14 patients with metastasis died 5–22 months after the surgery at the end of their observation. In a review of 54 patients with gastric small cell carcinoma [14], 3 patients without distant metastasis survived for more than 2 years after gastrectomy with dissection of regional lymph nodes. In our study, although the relapse rate was high, there were 2 long-term survivors who showed no relapse for more than 3 years after surgery. Although the effectiveness of surgery is limited, it remains one of the most important modalities for treating gastric PNEC.

For patients with gastric adenocarcinoma, adjuvant S-1 chemotherapy is effective [15], and chemoradiotherapy is effective for limited stages of SCLC [16]. Further studies that explore factors associated with relapse and assess the efficacy of adjuvant chemotherapy or chemoradiotherapy for gastric PNEC are needed. Prophylactic cranial irradiation is standard therapy in patients with limited SCLC, due to the high rate of brain metastasis [17]. In our study, no patients had brain recurrence; thus, it is unclear whether or not prophylactic cranial irradiation is effective for gastric PNEC.

There is no standard regimen for metastatic or recurrent gastric PNECs, which are typically treated according to the treatment guidelines for SCLC. The standard chemotherapy for SCLC is a combination regimen containing cisplatin. Noda et al. [8] reported that patients treated with cisplatin plus irinotecan had better outcomes than patients treated with cisplatin plus etoposide, with median survival times in the two groups of 12.8 and 9.4 months, respectively. On the other hand, there are few reports on chemotherapy for PNEC other than the chemotherapy used for SCLC. Moertel et al. [5] reported that a regimen containing cisplatin plus etoposide produced a good response rate (67%) in 18 patients with neuroendocrine carcinomas. Mitry et al. [6] obtained a response rate of 41.5% in 41 PNEC patients, with a PFS of 8.9 months and an overall survival of 15 months. These studies included only a few patients with gastric PNECs. A previous study showed good response of PNEC to combination chemotherapy with paclitaxel, carboplatin, and etoposide; however, the study included only 1 patient with gastric neuroendocrine carcinoma [7]. Kulke et al. [18] reported a very low response rate of 6.6% to cisplatin plus irinotecan for extrapulmonary NETs, although 78% (14/18) of their patients had well-differentiated NETs.

In our study, treatment with cisplatin plus irinotecan was effective against gastric PNECs, with an overall response rate of 75% and a PFS of 212 days. We consider the toxicity of this regimen tolerable. Of note, gastric PNECs often have components of adenocarcinoma [13], and cisplatin plus irinotecan has been shown to be effective against gastric adenocarcinoma [19]. Therefore, we consider this regimen suitable for gastric PNEC, although the present retrospective study has several limitations. We are now planning a prospective study of this cisplatin plus irinotecan regimen in PNEC patients.

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FGFR2 gene amplification and clinicopathological features in gastric cancer

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BACKGROUND: Frequency of *FGFR2* amplification, its clinicopathological features, and the results of high-throughput screening assays in a large cohort of gastric clinical samples remain largely unclear.

METHODS: Drug sensitivity to a fibroblast growth factor receptor (FGFR) inhibitor was evaluated *in vitro*. The gene amplification of the *FGFRs* in formalin-fixed, paraffin-embedded (FFPE) gastric cancer tissues was determined by a real-time PCR-based copy number assay and fluorescence *in situ* hybridisation (FISH).

RESULTS: *FGFR2* amplification confers hypersensitivity to FGFR inhibitor in gastric cancer cell lines. The copy number assay revealed that 4.1% (11 out of 267) of the gastric cancers harboured *FGFR2* amplification. No amplification of the three other family members (*FGFR1*, *3* and *4*) was detected. A FISH analysis was performed on 7 cases among 11 *FGFR2*-amplified cases and showed that 6 of these 7 cases were highly amplified, while the remaining 1 had a relatively low grade of amplification. Although the difference was not significant, patients with *FGFR2* amplification tended to exhibit a shorter overall survival period.

CONCLUSION: *FGFR2* amplification was observed in 4.1% of gastric cancers and our established PCR-based copy number assay could be a powerful tool for detecting *FGFR2* amplification using FFPE samples. Our results strongly encourage the development of FGFR-targeted therapy for gastric cancers with *FGFR2* amplification.

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Intensive investigations of anticancer treatments for gastric cancer have been done over the past three decades; however, the prognosis for patients with unresectable advanced or recurrent gastric cancer remains poor (Bittoni *et al*, 2010; Fujii *et al*, 2010), and new therapeutic modalities are needed.

Fibroblast growth factors (FGFs) and their receptors are considered to be associated with multiple biological activities, including fundamental developmental pathways, cellular proliferation, differentiation, motility and transforming activities (Itoh *et al*, 1994; Moffa *et al*, 2004; Grose and Dickson, 2005). Fibroblast growth factor signalling is also involved in many physiological roles in the adult organism, such as the regulation of angiogenesis and wound repair, and FGF receptors (FGFRs) are expressed on many different cell types and regulate key cell behaviours of cancer cells (Turner and Grose, 2010). Emerging evidence has demonstrated that the deregulation of FGF signalling is frequently observed in various solid cancers and haematological malignancies (Beenken and Mohammadi, 2009). The most well-known association with *FGFR* mutations is the *FGFR3* mutation observed in bladder cancer, in which somatic mutations in coding regions are observed in about 50% of all specimens (Cappellen *et al*, 1999; Turner and Grose, 2010). Other genetic alterations in *FGFR3* include gene amplification in bladder cancer and translocation in myeloma (Turner and Grose, 2010). Similarly, the deregulation of FGF signalling has been reported in various malignancies. Glioblastoma exhibits FGFR1 kinase domain gain-of-function mutations, and FGFR1 is abnormally activated in malignant prostate cells. In 8p11 myeloproliferative syndrome, translocations fuse different proteins in frame with the FGFR1 kinase domain, causing the constitutive dimerisation of the kinase (Giri *et al*, 1999; Rand *et al*, 2005; Beenken and Mohammadi, 2009). The *FGFR1* amplification has been reported in approximately 10% of breast cancers (Courjal *et al*, 1997) and oral squamous carcinomas, and has been also found at a low incidence in ovarian cancer, bladder cancer and rhabdomyosarcoma (Turner and Grose, 2010). *FGFR2* mutations are observed in 12% of endometrial cancers but are reportedly rare in gastric cancers (Jang *et al*, 2001; Dutt *et al*, 2008). The *K-sam* gene was first identified and characterised as an amplified gene in the human gastric cancer cell line KATO-III (Hattori *et al*, 1990; Ueda *et al*, 1999), and its product was later found to be identical to the bacteria-expressed kinase, or

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keratinocyte growth factor receptor, and FGF receptor 2 (FGFR2). *FGFR2* amplification has been found in diffuse-type gastric cancer-derived cell lines and the amplification was preferentially detected in diffuse-type gastric cancer. FGFR2 protein overexpression was detected using immunohistochemical staining in 20 of 38 advanced cases of diffuse-type gastric cancer (Hattori *et al*, 1996). FGFR2 protein expression was observed in 31% of the gastric carcinomas and was positively correlated with scirrhous cancer, a diffuse type, the invasion depth, the infiltration type and a poor prognosis (Toyokawa *et al*, 2009).

On the other hand, along with another group, we previously reported that *FGFR2* amplification confers hypersensitivity to FGFR inhibitor in gastric cancer cell lines both *in vitro* and *in vivo* (Nakamura *et al*, 2006; Takeda *et al*, 2007), strongly suggesting that *FGFR2* amplification may be a promising molecular target for the treatment of *FGFR2*-amplified gastric cancer. However, very limited information on *FGFR2* amplification is available regarding the frequency, the degree of the increase in the copy number, the histology and a high-throughput screening method in gastric cancer. In this report, we retrospectively studied these issues using formalin-fixed, paraffin-embedded (FFPE) samples in patients with gastric cancer who underwent surgery in an attempt to advance FGFR2-targeted therapy for gastric cancer.

MATERIALS AND METHODS

Cell culture

All of the gastric cancer cell lines used in this study were maintained in RPMI-1640 medium (Sigma, St Louis, MO, USA), except for IM95 (DMEM; Nissui Pharmaceutical, Tokyo, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY, USA), penicillin and streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. IM95 and OCUM1 were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan) and the others were provided from National Cancer Center Research Institute (Tokyo, Japan).

Patients

A total of 267 patients with histologically confirmed gastric cancer who had undergone surgery at the National Cancer Center Hospital between 1996 and 2006 were included in this study. All the patients in this series had an Eastern Cooperative Oncology Group performance status of 0 to 2 and had undergone surgery. Of these patients, one subject was excluded because an insufficient quantity of DNA was extracted from the patient's specimen. Thus, samples from the remaining 267 patients were analysed. This study was approved by the institutional review board of the National Cancer Center Hospital.

Isolation of genomic DNA

Genomic DNA samples were extracted from surgical specimens preserved as FFPE tissue using a QIAamp DNA Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Macro-dissection of the FFPE samples was performed to select a cancer region, which was marked by a pathologist after deparaffinisation. The DNA concentration was determined using the NanoDrop2000 (Thermo Scientific, Waltham, MA, USA).

Real-time reverse-transcription PCR (RT-PCR)

cDNA was prepared from the total RNA of each cultured cell line using a GeneAmp RNA-PCR kit (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR amplification was carried out using a Thermal Cycler Dice (Takara, Otsu, Japan) in accordance with the manufacturer's instructions under the following conditions: 95 °C for 5 min, and 50 cycles of 95 °C for 10 s and 60 °C for 30 s. The primers used for the real-time RT-PCR were as follows: *FGFR2*, forward 5′-GATAAATACTTCCAATGCAGAAGTGCT-3′ and reverse 5′-TGCCCTATATAATTGGAGACCTTACA-3′; *GAPDH*, forward 5′-GCACCGTCAAGGCTGAGAAC-3′ and reverse 5′-ATGGT GGTGAAGACGCCAGT-3′. *GAPDH* was used to normalise the expression levels in the subsequent quantitative analyses.

Immunoblotting

A western blot analysis was performed as described previously (Matsumoto *et al*, 2009). The following antibodies were used: monoclonal FGFR2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin antibody and HRP-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA).

Cell growth inhibitory assay

To evaluate growth inhibition in the presence of various concentrations of PD173074 (Sigma), we used an MTT assay and a previously described method (Kaneda *et al*, 2010). Briefly, the cells were seeded at a density of 2×10^3 cells per well in 96-well plates. After 24 h, PD173074 was added and the incubation was further continued for 72 h at 37 °C. The assay was conducted in triplicate.

Copy number assay for four FGFR family genes

The copy numbers for *FGFR 1–4* were determined using commercially available and pre-designed TaqMan Copy Number Assays according to the manufacturer's instructions (Applied Biosystems). The primer IDs used for *FGFRs* were as follows: *FGFR1*, Hs02862256_cn; *FGFR2*, HS05182482_cn (intron 14) and Hs05114211_cn (intron 12); *FGFR3*, Hs03518314_cn; and *FGFR4*, Hs01949336_cn. The *TERT* locus was used for the internal reference copy number. Human Genomic DNA (Takara) was used as a normal control. Real-time genomic PCR was performed in a total volume of 20 μl in each well, containing 10 μl of TaqMan genotyping master mix, 20 ng of genomic DNA and each primer. The PCR conditions were 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min; the resulting products were detected using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Data were analysed using SDS 2.2 software and CopyCaller software (Applied Biosystems).

Fluorescence *in situ* hybridisation analysis

The fluorescence *in situ* hybridisation (FISH) method was previously descried (Motoi *et al*, 2010). Probes designed to detect the *FGFR2* gene and the *CEN10p* on chromosome 10 were labelled with fluorescein isothiocyanate or Texas red and were designed to hybridise to the adjacent genomic sequence spanning approximately 0.33 and 0.64 Mb, respectively. The probes were generated from appropriate clones from a library of human genomic clones (GSP Laboratory, Kawasaki, Japan). Deparaffinised tissue sections were air dried and pre-treated with the GSP paraffin pre-treatment kit (GSP Laboratory). In all, 10 μl of fluorescent FISH probe was heated for 5 min at 73–75 °C in a waterbath for denaturation. The tissue sections were then placed in a denaturant solution (70% formamide/2 × saline sodium citrate (SSC) pH 7-8) in a 73–75 °C waterbath, denatured for 5 min, dehydrated in 70 and 100% ethanol for 1 min each at room temperature, and air-dried. Denatured probes were applied, and the specimens were covered with a coverglass and placed on a heated block at 45–50 °C. Then, the slides were sealed with rubber cement and placed in a pre-warmed humidified box overnight at 37 °C. Stringent washing was performed using 2 × SSC/0.3% NP-40 at room temperature and at 72 °C for 5 min and then with 2 × SSC at room temperature. The signals were observed using fluorescence microscopy, and the

FISH signals were evaluated by independent observers (TM and AK). After screening all the complete sections, images of the tumour cells were captured and recorded and the signals for 20 random nuclei were counted for an area where individual cells were recognised on at least 10 representative images. The positive result of copy number gain is determined as follows (FGFR2/CEN10p $\geqslant$ 2.0).

Statistical analysis

The statistical analyses of the clinicopathological features were performed using the Student *t*-test and the χ^2 test using PAWS Statistics 18 (SPSS Japan Inc., Tokyo, Japan). The overall survival (OS) curves were estimated using the Kaplan–Meier method.

RESULTS

FGFR2 amplification confers hypersensitivity to FGFR inhibitor in gastric cancer cell lines

We examined the growth inhibitory effect of PD173074 (0.004–80 μM) on four *FGFR2*-amplified (HSC-43, TU-KATPIII, SNU-16 and HSC-39) and four non-amplified (44As3, 58As1, IM95 and OCUM1) gastric cancer cell lines. The *FGFR2* amplification status of each cell line had already been examined using a CGH analysis (unpublished data). The mRNA and protein expressions of FGFR2 were overexpressed in the *FGFR2*-amplified cell lines (Figures 1A and B). A growth inhibitory assay showed that the IC_{50} values of the FGFR inhibitor PD173074 in *FGFR2*-amplified cells were 0.01–0.07 μM, whereas those in non-amplified cells were 2.6–13.2 μM, indicating that *FGFR2* amplification conferred an approximately 100-fold hypersensitivity to FGFR inhibitor in gastric cancer cell lines (Figure 1C).

FGFR2 amplification in clinical gastric cancer cell lines and surgical specimens

To develop a high-throughput method for detecting *FGFR2* gene amplification in a clinical setting, we verified a real-time PCR-based detection method, the TaqMan Copy Number Assay. The *FGFR2* copy number was 1.4–2.7 copies in the four non-amplified cell lines; however, the numbers in the four *FGFR2*-amplified cell lines were 28.2, 231.7, 88.2 and 36.3 copies, respectively (Figure 1D). In addition, another primer in intron 12 of *FGFR2* produced a very similar result ($R = 0.99$, Figure 1D). Collectively, these results suggested that a DNA copy number assay for *FGFR2* was a sensitive and reproducible method. We also examined the copy numbers of *FGFR1*, *FGFR3* and *FGFR4*, but no obvious gene amplification was observed in all of the eight cell lines (Figure 1D). Next, *FGFR2* amplification was evaluated using the copy number assay in 267 FFPE samples of primary gastric cancer specimens. *FGFR2* amplification of more than 5 copies was observed in 11

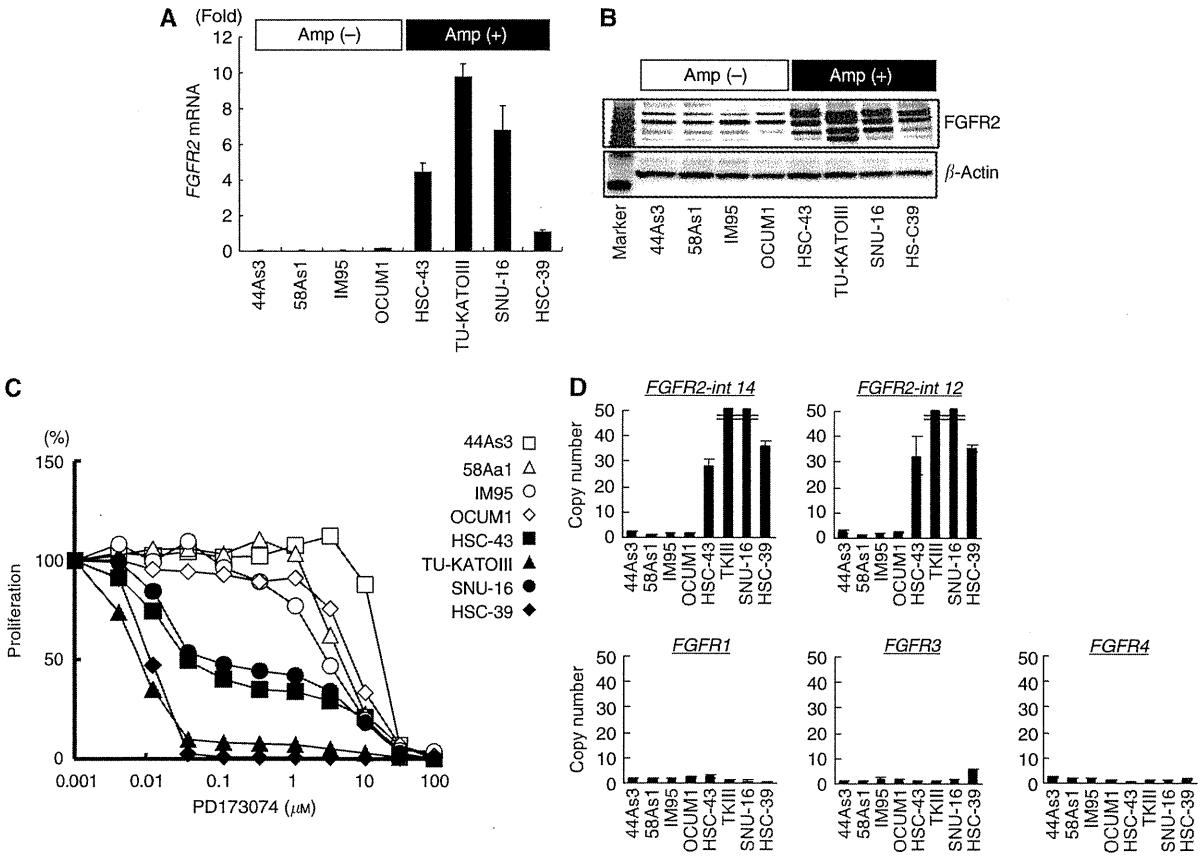

Figure 1 *FGFR2* amplification in gastric cancer cell lines. (**A**) The mRNA expression levels of *FGFR2* were determined using real-time RT–PCR for eight gastric cancer cell lines. *FGFR2* mRNA: normalised mRNA expression levels (*FGFR2*/*GAPDH* $\times 10^3$). (**B**) Western blot analysis for FGFR2 expression. β-Actin was used as an internal control. Marker, molecular marker. (**C**) Growth inhibition assay for the FGFR inhibitor PD173074, evaluated at the indicated concentrations using an MTT assay. (**D**) Evaluation of DNA copy number assay using gastric cancer cell lines. A TaqMan copy number assay was performed to determine the copy number using specific primers for the genomic loci of the *FGFR1–4* genes against DNA samples. Amp, gene amplification. FGFR2-int-14 and FGFR2-int-12, different primers for intron 14 or intron 12 of *FGFR2*.

cases (92.0, 63.0, 41.4, 19.9, 18.4, 13.7, 8.3, 6.2, 6.2, 5.7 and 5.6 copies), with a frequency of 4.1% (Figure 2A). The mean copy number in the non-amplified cases was 2.4 ± 0.6 copies. Meanwhile, no obvious gene amplification of *FGFR1*, *FGFR3* or *FGFR4* was observed (data not shown).

FISH analysis for *FGFR2* amplification

We used a FISH analysis to examine *FGFR2* amplification in the same samples to verify the results of the above PCR-based DNA copy number assay. Highly amplified TU-KATOIII cells showed numerous and large clustered signals, whereas non-amplified OCUM1 cells contained two normally paired signals (Figure 2B). A FISH analysis was performed on seven cases among 11 *FGFR2*-amplified cases and two non-amplified cases. The FISH analysis revealed that *FGFR2* was highly amplified in six of the seven *FGFR2*-amplified clinical samples (four showed multiple scattered signals and two showed large clustered signals), while the remaining sample exhibited a relatively low grade of amplification (FGFR2/CEN10p = 2.2, Figure 2B). The *FGFR2* signals in the G3 and G10 samples, which were determined not to be amplified based on the results of the DNA copy number assay, were not increased. These results clearly demonstrated the presence of *FGFR2*-amplified gastric cancers among clinical samples.

Clinicopathological features of *FGFR2*-amplified gastric cancer

We evaluated the clinicopathological features including age, sex, histology and pathological stage according to the *FGFR2* amplification status. Patients age with *FGFR2* amplification were significantly higher than the others, but sex and pathological stage were not associated with *FGFR2* amplification in this study (Table 1). Among the patients with *FGFR2* amplification, the

Table 1 Frequency of *FGFR2* amplification in gastric cancers and its association with clinical and pathologic factors

	FGFR2 (+)		FGFR2 (−)		
	n = 11	%	*n* = 256	%	*P*-value
Age					
Range	55–91		31–88		0.047
Median	67		63		
Gender					
Male	11	100	173	68	0.052
Female	0	0	83	32	
Pstage					
I	0	0	25	10	0.16[a]
II	0	0	32	13	
III	3	27	73	29	
IV	8	73	125	49	
Unknown	0	0	1	0	
Histology					
Tub1	0	0	41	16	0.55[b]
Tub2	2	18	51	20	
Pap	1	9	5	2	
Muc	2	18	8	3	
Sig	1	9	15	6	
Por1	0	0	28	11	
Por2	5	45	108	42	

Abbreviations: Amp = gene amplification; FGFR = fibroblast growth factor receptor; Muc = mucinous adenocarcinoma; Pap = papillary adenocarcinoma; Por = poorly differentiated adenocarcinoma; pStage = pathological stage; Sig = signet ring-cell carcinoma; Tub = tubular adenocarcinoma. [a]Comparison between pStage I+II and III+IV. [b]Comparison between intestinal (Tub1, Tub2 and Pap) and others. *P*-values were calculated using the *t*-test for age and the χ^2 test for the other variables.

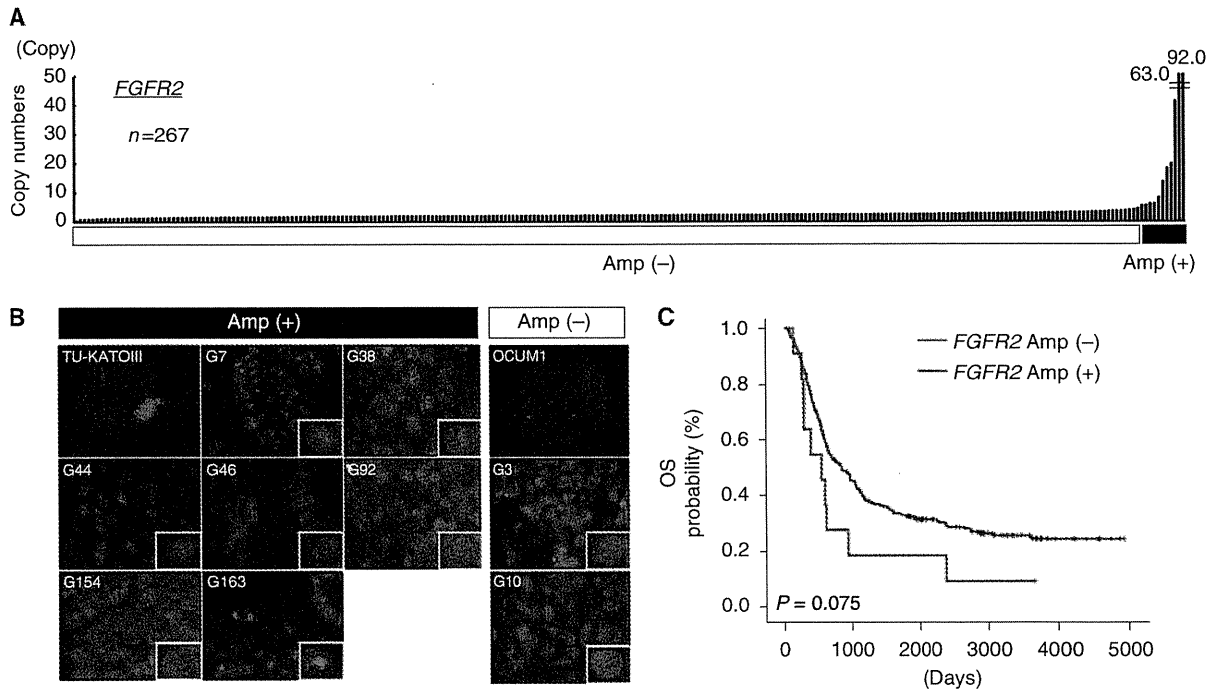

Figure 2 (**A**) Amplification of *FGFRs* in surgical specimens of gastric cancer. A TaqMan copy number assay for *FGFR2* was performed using DNA samples obtained from 267 FFPE samples. Human normal genomic DNA was used as a normal control. *FGFR2* amplification over 5 copies was observed in 11 cases (92.0, 63.0, 41.4, 19.9, 18.4, 13.7, 8.3, 6.2, 6.2, 5.7 and 5.6 copies). (**B**) Fluorescence *in situ* hybridisation analysis of *FGFR2*-amplified KATO-III cells, non-amplified OCUM1 cells and nine surgical specimens of gastric cancer. Green, signal of *CEN10P* locus; Red, signal of *FGFR2* locus; G3 ~ G92, sample numbers; Amp, gene amplification. High-power images are presented for a single cancer cell. (**C**) Overall survival in *FGFR2*-amplified gastric cancer. Kaplan–Meier curves for OS according to the *FGFR2* amplification status.

Table 2 Summary of *FGFR2*-amplified gastric cancers

No.	Age	Sex	Location	Size of lesion (cm)	Macroscopic type[a]	Lauren's classification	Histology	pStage	OS (days)	FGFR2 (CN)	FISH (type, copies)
G7	55	M	Lower	8.5 × 8	3	Diffuse	Muc > Por2, Sig	IV	612	41.4	LC, +++
G38	70	M	Upper	8.5 × 8	I + IIc	Intestinal	Pap > Tub1, Tub2, Por2	IIIa	591	92.0	MS, +++
G44	70	M	Lower	9.5 × 8	3	Diffuse	Por2 > Pap, Tub1, Muc	IIIa	938	5.6	Low, 2.2[b]
G46	75	M	Middle	10 × 6	4	Intestinal	Tub2 > Por2	IV	2380	13.7	MS, +++
G92	75	M	Middle	6.5 × 5.5	3	Diffuse	Por2 > Tub2	IV	280	19.9	MS, +++
G154	59	M	Middle	14 × 12	4	Diffuse	Por2	IV	132	5.7	MS, +++
G163	64	M	Lower	15 × 10	3	Diffuse	Muc > Sig > Tub2	IV	540	6.2	LC, +++
G203	64	M	Lower	10.5 × 6.5	4	Diffuse	Sig > Por2 > Muc	IV	283	8.3	ND
G271	91	M	Upper	7 × 6.5	2	Intestinal	Tub2 > Por1	IV	383	63.0	ND
G299	65	M	Middle	20 × 20	4	Diffuse	Por2 > Sig	IV	256	6.2	ND
G329	67	M	Middle	6.5 × 6	3	Diffuse	Por2 > Sig	IIIa	3642+	18.4	ND

Abbreviations: CN = copy number of *FGFR2* determined using a copy number assay; Diffuse = diffuse-type gastric cancer; FISH = fluorescence *in situ* hybridisation; FGFR2 = fibroblast growth factor receptor 2; Intestinal = intestinal-type gastric cancer; Location = tumor location in stomach; LC = large clustered signals; Low = low copy number gain; M = male; MS = multiple scattered signals; ND, not determined; No. = sample numbers; OS = overall survival; pStage = pathological stage; +++ = numerous *FGFR2* signals; + = patients. alive. [a]Macroscopic type, classification is based on the definitions of the Japanese Research Society for Gastric Cancer. [b]Ratio of FGFR2/CEN10p.

histologies of two cases were intestinal-type gastric cancer and one was unclassified type, while the others were diffuse-type (Table 2). The tumours were located in either the upper or lower stomach. These results are summarised in Table 2. Finally, we examined the prognostic impact of *FGFR2* amplification on OS after surgery. *FGFR2* amplification tended to be associated with a poorer outcome, compared with non-amplified cases, but no significant difference was observed in the current study (log-rank test, $P = 0.075$; Figure 2C).

DISCUSSION

To date, several studies have reported on the protein expression of FGFR2 and clinicopathological analyses using immunohistochemistry, with 20 of 49 (41%) and 42 of 134 (31%) gastric cancers expressing FGFR2 protein when evaluated using positive or negative staining (Hattori *et al*, 1996; Toyokawa *et al*, 2009). Regarding genomic alteration, the frequency of *FGFR2* amplification has been reported to be 3 out of 19 (16%, among diffuse-type gastric cancers) detected using comparative genomic hybridisation (CGH), 3 out of 57 (5%) detected using Southern blot analysis, and 2 out of 30 (7%) detected using CGH (Tsujimoto *et al*, 1997; Peng *et al*, 2003; Kim *et al*, 2010). These results suggest that the frequency of *FGFR2* amplification is around 5%, which is lower than the positive staining results obtained using immunohistochemistry. However, the frequency of amplification has not been determined in a large cohort. Our results indicated that the frequency of *FGFR2* amplification was 4.1% (11 out of 267), consistent with these previous reports on genomic alterations. To select a sub-population of gastric cancers sensitive to FGFR inhibitors in the future, gene amplification may be a more suitable biomarker than positive staining using immunohistochemistry based on the results of preclinical studies (Figure 1, Takeda *et al*, 2007).

In six cases, the copy number of *FGFR2* was larger than 10 copies and numerous signals were observed by the FISH analysis (Figure 2B), indicating that these gastric cancer cells harboured high levels of amplification, similar to the results obtained using gastric cancer cell lines. Preclinical studies suggest that these cases may be likely to respond to FGFR inhibitors. In the remaining case, *FGFR2* amplification was relatively low (4 ~ 8 copies, G44). Such cases with low levels of *FGFR2* amplification may require further investigation regarding their sensitivity to FGFR inhibitors in the future. Meanwhile, we used a copy number assay to detect gene amplification in FFPE samples. Although DNA extracted from FFPE samples was considered to be of low quality with a DNA degradation in general, a copy number assay was capable of detecting and screening amplification in the FFPE samples, which had been stored for as long as 10 years. The results were consistent with the results of FISH studies in several cell lines, with seven positive cases and two negative cases. Our findings suggest that a copy number assay is a powerful tool for detecting and screening gene amplification using FFPE samples.

Recently, trastuzumab in combination with chemotherapy has been regarded as a new standard option for patients with HER2-positive advanced gastric or gastro-oesophageal junction cancer (Bang *et al*, 2010). Therefore, the evaluation of both the HER2 and FGFR2 status before anti-cancer treatment may be needed in gastric cancer patients in the near future. Many small molecules of VEGFR2 tyrosine kinase inhibitors, categorised as anti-angiogenic agents, are now under clinical evaluation, and some of them, including sorafenib for hepatocellular carcinoma and sunitinib for renal cell carcinoma, are being clinically used as standard treatment options (Ellis and Hicklin, 2008). These compounds are also known to have a potential kinase inhibitory effect on FGFRs (Takeda *et al*, 2007; Turner *et al*, 2010), indicating that the development of these multi-kinase inhibitors may be a promising approach to the treatment of *FGFR2*-amplified gastric cancer. In addition to small molecular FGFR tyrosine kinase inhibitors, anti-FGFR antibodies, such as IMC-A1, PRO-001a and R3Mab, also offer promise as molecular-based drugs (Turner and Grose, 2010). We plan to conduct a prospective study in a cohort of Japanese patients with *FGFR2*-amplified gastric cancers.

In conclusion, we found that *FGFR2* amplification was observed in gastric cancer at a frequency of about 4.1%, and a copy number assay was a powerful tool for screening for *FGFR2* amplifications using FFPE samples. Our results warrant strong consideration of the development of FGFR inhibitors for the treatment of gastric cancers with *FGFR2* amplification.

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Conflict of interest

The authors declare no conflict of interest.

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Genotype-directed, dose-finding study of irinotecan in cancer patients with *UGT1A1*28* and/or *UGT1A1*6* polymorphisms

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Irinotecan-induced severe neutropenia is associated with homozygosity for the *UGT1A1*28* or *UGT1A1*6* alleles. In this study, we determined the maximum-tolerated dose (MTD) of irinotecan in patients with *UGT1A1* polymorphisms. Patients who had received chemotherapy other than irinotecan for metastatic gastrointestinal cancer were enrolled. Patients were divided into three groups according to *UGT1A1* genotypes: wild-type (*1/*1); heterozygous (*28/*1, *6/*1); or homozygous (*28/*28, *6/*6, *28/*6). Irinotecan was given every 2 weeks for two cycles. The wild-type group received a fixed dose of irinotecan (150 mg/m^2) to serve as a reference. The MTD was guided from 75 to 150 mg/m^2 by the continual reassessment method in the heterozygous and homozygous groups. Dose-limiting toxicity (DLT) and pharmacokinetics were evaluated during cycle 1. Of 82 patients enrolled, DLT was assessable in 79 patients (wild-type, 40; heterozygous, 20; and homozygous, 19). Dose-limiting toxicity occurred in one patient in the wild-type group, none in the heterozygous group, and six patients (grade 4 neutropenia) in the homozygous group. In the homozygous group, the MTD was 150 mg/m^2 and the probability of DLT was 37.4%. The second cycle was delayed because of neutropenia in 56.3% of the patients given the MTD. The $AUC_{0-24\ h}$ of SN-38 was significantly greater ($P < 0.001$) and more widely distributed in the homozygous group. Patients homozygous for the *UGT1A1*28* or *UGT1A1*6* allele can receive irinotecan in a starting dose of 150 mg/m^2, but many required dose reductions or delayed treatment in subsequent cycles. UMIN Clinical Trial Registration number: UMIN000000618. (*Cancer Sci* 2011; 102: 1868–1873)

Irinotecan, a semisynthetic camptothecin derivative with topoisomerase I-inhibiting activity,[1–3] entered clinical studies in the early 1990s and has been shown to be an effective anticancer drug against several malignancies. Irinotecan is a prodrug that is converted to its active metabolite, SN-38, by carboxylesterase. SN-38 is converted to an inactive metabolite, SN-38G, by UGTs. Irinotecan-associated adverse events, such as myelosuppression and diarrhea, are significantly correlated with the AUC of irinotecan, SN-38, and SN-38G.[1–3]

One of the isoforms of UGT, UGT1A1, is the main enzyme involved in SN-38 glucuronidation. Several studies have reported correlations between *UGT1A1* polymorphisms and irinotecan-associated adverse events,[4–6] and the efficiency of SN-38 glucuronidation is decreased in individuals homozygous for the *UGT1A1*28* allele.[7] In 2005, the US Food and Drug Administration revised the package insert for irinotecan (Camptosar, Yakult Honsha Co. Ltd., Tokyo, Japan), recommending that a reduced dose should be used in these individuals.[8] A subsequent meta-analysis suggested that genetic testing might not be necessary unless the dose of irinotecan is ≤150 mg/m^2; however, there was insufficient evidence for doses of approximately 150 mg/m^2,[9] and the recommendations for dose adjustments remain unclear.

Although *UGT1A1*28* is considered an important predictor of irinotecan-related toxicity, ethnic differences have been reported.[10,11] The allele frequency of *UGT1A1*28* is lower in Asians than in Caucasians, and grade 3–4 hematologic toxicity is associated with *UGT1A1*6* polymorphisms in Asians.[10] In addition, a recent Japanese study revealed that severe adverse events were associated with double heterozygosity (*UGT1A1*28/*6*).[11] Adverse events are related to the pharmacokinetic properties of the drug, and the AUC ratio of SN-38G to SN-38 ($AUC_{SN\text{-}38G}/AUC_{SN\text{-}38}$) was significantly reduced in *UGT1A1*28/*6* patients.[12,13] Thus, *UGT1A1*6* appears to be another important predictor of irinotecan-induced adverse events.

In this context, this study was designed to determine the MTD of irinotecan in patients with gastrointestinal cancer whose *UGT1A1* genotypes were *UGT1A1*28/*28*, **6/*6*, or **28/*6*. The CRM[14,15] was used to determine dose escalation and reductions. Compared with the traditional phase I design, the CRM can incorporate the actual drug doses into the analytical model and evaluate the frequency of adverse events more accurately, particularly in small groups of patients, such as those who are homozygous for *UGT1A1*28* or *UGT1A1*6*. Thus, the CRM was considered appropriate for our study objectives. We also investigated the pharmacokinetics and toxicity of irinotecan in patients with the *UGT1A1*28* and *UGT1A1*6* alleles.

Patients and Methods

Patients. Patients meeting the following criteria were eligible for the study: histologically confirmed and inoperable gastrointestinal cancer; ≥20 years old; Eastern Cooperative Oncology

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Group performance status of 0–1; no prior treatment with irinotecan; a wash-out period of 21 days after previous chemotherapy; adequate bone marrow function (leukocyte count 3000–12 000/mm^3; hemoglobin concentration ≥8.5 g/dL; platelet count ≥ 100 000/mm^3); serum creatinine ≤ 1.5 mg/dL; total bilirubin ≤2.0 mg/dL; and aspartate aminotransferase and alanine aminotransferase ≤100 IU/L. Written informed consent was obtained from all participants. Patients were excluded if they had severe ascites or had received chest or abdominal radiotherapy. The study protocol was approved by the Institutional Review Board at each institution. An independent Data and Safety Monitoring Committee evaluated safety throughout the study.

***UGT1A1* genotyping assay.** Genomic DNA was extracted from peripheral blood using a QIAamp blood kit (Qiagen, Hilden, Germany). An Invader UGT1A1 Molecular Assay kit (Third Wave Technologies, Madison, WI, USA) was used to genotype the *UGT1A1*28* and *UGT1A1*6* polymorphisms.

Classification of *UGT1A1* polymorphisms. We classified the *UGT1A1* polymorphisms into three groups: wild-type (**1/*1*), heterozygous (**28/*1*, **6/*1*), and homozygous (**28/*28*, **6/*6*, **28/*6*). The double heterozygous state (**28/*6*) was included within the homozygous group, taking into account the findings of previous studies.[11,13] A recent study found no evidence of alleles **28* and **6* existing on the same chromosome; patients harboring **28* and **6* on the same chromosome either do not exist or are extremely rare.[16] Therefore, concurrent **28* and **6* homozygosity was considered irrelevant.

Treatment schedule. Irinotecan was given i.v. over the course of 90 min of every 14-day cycle, for only two cycles. The wild-type group received a fixed dose of 150 mg/m^2 as a reference. This is a borderline dose between the low and medium dose levels, as proposed by Hoskins *et al.*,[9] and is the upper limit of the approved dose of irinotecan in Japan. The starting dose was 100 mg/m^2 in the heterozygous group and 75 mg/m^2 in the homozygous group. The dose was escalated in increments of 25 mg/m^2 up to 150 mg/m^2, as described below. The study treatment comprised two cycles, unless unacceptable toxicity developed during the first cycle, or the patient withdrew consent.

Safety was evaluated in the first and second cycles, and DLT was only assessed in the first cycle. Objective clinical evaluations, blood counts, and hepatic and renal function tests were carried out on days 1 and 8 of each cycle. Dose-limiting toxicity was defined as grade 4 neutropenia, grade 4 thrombocytopenia, febrile neutropenia (neutrophil count < 1000/mm^3 and fever ≥ 38.5°C), or grade 3 diarrhea. If DLT occurred in the first cycle, the dose was reduced by one dose level in the second cycle. Toxicity was evaluated according to the Common Terminology Criteria for Adverse Events version 3.0.

Pharmacokinetic assay. Venous blood for pharmacokinetic analysis was collected in sodium-heparinized, evacuated tubes on days 1 and 2 of the first cycle, before infusion of irinotecan, at the end of infusion, and at 1, 2, 4, 7, and 24 h after infusion. The plasma concentrations of intact irinotecan, SN-38, and SN-38G were determined by HPLC, as previously described.[17] The $AUC_{0-24\ h}$ was calculated using WinNonlin software version 5.0.1 (Pharsight, Mountain View, CA, USA).

Dose escalation/reductions: design and statistical considerations. Eligible patients underwent genotyping and were assigned to the wild-type group, heterozygous group, or homozygous group. They were then registered at the data center. All patients who received at least one dose of irinotecan without major protocol violations were included in the safety and pharmacokinetic analyses.

In the wild-type group, we estimated the probability of DLT occurring at a dose of 150 mg/m^2. A sample size of 40 was planned, assuming that the probability of DLT would be 10% (maximum 20%) with 95% confidence limits.

In the heterozygous and homozygous groups, dose escalation and reductions were carried out according to the CRM. We used a logistic regression model to determine the relationship between dose and toxicity. The model was updated based on the patients' responses, using a Bayesian approach. After enrolling the first patient, the doses given to subsequent patients were determined by the CRM. Each subsequent patient was treated at the dose level where the probability of DLT was closest to 30%. Dose-limiting toxicity was assessed in a maximum of three patients at the same time and dose. The dose was increased or decreased by 25 mg/m^2. The MTD was defined as the dose level at which nearest to 30% of patients were expected to have DLT. The recommended dose was determined based on the results obtained during the first two cycles. Simulation studies indicated that 10–20 patients were required to estimate the MTD. The decision to continue or stop the study was made after safety evaluation of the first 10 patients.

Because of a treatment-related death, patient enrolment was temporarily halted and the protocol was revised. The dose of irinotecan in the homozygous group was reduced by two levels in the second cycle if the patient had grade 3–4 neutropenia in the first cycle.

In accordance with the advice of the Data and Safety Monitoring Committee, medical experts and biostatisticians, all eligible patients in the homozygous group were included in the analysis of MTD, DLT, and the toxicity data, irrespective of protocol amendments.

The Cochran–Armitage trend test was used to analyze trends in grade 3–4 adverse events across the different genotypes. Fisher's exact test was used to compare the frequency of toxicity among the wild-type, heterozygous, and homozygous groups. Pearson's correlation coefficient was used to assess the relationships between laboratory test data and pharmacokinetic parameters during the first cycle. The association between pharmacokinetic parameters and genotype was evaluated using the Cochran–Armitage trend test. Levene's test was used to assess the homogeneity of variances in SN-38 among the genotypes. All analyses were carried out using SAS software version 8.2 (SAS Institute, Cary, NC, USA).

Results

Patient characteristics. Between November 2006 and October 2008, 82 patients were enrolled at 12 institutions and assigned to the wild-type (n = 41), heterozygous (n = 20; **28/*1* [n = 8], **6/*1* [n = 12]), or homozygous (n = 21; **28/*28* [n = 3], **6/*6* [n = 12], **28/*6* [n = 6]) groups. Toxicity and pharmacokinetic parameters were evaluated in 81 patients, excluding one patient in the homozygous group who withdrew consent before treatment. After the first dose, two patients were deemed ineligible. Therefore, 79 patients were eligible for DLT analysis (Table 1).

Dose escalation and identification of MTD. The first four patients in each of the heterozygous and homozygous groups showed no DLT, so the dose was increased to 150 mg/m^2 according to the CRM (Fig. 1). At 150 mg/m^2, DLT occurred in one patient in the wild-type group (grade 3 anorexia and fatigue) and in six patients in the homozygous group (**28/*28* [n = 1], **6/*6* [n = 4], **28/*6* [n = 1]) (grade 4 neutropenia, 6; grade 3 diarrhea, 1), but in no patients in the heterozygous group. Based on these data, the probability of DLT at 150 mg/m^2 was 2.5% in the wild-type group (1/40 patients; 95% CI, 0.1–13.2), 5.9% in the heterozygous group (0/16 patients; 80% CI based on the CRM, 2.2–11.2%), and 37.4% in the homozygous group (6/15 patients; 80% CI based on the CRM, 22.8–52.7%). In the homozygous group, the initial dose of irinotecan (150 mg/m^2) was determined to be the MTD, whereas the MTD in the heterozygous group was estimated to be >150 mg/m^2.

Table 1. Disposition and baseline characteristics of patients with gastrointestinal cancer who participated in this study (n = 82)

Characteristics	Wild-type group		Heterozygous group		Homozygous group	
	n	%	*n*	%	*n*	%
Patients enrolled	41	—	20	—	21	—
Consent withdrawn	0	—	0	—	1	—
Patients receiving study drug	41	—	20	—	20	—
Eligible	40	—	20	—	19	—
Not eligible	1	—	0	—	1	—
DLT analysis	40	—	20	—	19	—
Toxicity analysis	41	—	20	—	20	—
Pharmacokinetic analysis	41	—	20	—	20	—
Sex						
Male	22	53.7	10	50.0	13	65.0
Female	19	46.3	10	50.0	7	35.0
Age (years)						
Median	62	—	63	—	66	—
Range	21–88	—	47–80	—	38–78	—
ECOG performance status						
0	27	65.9	12	60.0	11	55.0
1	14	34.1	8	40.0	9	45.0
Adenocarcinoma histology						
Stomach	19	46.3	9	45.0	4	20.0
Colorectal	22	53.7	11	55.0	16	80.0
Total bilirubin (mg/dL)						
Median	0.5	—	0.6	—	0.8	—
Range	0.2–1.3	—	0.2–1.0	—	0.3–2.0	—
Direct bilirubin (mg/dL)						
Median	0.2	—	0.2	—	0.2	—
Range	0.0–0.5	—	0.1–0.3	—	0.1–0.5	—

Patients were divided into groups according to *UGT1A1* genotype: wild-type (*1/*1); heterozygous (*28/*1, *6/*1); or homozygous (*28/*28, *6/*6, *28/*6). DLT, dose-limiting toxicity; ECOG, Eastern Cooperative Oncology Group; —, not applicable.

Toxicity. The major adverse events in patients treated with 150 mg/m^2 irinotecan are listed in Table 2. The most frequently observed grade 3–4 toxicities were leukopenia and neutropenia. During the first cycle, hematologic toxicity was significantly associated with genotype ($P < 0.001$). Grade 3–4 neutropenia occurred in 9.8% of patients in the wild-type group, 18.8% of patients in the heterozygous group, and 62.5% of patients in the homozygous group. The frequency of severe neutropenia was significantly higher in the homozygous group than in the wild-type and heterozygous groups ($P < 0.001$). A similar trend was observed during the first two cycles (wild-type group, 22.0%; heterozygous group, 25.0%; homozygous group, 81.3%). Unlike hematologic toxicity, non-hematologic toxicity was not associated with genotype and was generally mild to moderate in severity (Table 2).

On *UGT1A1* diplotype analysis, grade 3–4 neutropenia and leukopenia occurred frequently in patients in the homozygous group (Table 3). Grade 3–4 diarrhea occurred in 1/9 patients (11.1%) with **6/*6*. The second cycle was delayed in 5/41 patients (12.2%) in the wild-type group, 4/16 patients (25.0%) in the heterozygous group, and 9/16 patients (56.3%) in the homozygous group. The reasons for delaying treatment were neutropenia in seven patients, infection and stomatitis in one, and diarrhea in one. In the homozygous group treated with 150 mg/m^2 irinotecan, the dose for the second cycle was reduced by two dose levels (or to 100 mg/m^2) in three patients and by one dose level (to 125 mg/m^2) in one patient. One patient who received a reduced dose of 100 mg/m^2 irinotecan developed grade 4 neutropenia again in the second cycle. Four of 16 patients (25.0%) in the homozygous group completed two cycles of therapy without needing to delay treatment or reduce the dose.

There was one treatment-related death in the homozygous group, which was caused by septic shock with grade 4 neutropenia after the second cycle of irinotecan at a dose of 150 mg/m^2. This patient, who was homozygous for *UGT1A1*28*, had no DLT in the first cycle, and the second cycle was delayed because of prolonged neutropenia.

Relationships between *UGT1A1* polymorphisms and pharmacokinetic profile and toxicity of irinotecan. The $AUC_{0-24\ h}$ of SN-38 was significantly higher in the homozygous group than in the wild-type or heterozygous groups ($P < 0.001$) and interpatient variability was also higher in the former group (Table 4). The $AUC_{0-24\ h}$ of SN-38G was significantly higher in the wild-type group than in the heterozygous or homozygous groups ($P = 0.001$). The $AUC_{SN\text{-}38G}/AUC_{SN\text{-}38}$ ratio was highest in the wild-type group, intermediate in the heterozygous group, and lowest in the homozygous group ($P < 0.001$).

The $AUC_{0-24\ h}$ of SN-38 was slightly higher in patients with the **28/*28* or **6/*6* genotypes than in patients with the **28/*6* genotype (Table 4). The $AUC_{SN\text{-}38G}/AUC_{SN\text{-}38}$ ratio was slightly lower in patients with the **6/*6* genotype than in those with the **28/*28* or **28/*6* genotypes.

The relationship between adverse events and pharmacokinetic parameters was also analyzed. The $AUC_{0-24\ h}$ of SN-38G was not correlated with hematologic toxicity. In contrast, the $AUC_{0-24\ h}$ of SN-38 was correlated with the frequency of grade 3–4 leukopenia and neutropenia ($r = 0.49$, $P < 0.001$). The $AUC_{SN\text{-}38G}/AUC_{SN\text{-}38}$ ratio also correlated with the frequencies of grade 3–4 leukopenia ($r = 0.25$, $P = 0.023$) and neutropenia ($r = 0.308$, $P = 0.005$).

Discussion

Irinotecan is generally given at a dose of 150 mg/m^2 every 2 weeks in Japanese patients with gastrointestinal cancer. This dose was determined based on the results of clinical trials in the 1990s[18,19] and is the upper limit of the approved dose in Japan.

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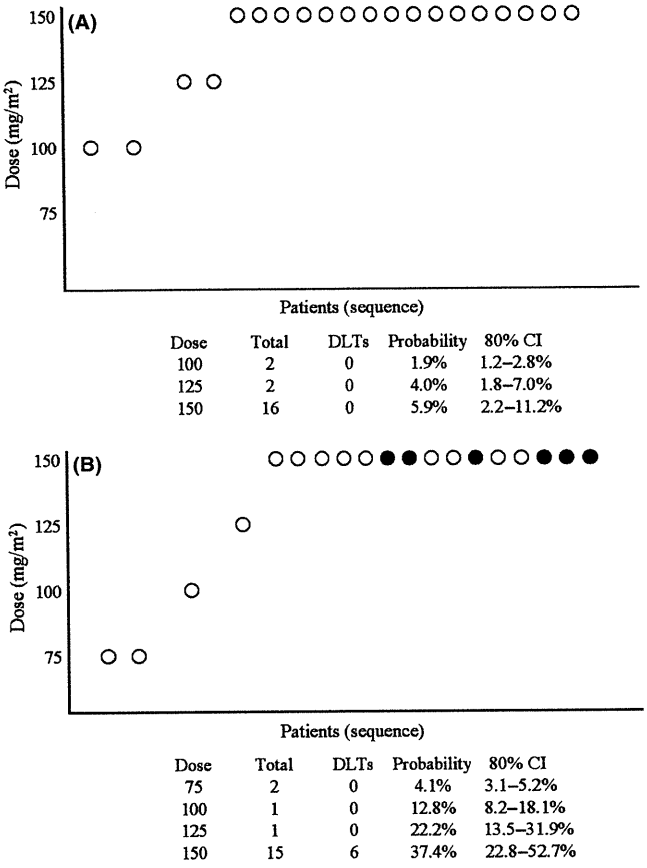

Fig. 1. Probability of dose-limiting toxicity (DLT) of irinotecan in patients with gastrointestinal cancer, determined using the continual reassessment method. Patients were grouped as heterozygous (A) or homozygous (B) according to their *UGT1A1* genotype. (○), Patients registered; (●), occurrence of DLT. CI, confidence interval.

The appropriate dosages of cytotoxic agents have been based on the concept of minimizing the risk of no response within the acceptable toxicity limits. However, genetic information has recently been obtained on the metabolism of CPT-11 and its related toxicities. Hoskins *et al.*[20] showed that the risk of severe hematologic toxicity is higher among patients with the *UGT1A1*28/*28* genotype than among those with the *UGT1A1*28/*1* or *UGT1A1*1/*1* genotypes at medium doses (150–250 mg/m^2) and at higher doses (>250–350 mg/m^2), but not at lower doses (100–125 mg/m^2). However, the results of a recently reported meta-analysis[21] showed that the *UGT1A1*28/*28* genotype was associated with an increased risk of neutropenia not only at medium or high doses of irinotecan, but also at low doses (relative risk [RR], 2.43; 80–145 mg/m^2). To verify these previous findings in a prospective manner, we needed to reset the MTD according to these genetic factors, so we used irinotecan at doses of 75–150 mg/m^2.

Our genotype-directed dose-finding study using the CRM showed that the principal DLT was neutropenia and the MTD of irinotecan was 150 mg/m^2 in Japanese patients carrying the *UGT1A1* variant alleles. In the heterozygous group, the MTD was estimated to be >150 mg/m^2. Our results also showed that the hematologic toxicity of irinotecan at 150 mg/m^2 was significantly more severe, and the $AUC_{0-24\ h}$ of SN-38 was significantly higher and more widely distributed in patients with two variant alleles than in those with one or no variant alleles. These findings are consistent with the results of previous studies.[12,13,22,23] As described above, *UGT1A1* genetic polymorphism is a factor that clearly affects pharmacokinetics, and individual variation in pharmacokinetics was greater in the homozygous group. The recommended dose could not be defined, because it was considered inappropriate to apply the dose obtained by increasing the number of cases to the general population. Moreover, the present study revealed that the $AUC_{0-24\ h}$ of SN-38 in the heterozygous group was similar to that in the wild-type group among patients treated with irinotecan at 150 mg/m^2. The *UGT1A1* polymorphisms were not related to diarrhea in our study, or in previous studies.[10,22,24] Thus, further studies are needed to determine the predictive factors for diarrhea.

When the *UGT1A1*28* and *UGT1A1*6* alleles were evaluated separately, the incidence of neutropenia and the $AUC_{0-24\ h}$ of SN-38 were similar in patients with a homozygous genotype. Although the frequency of patients with *UGT1A1*28/*28* has been reported to be small in Asia,[10–12,22] grade 3–4 neutropenia developed in all of our patients with the *UGT1A1*28/*28* genotype, and one patient died because of treatment-related sepsis. The $AUC_{0-24\ h}$ of SN-38 was also very high in these patients. Therefore, the *UGT1A1*28/*28* genotype is an important determinant of safety, even in Asian patients.

Several studies have addressed the issues of *UGT1A1* polymorphisms and the starting dose of irinotecan,[11,13,22] but most of these studies had limitations, such as small numbers of patients with the rare variant alleles, a retrospective design, or

Table 2. Common adverse events (grades 3–4) associated with 150 mg/m^2 irinotecan in patients with gastrointestinal cancer

Adverse events	First cycle								First and second cycles					
	Wild-type group (*n* = 41)		Heterozygous group (*n* = 16)		Homozygous group (*n* = 16)		*P**	*P***	Wild-type group (*n* = 41)		Heterozygous group (*n* = 16)		Homozygous group (*n* = 16)	
	G3/G4	%	G3/G4	%	G3/G4	%			G3/G4	%	G3/G4	%	G3/G4	%
Hematologic toxic effects														
Leukopenia	1/0	2.4	0/0	0.0	8/1	56.3	<0.001	<0.001	1/0	2.4	2/0	12.5	10/2	75.0
Neutropenia	4/0	9.8	3/0	18.8	4/6	62.5	<0.001	<0.001	9/0	22.0	3/1	25.0	6/7	81.3
Non-hematologic toxic effects														
Diarrhea	0/0	0.0	0/0	0.0	1/0	6.3	NS	NS	0/0	0.0	0/0	0.0	1/0	6.3
Fatigue	1/0	2.4	0/0	0.0	0/0	0.0	NS	NS	1/0	2.4	0/0	0.0	0/0	0.0
Anorexia	1/0	2.4	0/0	0.0	0/0	0.0	NS	NS	1/0	2.4	1/0	6.3	0/0	0.0

Patients were divided into groups according to *UGT1A1* genotype: wild-type (*1/*1); heterozygous (*28/*1, *6/*1); or homozygous (*28/*28, *6/*6, *28/*6). *Cochrane–Armitage trend test; **Fisher's exact test, wild-type/heterozygous group versus homozygous group. G, grade; NS, not significant.

Table 3. Association between *UGT1A1* genotype in patients with gastrointestinal cancer and the most common grade 3–4 adverse events during the first treatment cycle with 150 mg/m^2 irinotecan

Adverse events	*6/*1 (n = 9)		*28/*1 (n = 7)		*6/*6 (n = 9)		*28/*28 (n = 3)		*28/*6 (n = 4)	
	G3/G4	%	G3/G4	%	G3/G4	%	G3/G4	%	G3/G4	%
Hematologic toxic effects										
Leukopenia	0/0	0.0	0/0	0.0	5/1	66.7	2/0	66.7	1/0	25.0
Neutropenia	1/0	11.1	2/0	28.6	2/4	66.7	2/1	100	0/1	25.0
Non-hematologic toxic effects										
Diarrhea	0/0	0.0	0/0	0.0	1/0	11.1	0/0	0.0	0/0	0.0
Fatigue	0/0	0.0	0/0	0.0	0/0	0.0	0/0	0.0	0/0	0.0
Anorexia	0/0	0.0	0/0	0.0	0/0	0.0	0/0	0.0	0/0	0.0

G, grade.

Table 4. Relationship between *UGT1A1* genotype and pharmacokinetic parameters for 150 mg/m^2 irinotecan in patients with gastrointestinal cancer

Pharmacokinetic parameter	SN-38$_{0-24\ h}$ (ng × h/mL)	SN-38G$_{0-24\ h}$ (ng × h/mL)	AUC ratio
Wild-type group (n = 41)	264 ± 114	1266.8 ± 667.5	5.03 ± 2.25
Heterozygous group (n = 16)	279.6 ± 152.0	820.7 ± 378.7	3.25 ± 1.32
*UGT1A1*6/*1* (n = 9)	250.2 ± 70.4	723.1 ± 252.8	3.16 ± 1.49
*UGT1A1*28/*1* (n = 7)	317.3 ± 219.5	946.1 ± 490.6	3.35 ± 1.16
Homozygous group (n = 16)	509.8 ± 261.8	849.0 ± 561.9	1.85 ± 1.13
*UGT1A1*28/*6* (n = 4)	251.3 ± 89.5	557.8 ± 148.8	2.34 ± 0.82
*UGT1A1*6/*6* (n = 9)	564.9 ± 223.5	673.2 ± 304.1	1.21 ± 0.36
*UGT1A1*28/*28* (n = 3)	689.0 ± 327.0	1764.7 ± 631.4	3.10 ± 1.82
Cochrane–Armitage trend test	<0.001	0.001	<0.001
Levene's test	<0.001	0.310	0.013

Analyses were carried out between wild-type versus heterozygous or homozygous groups. AUC, area under the time–concentration curve; AUC ratio, AUC_{SN-38G}/AUC_{SN-38}; SN-38G, SN-38 glucuronide.

the inclusion of patients with various types of cancer. Even prospective studies have included patients who received heterogeneous treatments including irinotecan.[9,10,24] We focused on high-risk patients with relatively rare *UGT1A1* alleles to delineate the important pharmacogenetic determinants of irinotecan-induced neutropenia. To eliminate the potential effects of confounders such as diagnosis and concurrent therapy, we only included patients with gastric or colorectal cancer who received irinotecan monotherapy. We also evaluated the relationship between genotype and the pharmacokinetics of irinotecan and its major metabolites. We believe that these features of our study enhance the validity of our findings. The CRM used in our study offers important advantages over the conventional design with three patient cohorts.[25] We could enroll the patients promptly, and treat approximately 80% of patients at the MTD of irinotecan (150 mg/m^2), as initially estimated.

A previous dose-finding study suggested that the recommended dose of 180 mg/m^2 irinotecan in the FOLFIRI regimen was too low in patients with metastatic colorectal cancer who had the *UGT1A1*1/*1* or *UGT1A1*28/*1* genotype.[19] The incidence of grade 3–4 neutropenia was 24% in that study. In our study, the incidence of grade 3–4 neutropenia was 23% across the wild-type and heterozygous groups, supporting these earlier findings.

In the present study, only 25% of the patients in the homozygous group were able to complete two cycles of treatment at a dose of 150 mg/m^2 without treatment delays and dose reduction. Previous studies[12,20] revealed that low-dose irinotecan (100–125 mg/m^2) carried a low risk of neutropenia, even in patients with a *UGT1A1* homozygous genotype. Although the results of a meta-analysis[21] reported an increased risk of neutropenia not only at medium or high doses of irinotecan, but also at low doses in homozygous group, the results of the present study supported the report of Hoskin *et al.*[20] as, similarly, no DLT occurred at doses of 100 or 125 mg/m^2. This might be because retrospective studies were included and there was heterogeneity (such as cancer type, therapeutic line, regimen) in the meta-analysis,[21] whereas the present study was a prospective study using a single drug in a homogenous population of patients with colorectal cancer and gastric cancer. Moreover, the patients who required dose reduction from 150 to 125 or 100 mg/m^2 were able to receive subsequent treatment safely. Therefore, *UGT1A1* genetic polymorphism testing is useful, because a risk attributable to CPT-11 could be avoided by selecting another therapy, even in homozygous patients with a high risk of side-effects who have a poor performance status or a history of intensive treatment. Irinotecan at doses >150 mg/m^2 has been used in regimens such as cetuximab plus irinotecan (350 mg/m^2)[26] and FOLFIRI (180 mg/m^2) in colorectal cancer.[27] Our results suggest that starting treatment at such high doses of irinotecan would be very risky in patients who have two alleles of *UGT1A1*28* and/or *UGT1A1*6*. The *UGT1A1* genetic polymorphism is a solid factor that affects the pharmacokinetics and a factor for judging the risk of hemotoxicity. Because CPT-11 is used as the second- or third-line rather than the first-line therapy in patients with gastric cancer and colorectal cancer, it would be better to carry out genetic testing before therapy commenced and after a full explanation to all patients. Patients who have two alleles of *UGT1A1*28* and/or *UGT1A1*6* can receive irinotecan at a starting dose of 150 mg/m^2 and must be closely observed by carrying out observations and blood tests weekly, at least during the first cycle.

On the basis of the results obtained in our study, a nationwide close observational study is now ongoing to evaluate the safety and efficacy of irinotecan at a dose of ≤150 mg/m^2 after genetic testing.

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Disclosure Statement

The authors have no conflicts of interest to declare.

Abbreviations

AUC	areas under the time–concentration curves
CI	confidence interval
CRM	continual reassessment method
DLT	dose-limiting toxicity
FOLFIRI	folinic acid/fluorouracil/irinotecan
MTD	maximum tolerated dose
SN-38	7-ethyl-10-hydroxycamptothecin
SN-38G	SN-38 glucuronide
UGT	uridine diphosphate glucuronosyltransferases

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