the transmembrane region (PHAT matrix element difference = −6), and probable functional effects of Arg393Trp (*7) (PSIC score difference = 3.053), Tyr401Cys (*8) (3.382) and Asp786Glu (*9) (2.277), but no functional effects of *3 (1.446) and *5 (0.326).

In conclusion, the current study provided detailed information on *ABCC2* variations and haplotype structures in Japanese and also suggested a large ethnic difference in the frequencies of 3972C>T(Ile1324Ile) and 1446C>G (Thr482Thr) and their related haplotypes between Asians and Caucasians. This information would be useful for studies investigating the clinical significance of *ABCC2* alleles and haplotypes.

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# Impacts of excision repair cross-complementing gene 1 (ERCC1), dihydropyrimidine dehydrogenase, and epidermal growth factor receptor on the outcomes of patients with advanced gastric cancer

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Using laser-captured microdissection and a real-time RT–PCR assay, we quantitatively evaluated mRNA levels of the following biomarkers in paraffin-embedded gastric cancer (GC) specimens obtained by surgical resection or biopsy: excision repair cross-complementing gene 1 (ERCC1), dihydropyrimidine dehydrogenase (DPD), methylenetetrahydrofolate reductase (MTHFR), epidermal growth factor receptor (EGFR), and five other biomarkers related to anticancer drug sensitivity. The study group comprised 140 patients who received first-line chemotherapy for advanced GC. All cancer specimens were obtained before chemotherapy. In patients who received first-line S-1 monotherapy (69 patients), low MTHFR expression correlated with a higher response rate (low: 44.9% *vs* high: 6.3%; $P=0.006$). In patients given first-line cisplatin-based regimens (combined with S-1 or irinotecan) (43 patients), low ERCC1 correlated with a higher response rate (low: 55.6% *vs* high: 18.8%; $P=0.008$). Multivariate survival analysis of all patients demonstrated that high ERCC1 (hazard ratio (HR): 2.38 (95% CI: 1.55–3.67)), high DPD (HR: 2.04 (1.37–3.02)), low EGFR (HR: 0.34 (0.20–0.56)), and an elevated serum alkaline phosphatase level (HR: 1.00 (1.001–1.002)) were significant predictors of poor survival. Our results suggest that these biomarkers are useful predictors of clinical outcomes in patients with advanced GC.

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**Keywords:** gastric cancer; excision repair cross-complementing gene 1; dihydropyrimidine dehydrogenase; epidermal growth factor receptor; prognostic factor

Gastric cancer (GC) is the second leading cause of cancer-related deaths worldwide, annually accounting for 40–50 deaths per 100 000 population in Japan and 5–15 deaths per 100 000 population in Europe (Parkin, 2001). During the past decade, newly developed cytotoxic drugs have been included in treatment regimens for GC. These new regimens have better response rates, often at the cost of higher incidences of severe adverse events (Ajani, 2005). This situation has created a greater need for diagnostic techniques that can predict clinical outcomes such as tumour response and survival in GC (Ichikawa, 2006). Considerable evidence suggests that the intratumour gene expressions of drug-metabolising enzymes, DNA repair enzymes, or angiogenic enzymes are useful predictors of treatment outcomes such as survival and the response to anticancer drugs (Backus *et al*, 2000; Ulrich *et al*, 2003; Marsh and McLeod, 2004). However, the clinical significance of these biomarkers remains unclear, especially in GC.

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5-Fluorouracil (5-FU) and cisplatin are key drugs for the management of GC: Pharmacogenetic variability in metabolising enzymes of 5-FU and folate is a major determinant of the sensitivity to 5-FU and survival in GC (Lenz *et al*, 1996; Banerjee *et al*, 2002; Ichikawa *et al*, 2004; Napieralski *et al*, 2005). Several enzymes have key roles in the metabolic pathway of 5-FU and folate (Figure 1): thymidylate synthase (TS) is a target enzyme of 5-FU; dihydropyrimidine dehydrogenase (DPD) is a degrading enzyme of 5-FU; thymidine phosphorylase (TP) and orotate phosphoribosyl transferase (OPRT) are important metabolic enzymes; and dihydrofolate reductase (DHFR) and methylenetetrahydrofolate reductase (MTHFR) participate in folate metabolism. Lenz *et al* (1996) and Ichikawa *et al* (2004) have found that high TS mRNA expression in GC could predict poor clinical outcomes of treatment with 5-FU. Napieralski *et al* (2005) reported that high DPD expression in GC may correlate with poor survival and no response to 5-FU.

The cytotoxicity of cisplatin is attributed mainly to the induction of DNA intrastrand, interstrand, and DNA–protein crosslinks (Roberts and Thomson, 1979). Such DNA damage is

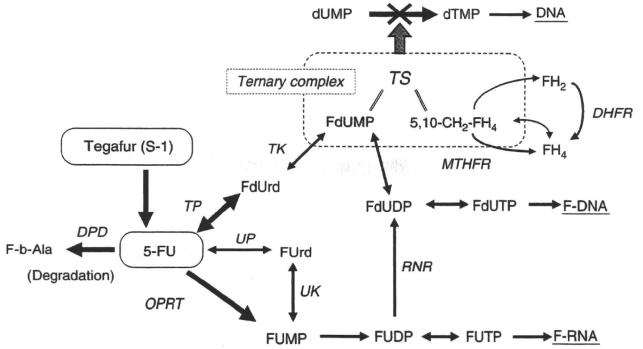


**Figure 1** 5-Fluorouracil and folate metabolic pathways. Genes examined in our study are shown in bold. DPD, dihydropyrimidine dehydrogenase; DHFR, dihydrofolate reductase; MTHFR, methylenetetrahydrofolate reductase; OPRT, orotate phosphoribosyl transferase; TS, thymidylate synthase; TP, thymidine phosphorylase; . The official Human Genome Organization gene nomenclature is used. Common or alternative names for each gene can be found at http://pharmacogenetics.wustl.edu.

thought to be repaired by the nucleotide excision pathway. Excision repair cross-complementing gene 1 (ERCC1) has a pivotal role in nucleotide excision repair and may promote the development of resistance to cisplatin (Dabholkar *et al*, 1992; Bramson and Panasci, 1993). Excision repair cross-complementing gene 1 is also associated with responses to cisplatin- and 5-FU-based chemotherapy in GC. Metzger *et al* (1998) reported that high ERCC1 expression in GC may be associated with poor survival and no response to cisplatin.

Other studies, however, have failed to confirm such correlations of the gene expressions of TS (Choi *et al*, 2001; Kwon *et al*, 2007), DPD (Ishikawa *et al*, 2000; Miyamoto *et al*, 2000), and ERCC1 (Napieralski *et al*, 2005) with the outcomes of chemotherapy. Further larger studies are thus required to confirm or refute previous claims.

This study was designed to further delineate the clinical implications of biomarkers and to identify potential predictors of the response to chemotherapy and survival in patients with GC. The epidermal growth factor receptor (EGFR) tyrosine kinase family and the vascular endothelial growth factor (VEGF) superfamily are also well-known mediators of tumour cell proliferation and tumour-related angiogenesis, which can influence tumour biology and survival (Carmeliet and Jain, 2000; Gamboa-Dominguez *et al*, 2004; Juttner *et al*, 2006). We tested the hypothesis that the clinical outcomes of chemotherapy (response rate, time to progression, and overall survival) in patients with advanced GC are related to the pretreatment intratumour mRNA levels of enzymes participating in critical pathways of drug resistance, such as 5-FU and folate metabolism (TS, DPD, TP, OPRT, DHFR, MTHFR), DNA repair (ERCC1), the EGFR signalling pathway (EGFR), and tumour-related angiogenesis (VEGF-A). We also compared the prognostic implications of these biomarkers with those of well-recognised prognostic factors (Chau *et al*, 2004; Lee *et al*, 2007).

## PATIENTS AND METHODS

### Patient eligibility

Patients with a diagnosis of histologically proven advanced GC were eligible for the study. Inclusion criteria were as follows: unresectable, locally-advanced, or metastatic disease; no prior chemotherapy and no prior adjuvant/neoadjuvant chemotherapy; specimens of primary gastric adenocarcinomas were obtained before the start of chemotherapy by surgical resection or biopsy at the National Cancer Center Hospital (Tokyo, Japan) or National Hospital Organization Shikoku Cancer Center (Matsuyama, Japan); first-line chemotherapy was received at either of the hospitals; radiographically measurable disease; and written informed consent. The tissue samples were collected retrospectively from patients who met these criteria. Measurable disease was assessed by computed tomography. Response was evaluated according to the standard UICC guidelines as complete response (CR), partial response (PR), no change (NC), or progressive disease (PD) (Hayward *et al*, 1978). Tumour response and survival times as of December 2006 were confirmed in all patients. The response rate was calculated as the ratio of (CR + PR)/(CR + PR + NC + PD). Written informed consent was obtained before treatment and evaluation of tumour samples. This study was approved by the institutional review boards of both hospitals.

### Clinical data

The following clinical data were included in analyses: performance status, liver and peritoneal metastases, and laboratory data at the start of chemotherapy, including leukocyte and lymphocyte counts and the serum levels of alkaline phosphatase (ALP), lactate dehydrogenase (LDH), albumin, C-reactive protein, and tumour markers (CEA, CA19-9).

### Chemotherapy

The following first-line chemotherapy regimens were administered to the patients in our study: S-1 monotherapy ($N=69$), cisplatin plus S-1 ($N=14$), cisplatin plus irinotecan ($N=29$), 5-FU monotherapy ($N=23$), and other regimens (5-FU plus methotrexate, $N=2$; paclitaxel, $N=2$; uracil/ftorafur, $N=1$). For S-1 monotherapy, patients received S-1 (40 mg m$^{-2}$ twice daily) on days 1–28 of a 42-day cycle. Treatment with cisplatin plus S-1 consisted of cisplatin (60 mg m$^{-2}$) on day 8 and S-1 (40 mg m$^{-2}$ twice daily) on days 1–21 of a 35-day cycle. Treatment with cisplatin plus irinotecan consisted of cisplatin (80 mg m$^{-2}$) on day 1 and irinotecan (70 mg m$^{-2}$) on days 1 and 15 of a 28-day cycle.

For 5-FU monotherapy, patients received 5-FU (800 mg $m^{-2}$ $day^{-1}$) as a continuous infusion on days 1–5 of a 28-day cycle.

### Laboratory methods

Ten-micrometre-thick sections obtained from identified areas with the highest tumour-cell concentration were mounted on uncoated glass slides. For histologic diagnosis, representative sections were stained with haematoxylin and eosin by standard methods. Before microdissection, sections were stained with nuclear fast red (American MasterTech Scientific, Lodi, CA, USA). The sections of interest were selectively isolated by laser-captured microdissection (PALM Microsystem, Leica, Wetzlar, Germany), according to standard procedures (Bonner *et al*, 1997). The dissected particles of tissue were transferred to a reaction tube containing 400 $\mu$l of RNA lysis buffer.

The samples were homogenised and heated at 92°C for 30 min. Fifty microlitres of 2 M sodium acetate was added at pH 4.0, followed by 600 $\mu$l of freshly prepared phenol/chloroform/isoamyl alcohol (250 : 50 : 1). The tubes were placed on ice for 15 min and then centrifuged at 13 000 r.p.m. for 8 min in a chilled (8°C) centrifuge. The upper aqueous phase was carefully removed. Glycogen (10 $\mu$l) and 300–400 $\mu$l of isopropanol were added. The tubes were chilled at −20°C for 30–45 min to precipitate the RNA. The samples were washed in 500 $\mu$l of 75% ethanol and air-dried for 15 min. The pellet was resuspended in 50 $\mu$l of 5 mM Tris. Finally, cDNA was prepared as described by Lord and colleagues (Lord *et al*, 2000).

Quantification of nine genes of interest and an internal reference gene ($\beta$-actin) was performed with a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System, TaqMan®, Perkin-Elmer (PE) Applied Biosystem, Foster City, CA, USA) using the standard curve method. The PCR reaction mixture consisted of 1200 nM of each primer, 200 nM of probe, 0.4 U of AmpliTaq gold polymerase, 200 nM each of dATP, dCTP, dGTP, and dTTP, 3.5 mM of $MgCl_2$, and 1 × Taqman buffer A containing a reference dye. The final volume of the reaction mixture was 20 $\mu$l (all reagents from PE Applied Biosystems, Foster City, CA, USA). Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 46 cycles of 95°C for 15 s and 60°C for 1 min. The primers and probes used are listed in Table 1. Gene expression values (relative mRNA levels) are expressed as ratios (differences between $C_t$ values) between the gene of interest and an internal reference gene ($\beta$-actin).

For each gene, we establish a usable $C_t$ range for the data and document the precision of the measurements within the usable range. For maximum accuracy, we demonstrate that the slopes of the plots of $\Delta C_t$ *vs* Log pg. RNA for target genes and the housekeeping gene (actin) demonstrate parallelism. Each replicate $C_t$ data point is the average of $C_t$ values obtained in three PCR reactions. To compare the results of two different TaqMan plates with each other, the same standardised samples are analysed on every plate.

### Statistical analysis

We examined the objective tumour response to chemotherapy, time to progression, and overall survival. Time to progression and overall survival were calculated as the period from the start of first-line chemotherapy until disease progression or death from any cause, respectively. If patients were lost to follow-up, data were censored at the date of the last evaluation.

To assess associations of gene expression levels with tumour response, time to progression, and overall survival, the expression levels of each gene were categorised into low and high values at optimal cutoff points. The maximal $\chi^2$ method (Halpern, 1982; Miller and Siegmund, 1982; Lausen and Schumacher, 1992) was used to determine which gene expression (optimal cutoff point) best segregated patients into poor- and good-outcome subgroups (in terms of likelihood of response and survival). To determine the corrected *P*-values on the basis of the maximal $\chi^2$ analysis, 2000 bootstrap-like simulations were used in univariate analyses to estimate the distribution of the maximal $\chi^2$ statistics under the null hypothesis of no association. The clinical laboratory data were treated as continuous variables. The estimates of hazard ratios (HRs) with 95% CIs, on the basis of a Cox proportional hazards model, were used to provide quantitative summaries of the gene expression data.

All reported *P*-values are two-sided, and the level of statistical significance was set at $P<0.010$. Variables for multivariate analysis were selected by the Stepwise Method, using a significance level of $<0.010$ for entering into or remaining in the model. All analyses were performed using the statistical software package R, version 2.4.1, and the SAS statistical package, version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

## RESULTS

A total of 140 patients were eligible for the study. Eighty-six patients (61%) were recruited at the National Cancer Center Hospital and 54 patients (39%) at the National Hospital Organization Shikoku Cancer Center. Chemotherapy began in July 1997 in the first patient and in June 2004 in the last patient. The demographic characteristics of the patients are shown in Table 2. There were 108 (77%) men and 32 (23%) women with a median age of 65 years. At the time of analysis, 131 (94%) patients had died and nine (6%) patients were alive.

**Table 1** Primer and probe sequences for quantitative RT–PCR

| Gene | Forward primer (5′-3′) | Reverse primer (5′-3′) | Taqman® probe (5′-3′) |
|---|---|---|---|
| TS | GCCTCGGTGTGCCTTTCA | CCCGTGATGTGCGCAAT | TCGCCAGCTACGCCCTGCTCA |
| DPD | AGGACGCAAGGAGGGTTTG | GTCCGCCGAGTCCTTACTGA | CAGTGCCTACAGTCTCGAGTCTGCCAGTG |
| TP | CCTGCGGACGGAATCCT | GCTGTGATGAGTGGCAGGCT | CAGCCAGAGATGTGACAGCCACCGT |
| OPRT | TAGTGTTTTGGAAACTGTTGAGGTT | CTTGCCTCCCTGCTCTCTGT | TGGCATCAGTGACCTTCAAGCCCTCCT |
| DHFR | GTCCTCCCGCTGCTGTCA | GCCGATGCCCATGTTCTG | TTCGCTAAACTGCATCGTCGCTGTGTC |
| MTHFR | CGGGTTAATTACCACCTTGTCAA | GCATTCGGCTGCAGTTCA | TGAAGGGTGAAAACATCACCAATGCCC |
| ERCC1 | GGGAATTTGGCGACGTAATTC | GCGGAGGCTGAGGAACAG | CACAGGTGCTCTGGCCCAGCACATA |
| EGFR | TGCGTCTCTTGCCGGAAT | GGCTCACCCTCCAGAAGGTT | ACGCATTCCCTGCCTCGGCTG |
| VEGF-A | AGTGGTCCCAGGCTGCAC | TCCATGAACTTCACCACTTCGT | TGATTCTGCCCTCCTCCTTCTGCCAT |
| $\beta$-Actin | GAGCGCGGCTACAGCTT | TCCTTAATGTCACGCACGATTT | ACCACCACGGCCGAGCGG |

Abbreviations: DHFR = dihydrofolate reductase; DPD = dihydropyrimidine dehydrogenase; EGFR = epidermal growth factor receptor; ERCC1 = excision repair cross-complementing gene 1; MTHFR = methylenetetrahydrofolate reductase; OPRT = orotate phosphoribosyl transferase; TP = thymidine phosphorylase; TS = thymidylate synthase; VEGF-A = vascular endothelial growth factor-A.

**Table 2** Patient characteristics

| Characteristic | Patients No. | Patients % |
|---|---|---|
| All patients | 140 | |
| *Sex* | | |
| Male | 108 | 77 |
| Female | 32 | 23 |
| *Age (years)* | | |
| Median | 65 | |
| Range | 18–87 | |
| *ECOG performance status* | | |
| 0 | 70 | 50 |
| 1 | 62 | 44 |
| 2 | 8 | 6 |
| *Metastatic site* | | |
| Lymph nodes | 87 | 62 |
| Peritoneum | 43 | 31 |
| Liver | 43 | 31 |
| Lung | 8 | 6 |
| Other | 9 | 6 |
| *Histological type* | | |
| Intestinal | 60 | 43 |
| Diffuse | 80 | 57 |
| *First-line chemotherapy regimen* | | *(Response rate[a] (95% CI))* |
| S-1 | 69 | 34.8 (23.7–47.2) |
| Cisplatin+S-1 | 14 | 35.7 (12.8–64.9) |
| Cisplatin+irinotecan | 29 | 44.8 (26.5–64.3) |
| 5-FU | 23 | 4.3 (0.1–22.0) |
| 5-FU+methotrexate | 2 | 0 |
| Paclitaxel | 2 | 50.0 (1.3–98.7) |
| Uracil/ftorafur (UFT®) | 1 | 0 |

Abbreviation: ECOG = Eastern Cooperative Oncology Group. [a]Response rate was calculated as the ratio of (CR+PR)/(CR+PR+NC+PD).

The chemotherapy regimens received by the patients and the response rates are also listed in Table 2. Many patients received S-1 monotherapy or cisplatin-based regimens as first-line treatment. The response rates with first-line chemotherapies in our study were comparable to those reported previously (Sakata *et al*, 1998; Boku *et al*, 1999; Ohtsu *et al*, 2003; Ajani *et al*, 2006).

## Gene expression levels of selected biomarkers, clinical data, and overall survival in all patients

Gene expression levels of selected biomarkers were quantifiable in 88.6–99.3% of the 140 tumours (Table 3). Gene expression cutoff values in terms of overall survival were defined by using the maximal $\chi^2$ method, and corrected *P*-values were calculated for each single gene. On univariate analyses, overall survival in the study group as a whole correlated with the expression levels of ERCC1, DPD, EGFR, and TS, the serum levels of LDH and ALP, and performance status (Table 4). Using these significant mRNA factors on univariate analyses, we performed combined analysis. Patients with low mRNA expressions of ERCC1, DPD, TS, and high expression of EGFR ($N=30$) had significantly longer overall survival than did the other patients ($N=106$) (median overall survival, 22.0 *vs* 11.2 months; $P<0.001$, log-rank test; Figure 2). Multivariate analysis with a Cox proportional hazards model demonstrated that high ERCC1 expression (HR: 2.38 (1.55–3.67)), high DPD expression (HR: 2.04 (1.37–3.02)), low EGFR expression (HR: 0.34 (0.20–0.56)), and an elevated serum ALP level (HR: 1.00 (1.001–1.002)) were significant predictors of poor survival (Table 4).

**Table 3** Gene expression levels of analysed biomarkers in all 140 patients

| Gene | No. of patients (%) | mRNA expression levels relative to β-actin ($\times 10^{-3}$) Median | Range |
|---|---|---|---|
| TS | 139 (99.3) | 2.81 | 0.84–16.05 |
| DPD | 134 (95.7) | 0.85 | 0.07–13.54 |
| TP | 139 (99.3) | 5.96 | 0.82–32.01 |
| OPRT | 138 (98.6) | 0.99 | 0.28–4.55 |
| DHFR | 124 (88.6) | 2.94 | 0.42–8.69 |
| MTHFR | 136 (97.1) | 1.24 | 0.25–8.20 |
| ERCC1 | 139 (99.3) | 1.03 | 0.22–6.22 |
| EGFR | 126 (90.0) | 1.24 | 0.12–57.78 |
| VEGF-A | 137 (97.9) | 4.89 | 1.07–30.23 |

Abbreviations: DHFR = dihydrofolate reductase; DPD = dihydropyrimidine dehydrogenase; EGFR = epidermal growth factor receptor; ERCC1 = excision repair cross-complementing gene 1; MTHFR = methylenetetrahydrofolate reductase; OPRT = orotate phosphoribosyl transferase; TP = thymidine phosphorylase; TS = thymidylate synthase; VEGF-A = vascular endothelial growth factor-A.

## Gene expression levels of selected biomarkers, tumour response, and time to progression in patients treated with S-1 monotherapy or cisplatin-based regimens as first-line chemotherapy

To better understand the relation between mRNA levels of selected biomarkers and treatment outcomes with each chemotherapy regimen, we performed subgroup analyses. Gene expression cutoff values that best segregated patients into poor- and good-response subgroups were defined by using the maximal $\chi^2$ method. In patients given first-line S-1 monotherapy, low MTHFR (low: 44.9% *vs* high: 6.3%, $P=0.006$) gene expression alone correlated with a better response (Table 5). Expressions of the other eight genes did not correlate with response. In patients treated with first-line cisplatin-based regimens (combined with S-1 or irinotecan), low ERCC1 (low: 55.6% *vs* high: 18.8%, $P=0.008$) gene expression alone correlated with a better response (Table 5). Expressions of the other eight genes did not show any correlation with response.

Gene expression cutoff values and the corrected *P*-values for time to progression analyses were determined by the same methods as those used in the analyses of overall survival. In patients given first-line S-1 monotherapy, expression levels of DHFR and EGFR were significantly associated with the time to progression (Table 6). When $2.89 \times 10^{-3}$ was used as the cutoff value for DHFR, the median time to progression was 6.1 months in the low-expression group and 4.0 months in the high-expression group (corrected log-rank $P=0.003$, HR: 2.43 (95% CI: 1.37–4.29)). DHFR gene expression correlated with TS expression, with a Spearman's rank correlation coefficient of 0.456 ($P<0.001$). When a cutoff value of $0.33 \times 10^{-3}$ was used for EGFR, the median time to progression was significantly longer in the high EGFR expression group (low: 2.8 months *vs* high: 5.3 months, $P=0.007$, HR: 0.31 (0.16–0.62)). The association between expression levels of TS, DPD, TP, OPRT, MTHFR, ERCC1, and VEGF-A and the time to progression did not show significant results (Table 6).

In patients who received cisplatin-based regimens as first-line chemotherapy, expression levels of DPD and MTHFR correlated with the time to progression (Table 6). At a DPD cutoff value of $1.55 \times 10^{-3}$, the median time to progression was 4.6 months in the

**Table 4** Univariate analysis and Cox regression multivariate analysis of overall survival in all patients included in this study: correlation with mRNA expression levels and clinical data

| | | | | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|---|---|---|
| **Factor[a]** | **Cut point** | **No. of patients** | **Median (months)** | **Hazard ratio (95% CI)** | ***P*** | **Hazard ratio (95% CI)** | ***P*** |
| LDH | Continuous | — | — | — | <0.001 | | |
| | Variable | | | 1.00 (1.000–1.001) | | | |
| ALP | Continuous | — | — | — | <0.001 | — | < 0.001 |
| | Variable | | | 1.00 (1.001–1.002) | | 1.00 (1.001–1.002) | |
| ERCC1 | $\leqslant 1.42 \times 10^{-3}$ | 103 | 14.3 | 1 | 0.002 | 1 | < 0.001 |
| | $> 1.42 \times 10^{-3}$ | 36 | 9.8 | 2.12 (1.41–3.18) | | 2.38 (1.55–3.67) | |
| DPD | $\leqslant 1.18 \times 10^{-3}$ | 93 | 14.5 | 1 | 0.003 | 1 | < 0.001 |
| | $> 1.18 \times 10^{-3}$ | 44 | 10.2 | 1.95 (1.34–2.83) | | 2.04 (1.37–3.02) | |
| PS | Continuous | — | — | — | 0.004 | | |
| | Variable | | | 1.55 (1.15–2.08) | | | |
| EGFR | $\leqslant 0.33 \times 10^{-3}$ | 21 | 8.2 | 1 | 0.005 | 1 | < 0.001 |
| | $> 0.33 \times 10^{-3}$ | 118 | 13.6 | 0.42 (0.26–0.69) | | 0.34 (0.20–0.56) | |
| TS | $\leqslant 2.61 \times 10^{-3}$ | 62 | 16.0 | 1 | 0.010 | | |
| | $> 2.61 \times 10^{-3}$ | 77 | 11.2 | 1.64 (1.15–2.34) | | | |

Abbreviations: ALP = alkaline phosphatase; DPD = dihydropyrimidine dehydrogenase; EGFR = epidermal growth factor receptor; ERCC1 = excision repair cross-complementing gene 1; LDH = lactate dehydrogenase; PS = performance status; TS = thymidylate synthase. Note: 'Cutoff point' for mRNA expression level was determined by the maximal $\chi^2$ method. [a]Factors with *P*-values of <0.010 in univariate analyses are listed in ascending order of *P*-values. The stepwise method was used to select factors for multivariate analysis.

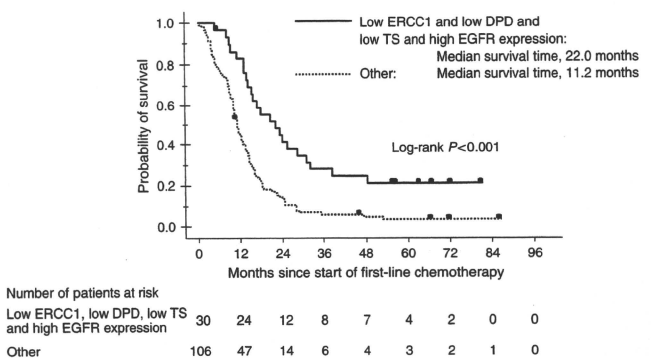


**Figure 2** Kaplan–Meier plot of overall survival for all patients according to ERCC1, DPD, TS, and EGFR mRNA expression levels.

low DPD expression group as compared with only 1.2 months in the high DPD expression group ($P = 0.008$, HR: 4.87 (1.75–13.53)). At a MTHFR cutoff value of $0.94 \times 10^{-3}$, the median time to progression was significantly longer in the high-expression group (low: 2.9 months *vs* high: 5.9 months, $P = 0.007$, HR: 0.17 (0.07–0.42)). The association between expression levels of the other seven genes and time to progression did not show significant results (Table 6).

## DISCUSSION

In this study, we analysed mRNA expression levels of nine genes involved in 5-FU and folate metabolism, DNA repair, and angiogenesis in primary tumours from 140 patients with advanced GC. Our goal was to determine whether such expression levels are related to treatment outcomes such as survival and response. We found that high DPD expression, high ERCC1 expression, and low EGFR expression in GC specimens were significant predictors of poor survival in advanced GC. Recently, several studies have reported that patients' genetic profiles are related to the outcomes of cancer therapy (van 't Veer *et al*, 2002; Ruzzo *et al*, 2006). In colorectal cancer, since many studies have examined molecular predictors of outcomes during the past decade, TS, DPD, and TP were newly included in 'ASCO 2006 tumour marker guidelines in gastrointestinal cancer' (Locker *et al*, 2006). Because sufficient supporting evidence is lacking, however, the guidelines recommend that these biomarkers should not yet be used clinically to predict prognosis or treatment response. Further studies are therefore needed to more clearly define the relation between mRNA expression levels and clinical outcomes.

Our study showed that gene expression levels of DPD (related to the pharmacokinetics of fluoropyrimidines) and ERCC1 (related to the pharmacodynamics of cisplatin) had significant impacts on the overall survival of patients with advanced GC. This finding is consistent with the results of previous investigations (Metzger *et al*, 1998; Terashima *et al*, 2002; Napieralski *et al*, 2005). S-1, an oral DPD inhibitory fluoropyrimidine, is a novel antitumour drug

**Table 5** Gene expression levels and tumour response in patients with advanced gastric cancer according to first-line chemotherapy

| | S-1 monotherapy ($N=69$) | | | | | Cisplatin-based regimens[a] ($N=43$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Factor** | **Total no. of patients** | **Cut point ($\times 10^{-3}$)** | **RR (%) in low group** | **RR (%) in high group** | ***P*** | **Total no. of patients** | **Cut point ($\times 10^{-3}$)** | **RR (%) in low group** | **RR (%) in high group** | ***P*** |
| TS | 66 | 3.67 | 45.2 (19/42) | 20.8 (5/24) | 0.044 | 43 | 3.43 | 50.0 (15/30) | 23.1 (3/13) | 0.103 |
| DPD | 65 | 0.83 | 25.9 (7/27) | 44.7 (17/38) | 0.119 | 42 | 0.84 | 28.0 (7/25) | 58.8 (10/17) | 0.041 |
| TP | 66 | 5.37 | 25.9 (7/27) | 43.6 (17/39) | 0.121 | 43 | 7.81 | 32.1 (9/28) | 60.0 (9/15) | 0.049 |
| OPRT | 65 | 0.61 | 0 (0/6) | 39.0 (23/59) | 0.059 | 43 | 0.94 | 57.1 (12/21) | 27.3 (6/22) | 0.029 |
| DHFR | 59 | 1.64 | 57.1 (4/7) | 28.8 (15/52) | 0.105 | 39 | 2.32 | 31.6 (6/19) | 45.0 (9/20) | 0.323 |
| MTHFR | 65 | 1.82 | **44.9 (22/49)** | **6.3 (1/16)** | **0.006** | 43 | 1.15 | 52.2 (12/23) | 30.0 (6/20) | 0.152 |
| ERCC1 | 65 | 0.92 | 50.0 (14/28) | 24.3 (9/37) | 0.033 | 43 | 1.18 | **55.6 (15/27)** | **18.8 (3/16)** | **0.008** |
| EGFR | 66 | 1.20 | 45.7 (16/35) | 25.8 (8/31) | 0.094 | 43 | 1.39 | 51.7 (15/29) | 21.4 (3/14) | 0.049 |
| VEGF-A | 65 | 2.70 | 54.5 (6/11) | 31.5 (17/54) | 0.104 | 43 | 6.52 | 53.8 (14/26) | 23.5 (4/17) | 0.022 |

Abbreviations: DHFR = dihydrofolate reductase; DPD = dihydropyrimidine dehydrogenase; EGFR = epidermal growth factor receptor; ERCC1 = excision repair cross-complementing gene 1; MTHFR = methylenetetrahydrofolate reductase; OPRT = orotate phosphoribosyl transferase; RR = response rate; TP = thymidine phosphorylase; TS = thymidylate synthase; VEGF-A = vascular endothelial growth factor-A. Note: 'Cutoff point' was determined by the maximal $\chi^2$ method. The level of significance was set at $P<0.010$. Significant values are shown in bold. [a]Cisplatin-based regimens: cisplatin+S-1 and cisplatin+irinotecan.

**Table 6** Univariate analyses of time to progression in patients with advanced gastric cancer treated with S-1 monotherapy or cisplatin-based regimens as first-line chemotherapy: correlation with mRNA expression levels

| | S-1 monotherapy ($N=69$) | | | | | Cisplatin-based regimens[a] ($N=43$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Factor** | **Cut point ($\times 10^{-3}$)** | **No. of patients** | **Median (months)** | **Hazard ratio (95% CI)** | ***P*** | **Cut point ($\times 10^{-3}$)** | **No. of patients** | **Median (months)** | **Hazard ratio (95% CI)** | ***P*** |
| TS | ⩽5.27 | 60 | 4.5 | 1 | 0.131 | ⩽3.36 | 29 | 5.4 | 1 | 0.140 |
| | >5.27 | 9 | 4.2 | 2.11 (0.97–4.55) | | >3.36 | 14 | 3.9 | 1.68 (0.87–3.24) | |
| DPD | ⩽1.57 | 51 | 4.9 | 1 | 0.080 | ⩽1.55 | 37 | 4.6 | 1 | **0.008** |
| | >1.57 | 17 | 4.0 | 1.90 (1.06–3.43) | | >1.55 | 5 | 1.2 | 4.87 (1.75–13.53) | |
| TP | ⩽5.58 | 31 | 4.0 | 1 | 0.207 | ⩽8.31 | 30 | 4.2 | 1 | 0.222 |
| | >5.58 | 38 | 5.1 | 0.72 (0.44–1.18) | | >8.31 | 13 | 6.2 | 0.62 (0.32–1.22) | |
| OPRT | ⩽1.44 | 48 | 4.2 | 1 | 0.223 | ⩽0.92 | 20 | 6.2 | 1 | 0.036 |
| | >1.44 | 20 | 4.2 | 1.37 (0.79–2.36) | | >0.92 | 23 | 3.9 | 1.93 (1.02–3.66) | |
| DHFR | ⩽2.89 | 29 | 6.1 | 1 | 0.003 | ⩽5.82 | 35 | 4.5 | 1 | 0.215 |
| | >2.89 | 33 | 4.0 | 2.43 (1.37–4.29) | | >5.82 | 4 | 14.6 | 0.41 (0.12–1.39) | |
| MTHFR | ⩽1.04 | 20 | 2.9 | 1 | 0.158 | ⩽0.94 | 10 | 2.9 | 1 | **0.001** |
| | >1.04 | 48 | 5.1 | 0.59 (0.34–1.01) | | >0.94 | 33 | 5.9 | 0.17 (0.07–0.42) | |
| ERCC1 | ⩽1.30 | 49 | 4.2 | 1 | 0.370 | ⩽1.12 | 24 | 4.2 | 1 | 0.318 |
| | >1.30 | 19 | 4.4 | 0.72 (0.41–1.27) | | >1.12 | 19 | 5.9 | 0.75 (0.41–1.39) | |
| EGFR | ⩽0.33 | 12 | 2.8 | 1 | **0.007** | ⩽0.81 | 16 | 3.9 | 1 | 0.158 |
| | >0.33 | 57 | 5.3 | 0.31 (0.16–0.62) | | >0.81 | 27 | 5.2 | 0.53 (0.28–1.03) | |
| VEGF-A | ⩽2.45 | 7 | 1.9 | 1 | 0.193 | ⩽7.86 | 34 | 3.8 | 1 | 0.130 |
| | >2.45 | 61 | 4.4 | 0.46 (0.21–1.02) | | >7.86 | 9 | 7.2 | 0.43 (0.20–0.93) | |

Abbreviations: DHFR = dihydrofolate reductase; DPD = dihydropyrimidine dehydrogenase; EGFR = epidermal growth factor receptor; ERCC1 = excision repair cross-complementing gene 1; MTHFR = methylenetetrahydrofolate reductase; OPRT = orotate phosphoribosyl transferase; TP = thymidine phosphorylase; TS = thymidylate synthase; VEGF-A = vascular endothelial growth factor-A. Note: 'Cutoff point' was determined by the maximal $\chi^2$ method. The level of significance was set at $P<0.010$. Significant values are shown in bold. [a]Cisplatin-based regimens: cisplatin+S-1 and cisplatin+irinotecan.

combining tegafur (FT: a prodrug of 5-FU), gimeracil (CDHP: 5-chloro-2,4 dihydropyridine), and oteracil (Oxo: potassium oxonate) (Shirasaka *et al*, 1993, 1996). CDHP inhibits DPD activity and therefore prevents fluoropyrimidine degradation (Shirasaka *et al*, 1996). Oxo is a gastrointestinal tract adverse effect modulator (Shirasaka *et al*, 1993). In Japan, S-1 as monotherapy or combined with cisplatin is a standard regimen for advanced GC (Sakata *et al*, 1998; Ajani *et al*, 2006). Boku *et al* (2007) reported the result of a randomised controlled trial showing that S-1 is a promising standard regimen as compared with 5-FU, and Narahara *et al* (2007) showed that S-1 plus cisplatin is superior to S-1 alone. A multinational phase III study comparing S-1 plus cisplatin with 5-FU plus cisplatin (control regimen) is now underway. In the future, S-1 combined with cisplatin may become a standard regimen not only in Japan but also worldwide. In our study, 129 patients (92%) received S-1- or 5-FU-based regimens, and 73 patients (52%) received cisplatin-based regimens as first-line or subsequent chemotherapy. Our results strongly suggest that tumours with high DPD and ERCC1 gene expression are unlikely to respond to current standard therapy, resulting in inadequate tumour control and poor outcomes. Patients with such tumours would require newly developed drugs and combined treatment modalities tailored to their specific needs.

Patients with low DHFR expression had a higher response rate and a longer time to progression while receiving S-1 monotherapy (Tables 5 and 6). DHFR is a key enzyme of folate metabolism. DHFR converts intracellular inactive dihydrofolate back to active tetrahydrofolate, which is reused in deoxythymidine-5′-monophosphate synthesis (Figure 1) and is crucial for 5-FU antitumour activity. Sowers *et al* (2003) reported that E2F transcription factors

may participate in the regulation of both TS and DHFR expression. We showed that a Spearman's correlation coefficient for TS/DHFR was 0.456 ($P<0.001$). Backus *et al* (2000) reported that low TS expression *in vitro* correlated with increased sensitivity to 5-FU. Several clinical studies have found that patients with low TS gene expression in primary GC correlate with a better tumour response and longer survival after 5-FU or S-1 treatment (Lenz *et al*, 1996; Ichikawa *et al*, 2004). The results of these studies suggest that low DHFR expression is associated with better clinical outcomes in patients given S-1 monotherapy, consistent with the results of our study. DHFR might thus be a candidate predictive biomarker of the response to S-1 treatment. Our data suggested that low DHFR expression may be an important determinant of tumour-cell sensitivity to S-1.

In patients who received S-1 monotherapy, low MTHFR gene expression also correlated with a better tumour response (Table 5). Some studies have reported that the *MTHFR 677T* mutation, linked to the reduced activity of MTHFR, increases chemosensitivity to 5-FU (Cohen *et al*, 2003; Sohn *et al*, 2004), whereas others have had inconsistent results (Etienne *et al*, 2004; Ruzzo *et al*, 2006). Although MTHFR and DHFR are key enzymes in folate metabolism, the role of MTHFR gene expression in the antitumour activity of 5-FU remains controversial.

In patients given cisplatin-based regimens as first-line chemotherapy, low ERCC1 mRNA expression alone correlated with a better tumour response, confirming a previously reported association (Metzger *et al*, 1998). With respect to EGFR gene expression, evidence supporting a correlation between mRNA expression levels and survival or time to progression in GC is scant. Vallbohmer *et al* (2006) reported that high mRNA expression of EGFR was associated with a better response as well as longer progression-free and overall survival in patients with colorectal cancer who received irinotecan therapy, which is partially in accord with our findings. In contrast, Gamboa-Dominguez *et al* (2004) found that strong membranous staining of EGFR on immunohistochemical analysis correlated with poor survival. The clinical implications of EGFR gene expression thus remain controversial.

In conclusion, our study provides evidence that high DPD, high ERCC1, and low EGFR gene expression levels in GC specimens and an elevated serum ALP level are risk factors for poor survival in patients with advanced GC. To the best of our knowledge, this is the first study showing that mRNA expression levels of molecular markers in primary GC had as much impact on survival outcomes as did well-recognised prognostic factors. The results of our analysis will hopefully provide a more rational basis for clinical decision-making, risk stratification of patients, and selection of management strategies as well as suggest benchmarks for future randomised controlled trials. Our relatively small sample size precludes drawing any firm conclusions, and candidate biomarkers must be validated in prospective studies. To confirm and extend the results of this exploratory study, larger studies are being planned in Japan.

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# Importance of *UDP-glucuronosyltransferase 1A1*6* for irinotecan toxicities in Japanese cancer patients

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**Abstract**

Recent pharmacogenetic studies on irinotecan have revealed the impact of *UDP glucuronosyltransferase (UGT) 1A1*28* on severe irinotecan toxicities. Although the clinical role of *UGT1A1*6*, which is specifically detected in East Asian patients, in irinotecan toxicities is suggested, clear evidence remains limited. To examine the impact of **6*, the association of *UGT1A1* genotypes with severe irinotecan toxicities was retrospectively investigated in Japanese cancer patients. A significant **6*-dependent increase in the incidence of grade 3 or 4 neutropenia was observed in 49 patients on irinotecan monotherapy ($p = 0.012$). This study further clarifies the clinical importance of **6* in irinotecan therapy in East Asians.


*Keywords:* UGT1A1; Pharmacogenetics; Irinotecan; SN-38

## 1. Introduction

Irinotecan, an anticancer prodrug, is widely applied for a broad range of carcinomas, including colorectal and lung cancers. The active metabolite, SN-38 (7-ethyl-10-hydroxycamptothecin), a topoisomerase I inhibitor, is generated by hydrolysis of the parent compound by carboxylesterases [1]. SN-38 is subsequently glucuronidated by uridine diphosphate glucuronosyltransferase 1As (UGT1As) such as 1A1, 1A7, 1A9 and 1A10, to form the inactive metabolite, SN-38 glucuronide (SN-38G) [2–5]. Among the UGT

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isoforms, UGT1A1 is thought to be a predominant contributor to SN-38G formation [2,6]. The dose-limiting toxicities in irinotecan therapy are severe diarrhea and leucopenia [7], and lowered UGT activity is well correlated with severe irinotecan toxicities [8]. Since Ando et al. first reported the significant relevance of *UGT1A1*28* – a repeat polymorphism in the TATA box (−40_−39insTA) – to severe neutropenia/diarrhea [9], a number of clinical studies, primarily conducted in Caucasian patients, have shown associations between *UGT1A1*28* and lowered SN-38G formation or severe neutropenia/diarrhea [10–13]. Based on these findings, the Food and Drug Administration (FDA) of the United States approved a revision of the label for Camptosar (irinotecan HCl) (NDA 20-571/S-024/S-027/S-028), recommending "a reduction in the starting dose by at least one level of irinotecan for the *UGT1A1*28* homozygous patients". Subsequently, the clinical application of *UGT1A1*28* testing was put into practice for irinotecan therapy in the United States.

To implement personalized irinotecan therapy in Asian countries, the racial differences in *UGT1A1* polymorphisms among Caucasians, African-Americans, and Asians must be taken into consideration [14]. For East Asians, the frequency of **28* is one third of that of Caucasians or African-Americans, and another low-activity allele **6* [211G>A(G71R)], which is not detected in Caucasians or African-Americans, shows the same frequency as the **28* allele. Clinical studies in Japanese cancer patients have demonstrated that significantly low area under concentration-time curve (AUC) ratios of SN-38G to SN-38 are observed in patients having **6* and/or **28* [15–17], suggesting the necessity of typing **6* in addition to **28*. A recent report on Korean lung cancer patients who received a combination therapy of irinotecan and cisplatin, showed a significant association of **6* homozygotes with severe neutropenia [18]. However, data on the role of **6* in irinotecan toxicities is still limited in terms of the various irinotecan-containing regimens. In the first study by Ando et al. on Japanese cancer patients, the association of **6* with irinotecan toxicities was not evident, but a possible enhancement of **28*-related toxicities by **6* was suggested [9]. Other studies in Japanese patients showed an additive effect of **6* on the lowered UGT activity by **28* [15–17]. A significant association of the genetic marker "**6 or *28*" with severe neutropenia was also shown in our previous study, but due to a lack of **6* homozygotes in our patient population, the effect of **6* alone was not confirmed [17].

In this study, to further demonstrate the clinical importance of **6* alone, *UGT1A1* genotypes were determined using DNA extracted from paraffin-embedded specimens (non-cancerous tissues) from 75 Japanese cancer patients by the pyrosequencing method [19,20], and the associations between *UGT1A1* genotype and severe irinotecan toxicities and serum total bilirubin levels were retrospectively analyzed.

## 2. Materials and methods

### 2.1. Patients and irinotecan treatment

In a post-marketing surveillance study conducted by Daiichi Pharmaceutical Co., Ltd. (currently Daiichi Sankyo Co., Ltd., Tokyo, Japan), irinotecan was prescribed to 297 patients with various types of cancers from 1995 to 2000 at the National Cancer Center Hospital. The patients were selected through standard clinical practice according to the drug label for indications and contraindications. Methanol-fixed, paraffin-embedded archival tissue specimens, which were necessary for high-quality extraction of DNA greater than 2 kb in size [21], were available for 75 of the 297 patients and were analyzed in this study. Irinotecan was administered by intravenous 30-min infusion as a single agent or in combination chemotherapy at a dose of 60 mg/m$^2$ (weekly or biweekly), 100 mg/m$^2$ (biweekly), or 150 mg/m$^2$ (biweekly). Profiles of the patients in this study, including cancer type, treatment history, and regimens, are summarized in Table 1. The pre-treatment levels of serum total bilirubin were determined by a kit (VL T-BIL, Azwell Inc., Osaka, Japan) according to an enzymatic method using bilirubin oxidase [22]. Toxicities were monitored during irinotecan therapy and graded according to the Common Toxicity Criteria version 2 of the National Cancer Institute.

Because the samples in this study were residual specimens remaining after histopathological diagnosis in the hospital and not collected specifically for research purposes, the samples and their clinical information were anonymized in an unlinkable fashion according to the Ethics Guidelines for Human Genome/Gene Analysis Research by the Ministry of Education, Culture, Sports, Science and Technology, Ministry of Health, Labour and Welfare, and Ministry of Economy, Trade and Industry of Japan. This study was approved by the ethics committees of the National Cancer Center and the National Institute of Health Sciences.

### 2.2. DNA extraction from paraffin-embedded tissue sections and genotyping of UGT1A1 polymorphisms

Three sections (20 μm of pathologically normal tissues around tumors) were deparaffinized twice by treat-

Table 1
Profiles of cancer patients in this study

| | | No. of patients |
|---|---|---|
| Patients genotyped (Male/female) | | 75 (51/24) |
| Age | | |
| Mean/range (y) | 50.7/34–75 | |
| Performance Status[a] | | |
| | 0/1/2 | 18/48/8 |
| Previous treatment | | |
| Surgery[a] | +/− | 71/3 |
| Chemotherapy[b] | +/− | 63/10 |
| Radiotherapy[b] | +/− | 9/64 |
| Combination therapy and tumor type [dose of irinotecan (mg/m$^2$)/(w or 2w)[c]] | | |
| Irinotecan monotherapy | Lung (60/w or 100/2w) | 4 |
| | Stomach (100/2w or 150/2w) | 5 |
| | Colon (100/2w or 150/2w) | 40 |
| With cisplatin | Lung (60/w or 100/2w) | 4 |
| | Stomach (60/2w) | 11 |
| With mitomycin C (MMC) | Stomach (150/2w) | 8 |
| | Breast (120/2w) | 1 |
| With 5-fluorouracil (5-FU) | Colon (150/2w) | 2 |
| Available data on serum bilirubin levels | | 37 |

[a] Data from one patient is lacking.
[b] Data from two patients are lacking.
[c] Weekly or biweekly.

ment with 1.5 ml of xylene at room temperature. After centrifugations, the residual pellet was then washed twice with 1.5 ml of ethanol. Finally, the pellet was dried at 37 °C for 15 min. DNA extraction was performed using a QIAamp tissue kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions with some modifications. Briefly, 540 μl of ATL lysis buffer and 60 μl of proteinase K (Qiagen) were added to each pellet, mixed thoroughly, and incubated at 56 °C for 3 h with a rotator. Any remaining tissue debris was removed by centrifugation, and the resulting supernatant was used for the extraction. Twelve microliters of RNase A (100 mg/ml) was added to the supernatant and incubated for 2 min at room temperature. Next, 600 μl of buffer AL was added and mixed thoroughly, and the mixture was incubated at 70 °C for 10 min. Six-hundred microliters of ethanol was added to the solution and mixed well, followed by extraction of DNA using a Qiagen DNA extraction column. The DNA was eluted in a final elution volume of 150 μl. The yield was determined using a NanoDrop spectrophotometer (NanoDrop Technology, Inc, Rockland, DE, USA) and the size of the extracted DNA was checked by agarose gel electrophoresis.

Genotyping of *UGT1A1*6* (211G>A, G71R), **28* (−364C>T, which is perfectly linked with −40_−39insTA in Japanese), and **60* (−3279T>G) were performed by pyrosequencing as described previously [19,20].

### 2.3. Association analysis and statistics

For association analysis, we focused on incidences of severe diarrhea and neutropenia (grade 3 or greater) observed during irinotecan-therapy. The incidence of severe diarrhea was very low, and the incidence of neutropenia was higher in combination therapy. Therefore, the association of neutropenia with *UGT1A1* genotypes was primarily evaluated in 49 patients with irinotecan monotherapy. As a parameter for in vivo UGT1A1 activity, serum total bilirubin levels taken at baseline from 37 patients were also used.

Statistical analysis for evaluation of the relationship between *UGT1A1* genotypes and severe neutropenia was performed using the chi-square test for trend using Prism version 4.0 (GraphPad Prism Software Inc., San Diego, CA). The gene-dose effect of the genetic marker "**6 or *28*" on serum total bilirubin levels was analyzed using the Jonckheere–Terpstra (JT) test in the SAS system (version 5.0, SAS Institute, Inc., Cary, NC). The *P*-value of 0.05 (two-tailed) was set as a significant level. Multivariate logistic regression analysis on neutropenia (grade 3 or greater) was performed using JMP software (version 6.0.0, SAS Institute, Inc., Cary, NC), including variables for age, sex, body surface area, performance status, concomitant disease, history of adverse reaction, irinotecan dosage, dosing interval, and *UGT1A1* genotypes. The variables in the final model for neutropenia were chosen using the forward and backward stepwise procedure at the significance level of 0.1.

## 3. Results

### 3.1. UGT1A1 diplotypes/haplotypes

The diplotypes and haplotypes (**1, *60, *6* and**28*) of *UGT1A1* exon 1 were analyzed in 75 Japanese cancer patients (Table 1) and their frequencies were summarized (Table 2). The haplotypes were assigned according to our previous definition [15]. It should be noted that the **60* haplotype does not harbor the **28* allele (−40_−39insTA), but most of the **28* haplotype does harbor the **60* allele (−3279T>G). In this study, the **28* homozygote was not present, and the frequency of haplotype **28* (0.113) was slightly lower than that found in our previous study (0.138) [17]. In contrast, the frequency of haplotype **6* (0.213) was higher than that found in the previous study (0.167) [17].

Table 2
Frequencies of *UGT1A1* diplotypes (A) and haplotypes (B) for cancer patients in this study

| | | Frequency |
|---|---|---|
| (A) Diplotype | No. of patients (N = 75) | |
| **1/*1* | 21 | 0.280 |
| **1/*60* | 9 | 0.120 |
| **60/*60* | 2 | 0.027 |
| **6/*1* | 14 | 0.187 |
| **6/*60* | 8 | 0.107 |
| **6/*6* | 4 | 0.053 |
| **28/*1* | 12 | 0.160 |
| **28/*60* | 3 | 0.040 |
| **28/*6* | 2 | 0.027 |
| **28/*28* | 0 | 0.000 |
| (B) Haplotype[a] | No. of chromosomes (N = 150) | |
| **1* | 77 | 0.513 |
| **60* | 24 | 0.160 |
| **6* | 32 | 0.213 |
| **28* | 17 | 0.113 |

[a] Haplotype definition follows the previous report [15]; **60*, −3279T>G without −40_−39insTA; **6*, 211G>A(G71R); **28*, −40_−39insTA.

### 3.2. Association of UGT1A1 genotypes with serum total bilirubin levels

Serum total bilirubin levels at baseline, a parameter of in vivo UGT1A1 activity, were available from 37 patients (treated by various regimens), and we analyzed their association with *UGT1A1* genotypes (Fig. 1). The median values of total bilirubin in **60/*1*, **28/*1* and **6/*1* heterozygotes were not significantly different from that of the wild type (**1/*1*). Higher median values were observed for the **6* homozygotes (**6/*6*) and the double heterozygotes of **6* and **28* (**6/*28*) than that of the wild type (**1/*1*), with increases of 1.9-fold and 2.2-fold, respectively. Since **6* and **28* are mutually independent and their reducing effects on UGT activity are equivalent [15,17], diplotypes were classified by the presence of "**6 or *28*" (indicated by "+" in Fig. 1). As shown in Fig. 1, a significant "**6 or *28*"-dependent increase in total bilirubin levels was observed ($p = 0.0088$, Jonckheere–Terpstra test).

### 3.3. Severe toxicities observed in this study

Incidences of severe diarrhea and neutropenia (grade 3 or greater) are shown in Table 3 for each irinotecan-containing regimen. Grade 3 diarrhea was observed in only 4 of the 75 subjects, and since the incidence of diarrhea was low (5.3%), an association analysis on diarrhea was not conducted. Regarding neutropenia, 26 patients experienced grade 3 or 4 neutropenia. Of these 26 patients, 90% experienced neutropenia within 2 months after starting irinotecan-therapy, and 70% within 2 weeks. Significant differences in neutropenia incidences were observed among the regimens used, and considerably high incidences were observed in the combination therapies. Accordingly, association of the *UGT1A1* genotypes with severe neutropenia was analyzed primarily in the patients who received irinotecan-monotherapy.

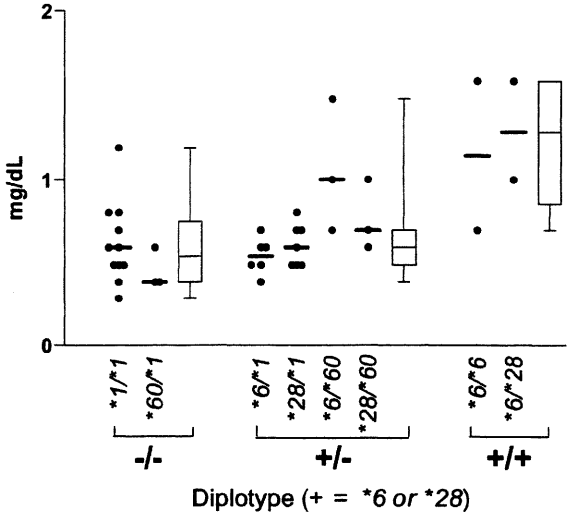


Fig. 1. Effects of *UGT1A1* genotypes on serum total bilirubin levels at baseline in Japanese cancer patients ($N = 37$). Each point represents a patient, and the median value of each diplotype is shown with a bar. All diplotypes are classified into −/−, +/−, and +/+ by the genetic marker, "*UGT1A1*6 or *28*", indicated by "+", and their distributions are shown by a box representing the 25–75 percentiles with a bar at the median and lines representing the highest and lowest values. A significant "**6 or *28*"-dependent increase in total bilirubin levels was observed ($p = 0.0088$, Jonckheere–Terpstra test).

Table 3
Severe toxicities observed in Japanese cancer patients

| Treatment | Diarrhea[a]/total (%) | Neutropenia[b]/total (%) |
|---|---|---|
| Total patients | 4/75 (5.3) | 26/75 (34.7) |
| Irinotecan alone | 1/49 (2.0) | 6/49 (12.2) |
| With CDDP | 2/15 (13.3) | 11/15 (73.3) |
| With MMC | 1/9 (11.1) | 8/9 (88.9) |
| With 5-FU | 0/2 (0.0) | 1/2 (50.0) |
| P-value[c] | NS | <0.0001 |

[a] Grade 3.
[b] Grade 3 or 4.
[c] Chi-squre test.

### 3.4. Association of UGT1A1 genotypes with neutropenia

Since significant associations of *UGT1A1*6* and **28* with increased total bilirubin levels (decreased UGT-activity) were once again confirmed in this study, we assessed the clinical relevance of these haplotypes, focusing on the effect of **6* on severe neutropenia. In the 49

patients who received irinotecan monotherapy, the incidence of grade 3 or 4 neutropenia was *6-dependently increased ($p = 0.012$ in the chi-square test for trend). Namely, incidences of severe neutropenia in the *6 heterozygotes (*6/*1,*6/*60, and *6/*28) and homozygotes (*6/*6) were 2.3-fold and 15-fold higher, respectively, than that seen in the non-*6 bearing patients (*1/*1, *60/*1, *28/*1, and *28/*60) (Table 4). In this study, no *28 heterozygotes (*28/*1 and *28/*60) experienced any severe neutropenia, and there were no *28 homozygotes enrolled. Therefore, the effect of *28 could not be determined. For the *60-bearing patients without *6 or *28 (only heterozygote, *60/*1), one patient among six experienced severe neutropenia, and no significant *60-dependent increase was observed (data not shown). Although no statistically significant association of the *28 heterozygotes with severe neutropenia was confirmed in this study, the incidence of discontinuation of irinotecan monotherapy was higher in the *28-bearing patients (91%, $N = 11$) than that in the non-*28 subjects (79%, N = 38), while *60- or *6-dependent increased discontinuation rates were not found (data not shown). For the patients with cisplatin-combination therapy, a higher incidence of severe neutropenia was observed in the *6-bearing patients (*6/*1, *6/*60, and *6/*6) (100%, $N = 3$) than that in the non-*6 bearing subjects (*1/*1, *60/*1, *60/*60, and *28/*1) (66.7%, $N = 12$).

### 3.5. Multivariate analysis of neutropenia

In order to further clarify the clinical impact of *6 on irinotecan toxicities, multivariate logistic regression analysis on grade 3 or 4 neutropenia was conducted using variables, including *UGT1A1* genotypes and patient background factors, described in Section 2. The final model revealed a significant association of *6 with the incidence of grade 3 or 4 neutropenia at an odds ratio of 5.87 (Table 5).

## 4. Discussion

The clinical application of the genetic test for *UGT1A1*28* prior to irinotecan therapy has been in practice in the United States since 2005, which was based on cumulative evidence supporting the significant association of *28 with severe irinotecan toxicity [9–13]. Most of the evidence was obtained in Caucasian patients, where *28 is relatively frequent (30–40%) [14]. Although additive effects of another low activity allele, *6, which is specific for East Asians, has been also suggested [9,15–17], direct evidence in Japanese patients has remained limited. In this study, we clearly showed the significant correlation of *6 to grade 3 or 4 neutropenia in Japanese cancer patients who received irinotecan monotherapy. An increased incidence of severe neutropenia was also observed in the *6-bearing patients using cisplatin combination therapy. This finding is in accordance with a report on Korean lung cancer patients who received a combination therapy of irinotecan and cisplatin, which showed a significant association of *6 homozygotes with grade 4 neutropenia [18]. Since combination therapies using irinotecan may cause higher incidences of severe toxicities, the *UGT1A1* polymorphisms should be carefully considered in regimens that include irinotecan.

Since the alleles *6 and *28 are mutually independent [15] and their effects on the UGT activities were shown to be equivalent, the usefulness of the genetic marker "*6 or *28" for personalized irinotecan therapies has been suggested [17]. This was also supported in the current study, which showed a "*6 or *28"-dependent increase in serum total bilirubin levels (Fig. 1). Because of the low frequency of *28 without homozygotes among our subjects, the influence of *28 on toxicities was not clearly demonstrated, as in the case of the Korean patients where the allele frequency of *1A1*6* (23.5%) was much higher than that of *1A1*28* (7.3%) [18]. However, in the current study, the double heterozygotes of *6 and *28 (*6/*28) showed increases in serum total bilirubin levels (Fig. 1). Moreover, a higher incidence of severe neutropenia in the *6/*28 patients was observed, although the patient number was small ($N = 2$) (Table 4). This finding also indi-

Table 4
Association of *UGT1A1* genotypes with severe neutropenia (grade 3 or 4) in irinotecan monotherapy

| Diplotype[b] | Neutropenia[a]/total (%) | Effect of *6 (%) | |
|---|---|---|---|
| –/– | 1/20 (5.0) | non-*6/non-*6 | (3.4) |
| *28/– | 0/9 (0.0) | | |
| *6/– | 3/16 (18.8) | *6/non-*6 | (22.2) |
| *6/*28 | 1/2 (50.0) | | |
| *6/*6 | 1/2 (50.0) | *6/*6 | (50.0) |
| P-value[c] | | 0.012 | |

[a] Grade 3 or 4.
[b] "–" represents "*1 or *60".
[c] Chi-square test for trend.

Table 5
Multivariate logistic regression analysis of severe neutropenia (grade 3 or 4) in irinotecan monotherapy

| Variable | Coefficient | SE | *P*-value | Odds ratio | (95% Confidence limit) |
|---|---|---|---|---|---|
| *UGT1A1*6* | 1.77 | 0.809 | 0.0289 | 5.87 | (1.37–39.6) |

$R^2 = 0.157$, Intercept = 3.15, $N = 49$.

cates the importance of "*6 or *28" in severe neutropenia, and in fact, a gene-dose effect of "*6 or *28" ($p = 0.04$ in the chi-square test for trend) and its significant contribution in multivariate analysis ($p = 0.0326$) were also confirmed (data not shown).

For the *60 haplotype (-3279T>G without -40_-39insTA), no association of *60 with severe neutropenia was observed in this study, which coincides with reports of other studies on Japanese cancer patients [17,23]. As for the *27 allele [686C>A(P229Q)], it was linked with the *28 allele and the haplotype was defined as the *28 subtype,*28c [15]. One *28c-heterozygous patient with irinotecan monotherapy showed no severe neutropenia, suggesting a small contribution of the *27 allele (data not shown).

In this study, the association between *UGT1A1* genotypes and antitumor activity was difficult to evaluate because of the small number of subjects stratified into each tumor type. Further clinical studies are needed to establish methods for selection of the appropriate regimen or dosage based on the *UGT1A1* genotypes, where a balance between toxicity and antitumor effect should be considered.

In conclusion, this study demonstrated the significant association of *UGT1A1*6* with severe irinotecan-mediated neutropenia. The current data also supported the usefulness of the genetic marker "*6 or *28" for personalized irinotecan therapy in Japanese, and likely East Asian, patients.

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# Combination of $O^6$-methylguanine-DNA methyltransferase and thymidylate synthase for the prediction of fluoropyrimidine efficacy

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ABSTRACT

We investigated the correlation between the response to fluoropyrimidines as first-line therapy and the expressions of genes in patients with primary colorectal cancer (CRC). The study group comprised 92 patients with metastatic CRC. Total RNA was isolated from laser-captured tumour cells in surgically resected primary lesions, and gene expression was quantitatively evaluated by real-time RT-PCR assay. Low thymidylate synthase (TS), low γ-glutamyl hydrolase, high reduced folate carrier 1, high $O^6$-methylguanine-DNA methyltransferase (MGMT) and low cyclin E expressions were associated with a good response ($P = 0.0030$, 0.0250, 0.0120, 0.0030 and 0.0020, respectively) on univariate analysis. On multivariate logistic regression analysis, TS and MGMT remained independent predictors of the response. The clinical response rates were 63.2% in the low TS or high MGMT group and 14.3% in high TS and low MGMT group ($P < 0.0001$). The combination of high TS and low MGMT expression is a significant predictor of a poor response to fluoropyrimidine treatment.



## 1. Introduction

The median survival time of patients with colorectal cancer (CRC) has improved in the past 10 years because of the development of new agents with advantages over 5-fluorouracil (5-FU), including irinotecan hydrochloride (CPT-11) and oxaliplatin. CPT-11 or oxaliplatin monotherapy, however, was not shown to be more effective than bolus 5-FU/leucovorin (LV) in terms of response and median survival time. CPT-11 or oxaliplatin plus bolus or infusional 5-FU/LV regimens were found to be clearly more effective than 5-FU/LV, resulting in a doubling of the tumour response rate and prolongation of median survival time by 2–3 months. Regimens combining CPT-11 or oxaliplatin with fluoropyrimidines are now key first- and second-line chemotherapies for CRC. Response rates with these regimens, however, remain around 40–50%, prompting investigations of molecular predictors of the response to specific chemotherapeutic regimens. In this study, we evaluated molecular markers that could be used to predict the clinical outcomes of treatment with fluoropyrimidine-based

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regimens, now widely used to treat CRC. Because data on CPT-11-based regimens not including fluoropyrimidines will be difficult to obtain in the future, we also investigated such regimens used for second-line treatment in this study.

Evaluations of regimens including fluoropyrimidines alone as well as those including CPT-11 without fluoropyrimidines are required to produce benchmarks for predicting the efficacy of combined treatment with fluoropyrimidines and CPT-11.

Many potential predictors of the response to fluoropyrimidines have been reported. Several enzymes involved in the targeting, metabolism and catabolism of fluoropyrimidines have been extensively studied, including thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD).[1–3] The enzymes concerning folinic acid metabolism and transport are also the important factors involved in the efficacy of biochemical modulation of 5-FU by LV; among these being folylpolyglutamate synthetase (FPGS), γ-glutamyl hydrolase (GGH) and reduced folate carrier 1 (RFC1).[4,5]

The role of molecular markers in predicting the response to CPT-11-based chemotherapy remains largely unclear, as compared with oxaliplatin-based chemotherapy for CRC, for which several promising markers have been identified.[6] Recently, comprehensive analysis based on the microarray gene expression also have been performed to clarify the predictive markers for CPT-11/5-FU/LV treatment.[7] DNA topoisomerase I (TOPO I) may be a useful predictor of the response to CPT-11-based treatments in colon cancer cell lines as well as in patients with metastatic CRC.[8] Factors involved in DNA-repair systems, such as excision repair cross-complementing 1 (ERCC1) and $O^6$-methylguanine-DNA methyltransferase (MGMT), have also been investigated recently with respect to their role in resistance to CPT-11.[9,10] The relations between response and factors involved in drug detoxification, such as glutathione S-transferase pi (GSTpi), have been studied for many chemotherapeutic agents, including CPT-11.[9,10] On the other hand, epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and cell-cycle-regulation genes, such as cyclin E, have been reported to be related to the outcomes of patients with CRC.[11]

To gain further insight into potentially useful markers, we investigated the correlation between clinical response and the expressions of genes involved in the targeting, metabolism or catabolism of fluoropyrimidines, drug detoxification, cell cycles and DNA-repair systems in patients with metastatic or recurrent CRC who received first-line fluoropyrimidine-based regimens with or without LV or second-line CPT-11-based regimens.

## 2. Patients and methods

### 2.1. *Patient selection and chemotherapy*

This clinical–biological correlative study was performed retrospectively in a consecutive series of patients who underwent surgery for primary CRC at our hospital between 1996 and 2003 and received first-line fluoropyrimidine-based regimens for metastatic or recurrent CRC. Their responses to treatment and outcomes were confirmed. Patients who received second-line CPT-11-based chemotherapy were analysed as a subgroup. Approval for this study was obtained from the institutional review board of the National Cancer Center Hospital, Tokyo.

Fluoropyrimidines included 5-FU/l-LV (5-FU 600 mg/m$^2$ bolus and l-LV 250 mg/m$^2$ div, weekly × 6, q 8 weeks), continuous infusion of 5-FU (5-FU 250 mg/m$^2$/day), uracil-tegafur (UFT)/LV (UFT 300 mg/day and LV 75 mg/day per os, 4 weeks on and 1 week off), UFT alone (UFT 300 mg/day per os, 4 weeks on and 1 week off) and TS-1 (TS-1 80, 100 or 120 mg/day per os, 4 weeks on and 2 weeks off). CPT-11-based chemotherapy included CPT-11 alone (CPT-11 150 mg/m$^2$ div, biweekly) and CPT-11/mitomycin C (CPT-11 150 mg/m$^2$ div and mitomycin C 5 mg/m$^2$ bolus, biweekly).

### 2.2. *Clinical evaluation and response criteria*

Clinical response was evaluated every 6–8 weeks by CT imaging. Responders to treatment were classified as those patients whose tumours shrank by 50% or more, as estimated on two observations not less than 6 weeks apart. More precisely, a complete response (CR) was defined as the complete disappearance of all evidence of tumour, while a partial response (PR) was defined as a greater than 50% decrease in the sum of the products of the largest perpendicular diameters of all measurable lesions, without the occurrence of new lesions. Amongst those classified as non-responders, stable disease (SD) was defined as a change of less than 25% in tumour size, and progressive disease (PD) was defined as an increase of greater than 25% in the area of the measurable tumour deposits or the appearance of new lesions. Time to progression (TTP) during first-line or second-line chemotherapy was defined as the period from the starting date of chemotherapy to the date on which progression was confirmed.

### 2.3. *Laboratory methods*

Ten-micrometre-thick sections of resected primary CRC tumours were obtained from identified areas with the highest tumour concentration and were then mounted on uncoated glass slides. For histologic diagnosis, representative sections were stained with haematoxylin and eosin by standard methods. Before microdissection, sections were stained with nuclear fast red (NFR, American MasterTech Scientific, Lodi, CA). The sections of interest were selectively isolated by laser capture microdissection (P.A.L.M. Microsystem, Leica, Wetzlar, Germany), according to standard procedures.[12] The dissected particles of tissue were transferred to a reaction tube containing 400 μl of RNA lysis buffer.

The samples were homogenised and heated at 92 °C for 30 min. Fifty microlitres of 2 M sodium acetate was added at pH 4.0, followed by 600 μl of freshly prepared phenol/chloroform/isoamyl alcohol (250:50:1). The tubes were vortexed for 15 s, placed on ice for 15 min, and then centrifuged at 13,000 rpm for 8 min in a chilled (8 °C) centrifuge. The upper aqueous phase was carefully removed and placed in a 1.5-mL centrifuge tube. Glycogen (10 μl) and 300–400 μl of isopropanol were added and the samples were vortexed for 10–15 s. The tubes were chilled at −20 °C for 30–45 min to precipitate the RNA. The samples were then washed in 500 μl of 75% v/v ethanol and air-dried for 15 min. The pellet was

resuspended in 50 μl of 5 mM Tris. Finally, cDNA was prepared as described by Lord and colleagues.[13]

Quantification of the 12 genes of interest and an internal reference gene (β-actin) was done using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System, TaqMan®, Perkin–Elmer [PE] Applied Biosystems, Foster City, CA). The PCR reaction mixture consisted of 1200 nM of each primer, 200 nM of probe, 0.4 U of AmpliTaq gold polymerase, 200 nM each of dATP, dCTP, dGTP and dTTP, 3.5 mM of $MgCl_2$ and 1 × Taqman buffer A containing a reference dye. The final volume of the reaction mixture was 20 μl (all reagents from PE Applied Biosystems, Foster City, CA). Cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 46 cycles of 95 °C for 15 s and 60 °C for 1 min. The primers and probes used are listed in Table 1. Gene expression values (relative mRNA levels) are expressed as ratios (differences between Ct values) between the gene of interest and the internal reference gene (β-actin).

### 2.4. *Statistical analysis*

To evaluate the association of gene expressions with response and TTP, gene expression levels were categorised into low and high values. To determine cutoff values, the maximally selected $\chi^2$ method was employed.[14–16] For each observed value, patients were classified as falling below or equal to that value, or above that value. The maximally selected $\chi^2$-test statistic was used to compare the response rates of the two resulting groups of patients (below or equal to the value versus above the value). The value that yielded the largest $\chi^2$-test statistic (the maximal $\chi^2$ statistic) was selected as the optimal cutoff point. To determine the P-value associated with the maximal $\chi^2$ statistic, we performed 2000 bootstrap-like simulations. For each simulation, a randomly selected value was drawn (with replacement) from the set of observed values and assigned to each of the observed responses; the maximal $\chi^2$ statistic was calculated based on this set of randomly matched values and responses. The corrected P-value was calculated as the proportion of the 2000 simulated maximal statistics that was larger than the original maximal $\chi^2$ statistic. This analysis was repeated using the log-rank test to compare TTP. If promising significant predictive variables were found on this analysis, multivariate logistic regression analysis was preformed for the response to fluoropyrimidines. Stepwise variable selection was done using a significance level of 0.01 for entering into or remaining in the model.

All reported P-values are two-sided, and the level of significance was set at $P < 0.05$, except for stepwise variable selection. All analyses were performed using the statistical software package R, version 2.4.1 and the SAS statistical package, version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

## 3. Results

### 3.1. *Demographics and patients assessed for response and TTP*

A total of 92 Japanese patients (54 men and 38 women; median age, 61 years; range 27–77 years) were evaluated (Table 2). Seventy of these patients (43 men and 27 women; median age, 62 years; range 27–77 years) had received 5-FU/LV regimens, and 63 had received CPT-11 as second-line chemotherapy; 43 patients with CPT-11 and 20 patients with CPT-11/ mitomycin C. Gene expression levels of TS, DPD, FPGS, GGH, RFC1, TOPO I, ERCC1, MGMT, GSTpi, EGFR, VEGF and cyclin E were assessed in all patients, and the relations of these levels to response and TTP were examined.

The response to first-line fluoropyrimidine-based chemotherapy was CR in 2 patients (2%), PR in 39 (42%), NC in 30 (33%) and PD in 21 (23%). The median TTP was 5.1 months. The response to first-line 5-FU/LV treatment was CR in 2 patients (3%), PR in 31 (44%), NC in 21 (30%) and PD in 16 (23%). The median TTP was 5.0 months. For second-line CPT-11-based chemotherapy, the response was PR in 9 patients (14%), NC in 32 (50%) and PD in 22 (36%). The median TTP was 3.5 months.

**Table 1 – Primers and probes**

| Gene | GenBank Accession | Forward primer (5′–3′) | Reverse primer (5′–3′) | Taqman probe (5′–3′) |
|---|---|---|---|---|
| β-Actin | NM_001101.2 | GAGCGCGGCTACAGCTT | TCCTTAATGTCACGCACGATTT | ACCACCACGGCCGAGCGG |
| TS | NM_001071.1 | GCCTCGGTGTGCTTTCA | CCCGTGATGTGCGCAAT | TCGCCAGCTACGCCCTGCTCA |
| DPD | NM_000110.2 | AGGACGCAAGGAGGGTTTG | GTCCGCCGAGTCCTTACTGA | CAGTGCCTACAGTCTCGAGTCTCGCCAGT |
| FPGS | M98045 | GGCTGGAGGAGACCAAGGAT | CATGAGTGTCAGGAAGCGGA | CAGCTGTGTCTCCATGCCCCCCTAC |
| GGH | NM_003878 | GCGAGCCTCGAGCTGTCTA | AATATTCCGATGATGGGCTTCTT | ACCCCACGGCCGACACCGC |
| RFC1 | NM_194255.1 | CATCGCCACCTTTCAGATT | TGGCAAAGAACGTGTTGAC | CCCGAAGACCAGGGCACAGA |
| TOPO I | NM_003286 | TGTAGCAAAGATGCCAAGGT | TGTTATCATGCCGGACTTCT | GCTTCTCCTCCTCCAGGACATAAGTGGA |
| ERCC1 | NM_001983.2 | GGGAATTTGGCGACGTAATTC | GCGGAGGCTGAGGAACAG | CACAGGTGCTCTGGCCCAGCACATA |
| MGMT | NM_002412 | CGTTTTCCAGCAAGAGTCGTT | GAAATCACTTCTCCGAATTTCACA | TCAGCAGCTTCCATAACACGTGTCTGG |
| GSTpi | X06547 | CCTGTACCAGTCCAATACCATCCT | TCCTGCTGGTCCTTCCCATA | TCACCTGGGCCGCACCCTTG |
| EGFR | X00588 | TGCGTCTCTTGCCGGAAT | GGCTCACCCTCCAGAAGGTT | ACGCATTCCCTGCCTCGGCTG |
| VEGF | NM_003376.4 | AGTGGTCCCAGGCTGCAC | TCCATGAACTTCACCACTTCGT | TGATTCTGCCCTCCTCCTTCTGCCAT |
| Cyclin E | NM_001238 | CAGCTTATTGGGATTTCATCTTT | ATACGCAAACTGGTGCAACT | TGCAGCCAAACTTGAGGAAATCTATCC |

TS: thymidylate synthase, DPD: dihydropyrimidine dehydrogenase, FPGS: folylpolyglutamate synthetase, GGH: γ-glutamyl hydrolase, RFC1: reduced folate carrier 1, TOPO I: DNA topoisomerase I, ERCC1: excision repair cross-complementing 1, MGMT: $O^6$-methylguanine-DNA methyltransferase, GSTpi: glutathione S-transferase pi, EGFR: epidermal growth factor receptor, VEGF: vascular endothelial growth factor.