Table 2. Summary of *ABCC2* variations detected in this study

SNP ID					Position				
This Study	dbSNP (NCBI)	JSNP	Reference	Location	NT_030059.12	From the translational initiation site or from the end of the nearest exon	Nucleotide change	Amino acid change	Frequency (total = 472)
MPJ6_AC 2082			8	5′-Flanking	**20289354**	−1774	acttatcttgttG/_ttttttttttt		0.343
MPJ6_AC 2078[a]				5′-Flanking	**20289538**	−1590	tttaattttgttaG/Atgtatgtttgct		0.002
MPJ6_AC 2079			8, 10, 17	5′-Flanking	**20289579**	−1549	tcettatagtatG/Attgtggatatta		0.203
MPJ6_AC 2080			9, 17	5′-Flanking	**20290105**	−1023	tgggaggccaagG/Acagaaggattgt		0.343
MPJ6_AC 2081			10, 17	5′-Flanking	**20290109**	−1019	aggccaaggcagA/Gaggattgttgaa		0.203
MPJ6_AC 2028[a]				5′-Flanking	**20290395**	−733	acagtttctagcG/Tactgatgccacc		0.004
MPJ6_AC 2029				5′-Flanking	**20290395**	−733	acagtttctagcG/Aactgatgccacc		0.002
MPJ6_AC 2030[a]				5′-Flanking	**20290715**	−413	ttgcagcagaagC/Tgaaactgcacat		0.002
MPJ6_AC 2003		**ssj0000371**	9, 12, 15–18, 20, 26	Exon 1	**20291104**	−24	tagaagagtcttC/Tgttccagacgca		0.174
MPJ6_AC 2004			18	Exon 1	**20291105**	−23	agaagagtcttcG/Attccagacgcag		0.006
MPJ6_AC 2031		**ssj0000386**	17, 26	Intron 3	**20301785**	IVS3 −49	ctccccctcagtcC/Ttcggttagtggc		0.203
MPJ6_AC 2032[a]				Intron 6	**20302837**	IVS6 +86	tatttttattattT/Attttttgagat		0.076
MPJ6_AC 2033[a]				Exon 7	**20305479**	732	caagtttgaaacG/Acacatgaagaga	Thr244Thr	0.002
MPJ6_AC 2066[a]				Intron 7	**20307421**	IVS7 −69	tcacaggctgacC/Gaccctggagctg		0.002
MPJ6_AC 2067[a]				Intron 7	**20307423**	IVS7 −67	acaggctgaccaC/Acctggagctgct		0.002
MPJ6_AC 2035[a]				Exon 9	**20308814**	1177	ggtgtaaaagtaC/Tggacagctatca	Arg393Trp	0.002
MPJ6_AC 2068[a]				Exon 9	**20308839**	1202	tggcttctgtatA/Gtaagaaggtaag	Tyr401Cys	0.002
MPJ6_AC 2036[a]				Intron 9	**20308859**	IVS9 +13	gtaagcagaataC/Tggcaggtatcac		0.002
MPJ6_AC 2037[a]				Exon 10	**20312319**	1227	gaccctatccaaC/Tttggccaggaag	Asn409Asn	0.002
MPJ6_AC 2009		**ssj0000388**	17, 18, 20, 23–26	Exon 10	**20312341**	1249	aaggagtacaccG/Atggagaaacag	Val417Ile	0.097
MPJ6_AC 2010			18	Exon 10	**20312549**	1457	ccaagagtaagaC/Tcattcaggtaaa	Thr486Ile	0.019
MPJ6_AC 2069[a]				Intron 11	**20315600**	IVS11 −67	taaaacatgggtG/Agatcagatacac		0.002
MPJ6_AC 2038		**ssj0000390**	26	Intron 12	**20315952**	IVS12 +148	ccgccccatgccA/Gctttcctcctctt		0.210
MPJ6_AC 2039[a]				Intron 13	**20318344**	IVS13 −73	tcatggactaacG/Aacaaagtcaaaa		0.002
MPJ6_AC 2070[a]				Intron 14	**20318515**	IVS14 +14	taaataaatttgG/Taagttgcttccc		0.002
MPJ6_AC 2040[a]				Intron 14	**20318521**	IVS14 +20	aatttggaagtt(del/Ins)[b]cagcaaactga		0.002
MPJ6_AC 2071[a]				Intron 14	**20318594**	IVS14 +93	agcaaactgagaG/Tagagtgtggaga		0.002
MPJ6_AC 2041[a]				Intron 14	**20319757**	IVS14 −62	cggagagagacaC/Tgtgagggcagac		0.002
MPJ6_AC 2042[a]				Intron 14	**20319758**	IVS14 −61	ggagagagacacG/Atgagggcagaca		0.006
MPJ6_AC 2043		**ssj0000393**	26	Intron 15	**20320054**	IVS15 +169	aaagcaaaggttT/Ctcagccccttcc		0.210
MPJ6_AC 2044[a]				Intron 15	**20321170**	IVS15 −131	gtcttgtatatcC/Gaaggcaaatttt		0.004
MPJ6_AC 2045[a]				Intron 16	**20325422**	IVS16 −169	ttgagtcctgagA/Tgtggaataacta		0.004
MPJ6_AC 2046		**ssj0000396**	17	Intron 16	**20325486**	IVS16 −105	tgcacagttattC/Taaatttaagctc		0.214
MPJ6_AC 2072[a]				Exon 18	**20327159**	2358	tcttctagatgaC/Acccctgtctgca	Asp786Glu	0.002
MPJ6_AC 2012			18, 20, 23	Exon 18	**20327167**	2366	atgaccccctgtC/Ttgcatggatgc	Ser789Phe	0.008
MPJ6_AC 2073[a]				Intron 19	**20327555**	IVS19 +3	gaagccacaggtA/Gtgtaagaaggat		0.002
MPJ6_AC 2047[a]				Intron 19	**20327645**	IVS19 +93	agtatccagtgaA/Tctagatttggaa		0.002
MPJ6_AC 2048				Intron 20	**20338745**	IVS20 +29	gctggcagcccctC/Agtcagctctata		0.002
MPJ6_AC 2049[a]				Exon 21	**20339052**	2801	ccttgaaaactcG/Agaatgtgaatag	Arg934Gln	0.002
MPJ6_AC 2015		**ssj0000398**	8, 18, 26	Exon 22	**20339944**	2934	aggattgttttcG/Aatattccttcatc	Ser978Ser	0.040
MPJ6_AC 2050[a]				Exon 22	**20340061**	3051	cgactatccagcA/Gtctcagagggac	Ala1017Ala	0.002
MPJ6_AC 2051[a]				Exon 23	**20340337**	3181	cacaagcaactgC/Ttgaacaatatcc	Leu1061Leu	0.002
MPJ6_AC 2052		**ssj0000399**	17, 26	Intron 23	**20340470**	IVS23 +56	ggatcttttctgaC/Tagggaggaatta		0.222
MPJ6_AC 2074[a]				Exon 24	**20342724**	3320	ttacatgcttccT/Gggggataatcag	Leu1107Arg	0.002
MPJ6_AC 2053				Intron 24	**20342843**	IVS24 +25	atggctaagtcaT/Ccctttcttcctc		0.030
MPJ6_AC 2075[a]				Intron 24	**20342880**	IVS24 +62	agcccagcctctT/Ctcctgagaatct		0.002
MPJ6_AC 2054				Intron 24	**20342926**	IVS24 +108	cactcactcctcC/Tcctcagcagctt		0.023
MPJ6_AC 2055[a]				Intron 24	**20344318**	IVS24 −56	agaaaggaggaaG/Aatggtgatgcc		0.002
MPJ6_AC 2056[a]				Intron 26	**20352061**	IVS26 −21	atgatgattttcA/Ggtcttctggttt		0.002
MPJ6_AC 2057[a]				Intron 27	**20352227**	IVS27 +44	ggcaaaaacaacA/Gtgcaactccttc		0.008
MPJ6_AC 2058		**ssj0000404**	17, 26	Intron 27	**20352307**	IVS27 +124	aaagtttccttttC/Gctctaactcaaa		0.222
MPJ6_AC 2076			26	Exon 28	**20352688**	3927	ccaagtgcggtaC/Tcgacctgagctg	Tyr1309Tyr	0.002
MPJ6_AC 2022		**ssj0000407**	8, 12, 13, 17, 18, 20, 26	Exon 28	**20352733**	3972	cacttgtgacatC/Tggtagcatggag	Ile1324Ile	0.216
MPJ6_AC 2059[a]				Intron 28	**20352920**	IVS28 +172	agggaaggatagC/Tagccagggatca		0.004
MPJ6_AC 2060[a]				Intron 29	**20354201**	IVS29 +136	cttgagctagttC/Tcctaggatggac		0.002
MPJ6_AC 2061		**ssj0000408**	26	Intron 29	**20354219**	IVS29 +154	gatggacacgtcA/Gtttccagaactt		0.367
MPJ6_AC 2062		**IMS-JST090926**	17	Intron 29	**20355209**	IVS29 −35	cttttctggcatG/Aagccccaacagc		0.015
MPJ6_AC 2063[a]				Intron 30	**20358793**	IVS30 −92	gggggttttgaA/Gagtctgatctgg		0.008
MPJ6_AC 2064		**IMS-JST185750**		Intron 30	**20358832**	IVS30 −53	ccccctgccctgC/Tgtctttccttgg		0.051
MPJ6_AC 2077[a]				3′-UTR	**20359975**	*61[c]	taattttattttT/Gtataaaatacag		0.002
MPJ6_AC 2065[a]				3′-Flanking	**20360190**	*193+83[c]	ttattccttgcC/Gtttcatttctgt		0.002a8

[a]Novel genetic variation

[b]delGCTTCCCAAACTTATTCGCAGTACTGGTGCCAGAATTTTGATAATACAAGAGCTTAGTAG/insTATTTACCT

[c]Numbered from the termination codon.

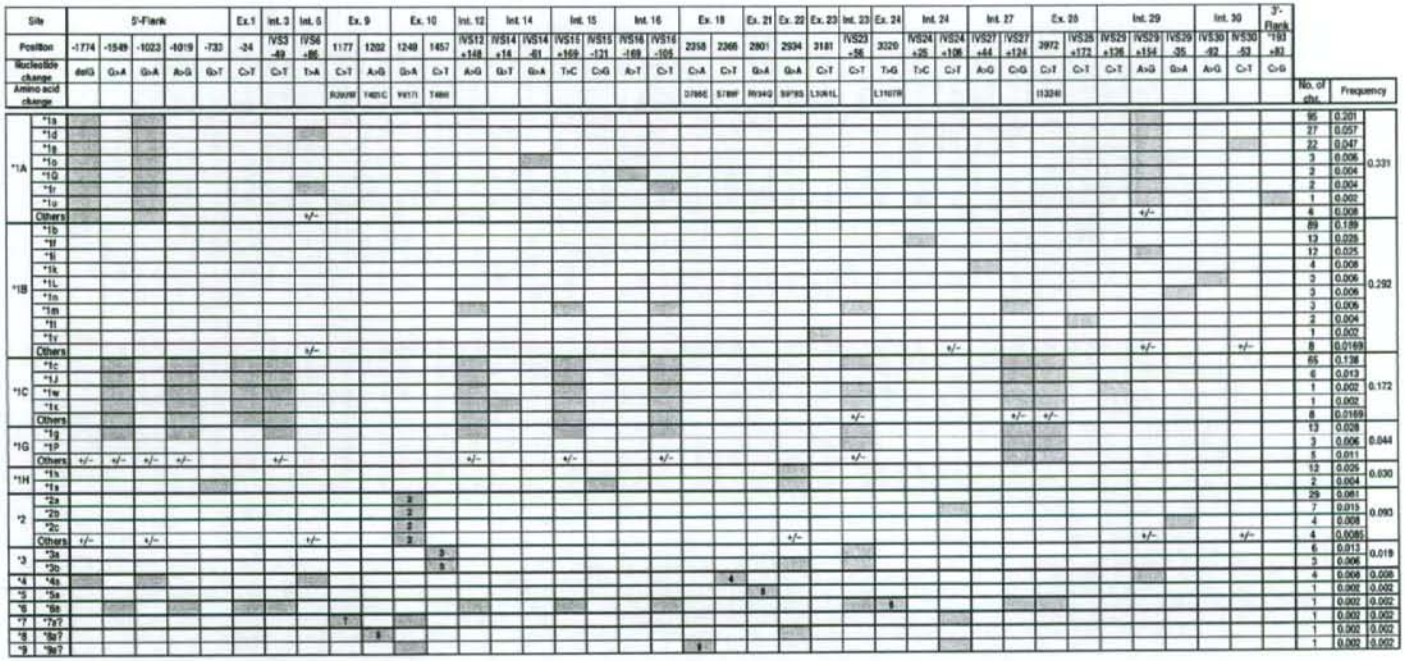

Fig. 2. *ABCC2* **haplotypes in 236 Japanese subjects**
The *1 groups (without nonsynonymous substitutions) were classified into *1A (harboring −1774delG), *1C (harboring −24C>T), *1G [harboring 3972C>T (Ile1324Ile) without −24C>T], *1H [harboring 2934G>A (Ser978Ser)] and *1B [without the common variations]. Marker SNPs for *2 to *9 are indicated by numbers. Rare and ambiguous haplotypes (n = 1) are shown with "?" or grouped into "others".

any amino acid substitution were assigned as the *1 group and named with small alphabetical letters in descending frequency order (*1a to *1x). Haplotypes with nonsynonymous variations were assigned from *2 to *9 groups, and their subtypes were named with small alphabetical letters. The haplotypes (*7a to *9a) were inferred in only one patient and described with "?" due to their ambiguity. Also, ambiguous rare haplotypes in the *1 and *2 groups were classified as "Others" in **Figure 2**. The *1 haplotypes were further classified into the *1A, *1B, *1C, *1G and *1H groups (capital alphabetical letters of the most frequent haplotypes were used) according to the common tagging SNPs, such as −1774delG, −24C>T, 3972C>T (Ile1324Ile), and 2937G>A (Ser978Ser).

The most frequent *1 group, *1A, harbors the common SNPs −1774delG and −1023G>A in the 5′-flanking region and mostly IVS29+154A>G, and the frequency of *1A (0.331) is almost the same as that in healthy Koreans (0.323) reported by Choi *et al.*[8] They have shown that −1774delG reduced promoter activity both at the basal level and after induction by chenodeoxycolic acid (CDCA), a component of bile acids, and that the haplotype bearing −1774delG is associated with chemical-induced hepatitis (cholestatis and mixed types).[8] Therefore, it is possible that *1A can affect the pharmacokinetics or pharmacodynamics of MRP2-transported drugs.

The *1B group haplotypes (0.292 frequency) harbor no or any intronic or synonymous variations the functions of which are unknown. The functional significance of variations in the *1B group, including the most frequent SNP IVS24+25T>C, needs further confirmation.

The third group *1C (0.172 frequency) harbors the known common SNPs −1549G>A, −1019A>G, −24C>T, IVS3–49C>T, and 3972C>T (Ile1324Ile), except for one rare ambiguous haplotype lacking 3972C>T (Ile1324Ile). The *1C haplotypes also harbor IVS12+148A>G, IVS15+169T>C and IVS16–105C>T. The haplotypes bearing −1549G>A, −24C>T and 3972C>T (Ile1324Ile) are commonly found in Korean populations (frequency 0.14–0.25)[6] and Caucasians (0.14–0.17).[10,14,21] The functional importance of the tagging SNP in the *1C group, −24C>T, has been reported by several researchers; *e.g.*, reduced promoter activity,[8,11] reduced mRNA expression in the kidney,[11] association with chemical-induced hepatitis (hepatocellular type),[8] and influence on irinotecan-pharmacokinetics and pharmacodynamics.[12,16] For other SNPs in the *1C group, functional alterations *in vitro* have not been shown; no change in promoter activity by −1549G>A, no influence of IVS3–49C>T on splicing, and no change induced by 3972C>T (Ile1324Ile) on MRP2 expression or transporter activity.[8] Although −24C>T caused reduced promoter activity in the absence of the bile acid CDCA,[8,11], enhanced promoter activity of −24C>T under induction by CDCA has been demonstrated.[8] Therefore the function of this SNP might depend on cholestatic status.

Our data demonstrated that −1019A>G was closely associated with the other *1C SNPs (complete linkage with −1549G>A). The close linkage between −1019A>G and −1549G>A was also observed in Caucasians, but their linkages with −24C>T and 3972C>T were relatively weak.[14] In contrast, another study on Caucasians reported that −1019A>G was exclusive to −1549G>A, −24C>T and 3972C>T.[10] Although the reasons for these discrepancies are not clear, some ethnic differences might exist in the 5′-flanking region.

The *1G group harbors 3972C>T (Ile1324Ile) but not −24C>T. Caucasians have haplotypes bearing 3972C>T (Ile1324Ile) without −24C>T at frequencies of 0.15–0.20.[10,21] In contrast, the frequency of the corresponding haplotype group in our study (*1G) was much lower (0.044). Although no *in vitro* effect of 3972C>T (Ile1324Ile) was shown,[8] its *in vivo* association with increased area under the concentration-time curve of irinotecan and its metabolites was reported in Caucasians.[13]

The *1H group (*1h and *1s) harbors a synonymous substitution of 2934G>A (Ser978Ser) (0.03 frequency). No influence of 2934G>A(Ser978Ser)on MRP2 expression or transport activity has been shown.[8]

As for haplotypes with nonsynonymous substitutions, eight haplotype groups (*2 to *9) were identified. The *2 [including 1249G>A (Val417Ile)] was the most frequent among them, and its frequency (0.093) was similar to those for Asians (0.10–0.13)[8,12,20] and slightly lower than those for Caucasians (0.13–0.22).[9,10,14,15,21] The haplotype frequencies of *3 [harboring 1457C>T (Thr486Ile)] and *4 [2366C>T (Ser789Phe)] were 0.019 and 0.008. Other rare haplotypes with novel nonsynonymous variation, *5 [2801G>A (Arg934Gln)], *6 [3320T>G (Leu1107Arg)], *7 [1177C>T (Arg393Trp)], *8 [1202A>G (Tyr401Cys)], and *9 [2358C>A (Asp786Glu)] were found each in only one subject as heterozygote at a 0.002 frequency. No functional significance of the marker SNP [1249G>A (Val417Ile)] of *2 has been shown *in vitro*,[8,23] but its *in vivo* associations with lower MRP2 expression in the placenta[24] and chemical-induced renal toxicity[25] have been reported. The variation 2366C>T (Ser789Phe) (*4) has been shown to cause reduced MRP2 expression and alter localization *in vitro*,[23] but clinical data are limited. Functional changes in *3 [1457C>T (Thr486Ile)] and *5 to *9 (novel nonsynonymous variations) are currently unknown. Possible effects of these amino acid substitutions were speculated using PolyPhen analysis (http://genetics.bwh.harvard.edu/pph); its prediction is based on the analysis of substitution site [e.g., a substitution in transmenbrane domain is assessed by the predicted hydrophobic and transmembrane (PHAT) matrix score], likelihood of the substitution assessed by the position-specific independent count (PSIC) profile scores, and protein 3D structures. This analysis predicted a possible functional change of Leu1107Arg (*6) due to substitution in

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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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

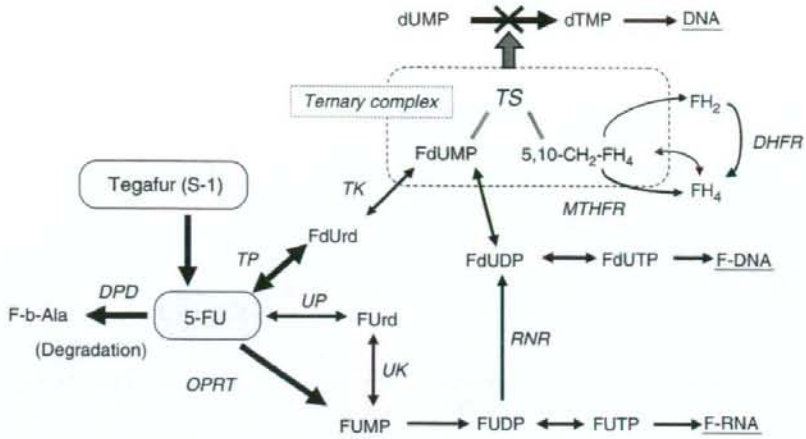

Figure 1 5-Fluorouracil and folate metabolic pathways. Genes examined in our study are shown in bold. DPD, dihydropyrimidine dehydrogenase; DHFR, dihydrofolate reductase; MTHFR, methylenetetrahydrofolate reductase; OPRT, orotate phosphoribosyl transferase; TS, thymidylate synthase; TP, thymidine phosphorylase; . 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 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 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 μl) and 300–400 μl 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 μl of 75% 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 (Lord *et al*, 2000).

Quantification of nine genes of interest and an internal reference gene (β-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 μl (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 (β-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 ΔC_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 χ^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 χ^2 analysis, 2000 bootstrap-like simulations were used in univariate analyses to estimate the distribution of the maximal χ^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
β-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 χ^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 χ^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 2.89×10^{-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 0.33×10^{-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 1.55×10^{-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 χ^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.

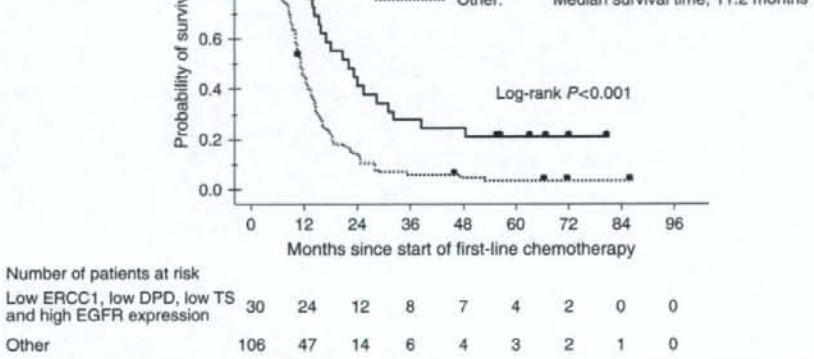

Figure 2 Kaplan–Meier plot of overall survival for all patients according to ERCC1, DPD, TS, and EGFR mRNA expression levels.

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 0.94×10^{-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 χ^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 χ^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.

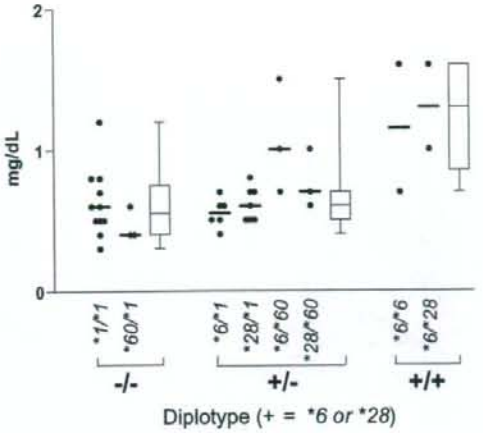

Fig. 1. Effects of *UGT1A1* genotypes on serum total bilirubin levels at baseline in Japanese cancer patients ($N = 37$). Each point represents a patient, and the median value of each diplotype is shown with a bar. All diplotypes are classified into −/−, +/−, and +/+ by the genetic marker, "*UGT1A1*6 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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