Table 1. continued

Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified region[a]
Exon 17	GTGGAATAACTACAAGCACG	TCAACTAGATTACCCCTGTGT[b]	
Exon 18	GGTGACAAGCAACAAAACTA	CCACCATCTTCCCTGTCTTA	
Exon 19	GATGCTCATGTAGGAAAACA	TTTACCATTCCACCCATGGC	
Exon 20	GGCTTCTCTCTCCTTGTTCA	CAAAGAAACAAAGGAAGAGC	
Exon 21	TGACTGTGACATCTGCTTGC[b]	GGACAGAGGACATATTGCTCC[b]	
Exon 22	GCATTGTATTTCAGCATTGT[b]	GATATTTGATGCATGGACGA	
Exon 23	GAATCTGTCTGGACCCTGTA	GTCTAGGGGGACATAATAAT	
Exon 24	ACACACAGAATCCAACAGAT	TCAACATATGACTAAATGGC	
Exon 25	GGAGCCTCTCATCATTCTGC	TTTCACACCACTAGCCATGC	
Exon 26	CCGATCAAGTCAAACCCTCT	TTTGAACCTCAGTCTTCTTT	
Exon 27	TTTCCTTACTCCCTTGTAGA	AAACTTTAGGGACCCATTAT	
Exon 28	CTGCTACCCTTCTCCTGTTC	CCTTCCCTCTGATACTGTGT	
Exon 29	TACCTCCTGTGACTGTGAAT	CAGCCACAAATGCATATTACC[b]	
Exon 30	GCCAGTCCTATCCACCATCT	AACACGAGGAACACGAGGAG	
Exon 31	GATCTGGAACATGAAAATGG	TTTTGGCCAGATTACTTGAC	
Exon 32	GCTCATTGATTTTCACTGCT	AAGGCAAAGGAATAATTATCG	

[a]The reference sequence is NT_030059.12.
[b]The same primer that was used for the 2nd PCR.

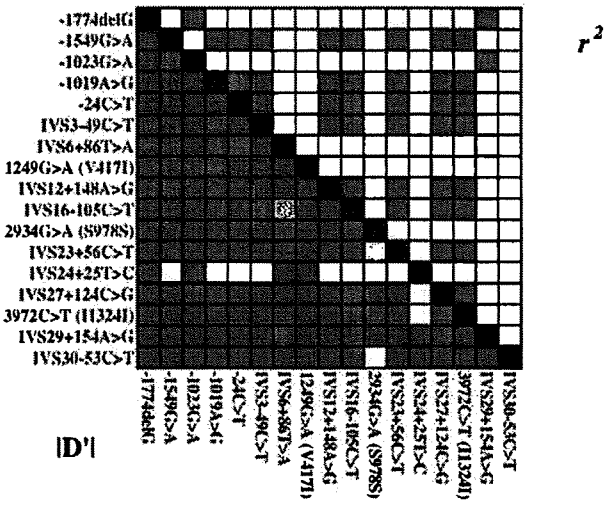

Fig. 1. Linkage disequilibrium (LD) analysis of *ABCC2*
Pairwise LD (r^2 values and |D′|) of polymorphisms detected in no less than 3% of allele frequencies is shown as a 10-graded blue color.

(**Table 2**). All detected variations were in Hardy-Weinberg equilibrium ($p > 0.05$). Novel variations consisted of 5 non-synonymous and 4 synonymous variations in the coding region, 22 in the intronic regions, 3 in the 5′-flanking region, 1 in the 3′- flanking region, and 1 in the 3′-UTR. The novel non-synonymous variations were 1177C>T (Arg393Trp), 1202A>G (Tyr401Cys), 2358C>A (Asp786Glu), 2801G>A (Arg934Gln), and 3320T>G (Leu1107Arg), and their frequencies were 0.002. No statistically significant differences were found in the allele frequencies of all variations between 177 cancer patients and 59 healthy subjects ($P > 0.05$, Fisher's exact test), although a larger number of subjects would be needed to conclude.

The frequency of the known common SNP −24C>T (0.173) was comparable to those reported in Asians (0.17–0.25)[8,12,20] and Caucasians (0.15–0.23)[9,10,14,15,21]. The allele frequency of another common SNP, 3972C>T (Ile1324Ile) (0.216), was also comparable to those in Asians (0.22–0.30)[8,12,20] but lower than those in Caucasians (0.32–0.37)[9,10,14,15,21]. The other major variations in the 5′-flanking region, −1774delG and −1549G>A, were found at frequencies of 0.343 and 0.203, respectively, and these values were similar to those obtained in Koreans (0.34 and 0.21, respectively).[8] However, the relatively frequent SNPs 1446C>G (Thr482Thr) (allele frequency = 0.125), IVS15-28C>A (0.333) and IVS28+16G>A (0.167) in Caucasians[17] were not detected in our study.

The LD profile of the *ABCC* variations (no less than 3% allele frequency) is shown in **Figure 1**. As assessed by r^2 values, close linkages were observed among −1774delG, −1023G>A and IVS29+154A>G, and among −1549G>A, −1019A>G, −24C>T, IVS3-49C>T, IVS12+148A>G, IVS15+169T>C, IVS16-105C>T, IVA23+56C>T, IVS27+124C>G, and 3972C>T (Ile1324Ile). It must be noted that complete linkage was observed between −1549G>A and −1019A>G in our population. In |D′| values, strong LD was also observed almost throughout the region analyzed. Overall, since close associations between the variations were observed throughout the entire *ABCC2* gene, the region sequenced was analyzed as a single LD block for the haplotype inference.

The *ABCC2* haplotype structures were analyzed using 61 detected genetic variations and a total of 64 haplotypes were identified/inferred. **Figure 2** summarizes the haplotypes and their grouping. Our nomenclature system is based on the recommendation of Nebert.[22] Haplotypes without

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

SNP ID			Reference	Location	Position		Nucleotide change	Amino acid change	Frequency (total = 472)
This Study	dbSNP (NCBI)	JSNP			NT_030059.12	From the translational initiation site or from the end of the nearest exon			
MPJ6_AC 2082			8	5′-Flanking	**20289354**	−1774	acttatcttgttG/_ttttttttttt		0.343
MPJ6_AC 2078[a]				5′-Flanking	**20289538**	−1590	tttaatttgttaG/Atgtatgcttgct		0.002
MPJ6_AC 2079			8, 10, 17	5′-Flanking	**20289579**	−1549	tccttatagtatG/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	tagaagagtcttcG/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	ctcccctcagtcC/Ttcggttagtggc		0.203
MPJ6_AC 2032[a]				Intron 6	**20302837**	IVS6 +86	tattttattattT/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/Ttcggccaggaag	Asn409Asn	0.002
MPJ6_AC 2009		**ssj0000388**	17, 18, 20, 23–26	Exon 10	**20312341**	1249	aaggagtacaccG/Attggagaaacag	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/Gcttttcctcctt		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	atgacccccctgtC/Ttgcagtggatgc	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	aggattgtttttcG/Aatattcttcatc	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	ggatctttctgaC/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/Ccctccttcctc		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/Aatggtggatgcc		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	aaagtttccttC/Gctctaactcaaa		0.222
MPJ6_AC 2076			26	Exon 28	**20352688**	3927	ccaagtgcggtaC/Tcgacctgagcag	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]	ttattccttttgcC/Gtttcatttctgt		0.002a8

[a]Novel genetic variation

[b]delGCTTCCCAAACTTATTCGCAGTACTGGTGCCAGAATTTTGATAATACAAGAGCTTAGTAG/insTATTTACCT

[c]Numbered from the termination codon.

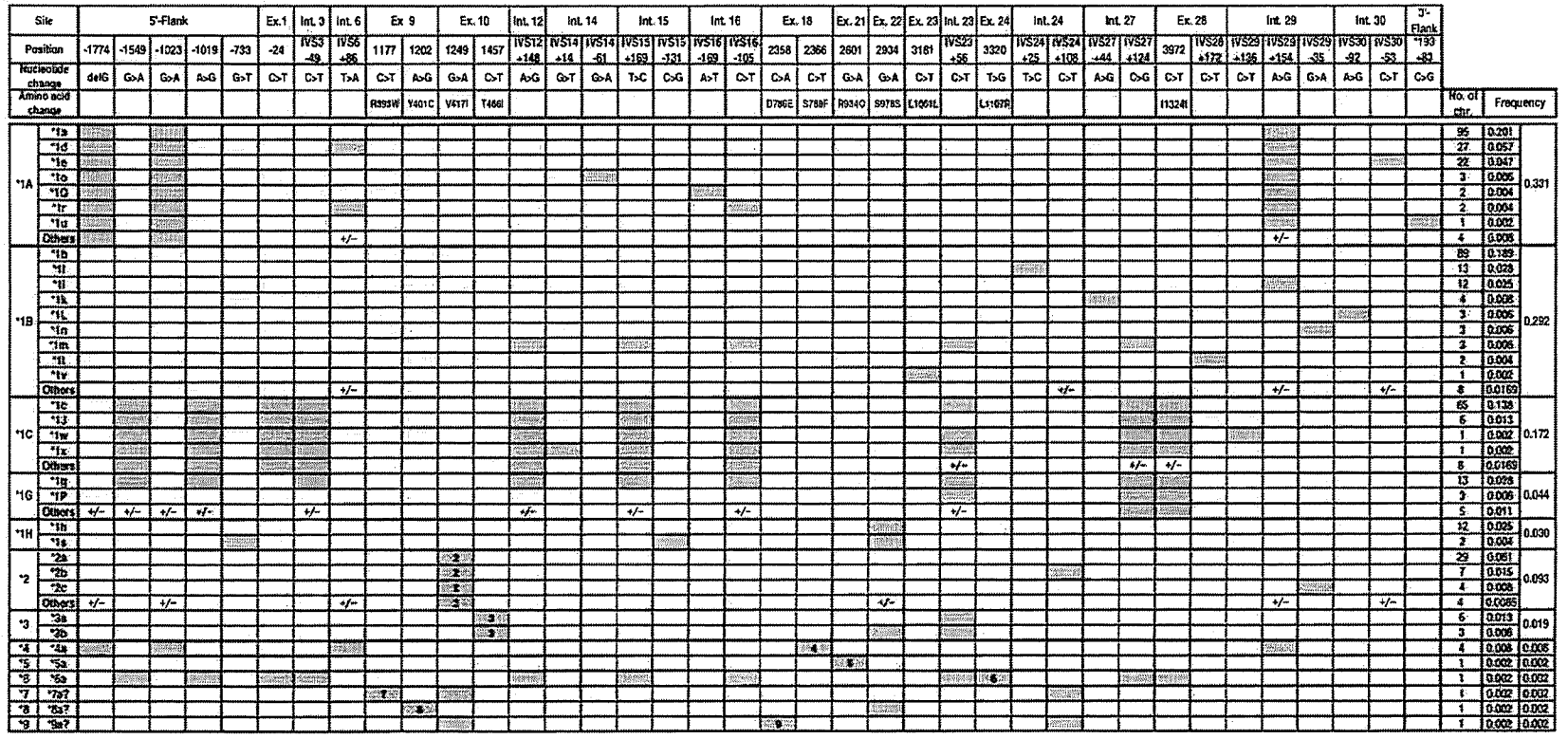

Fig. 2. ***ABCC2*** **haplotypes in 236 Japanese subjects**

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)[8] 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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References

1) Jedlitschky, G., Hoffmann, U. and Kroemer, H. K.: Structure and function of the MRP2 (ABCC2) protein and its role in drug disposition. *Expert Opin. Drug Metab. Toxicol.*, **2**: 351–66 (2006).
2) Wada, M.: Single nucleotide polymorphisms in *ABCC2* and *ABCB1* genes and their clinical impact in physiology and drug response. *Cancer Lett.*, **234**: 40–50 (2006).
3) Huang, Y.: Pharmacogenetics/genomics of membrane transporters in cancer chemotherapy. *Cancer Metastasis Rev.*, **26**: 183–201 (2007).
4) Taniguchi, K., Wada, M., Kohno, K., Nakamura, T., Kawabe, T., Kawakami, M., Kagotani, K., Okumura, K., Akiyama, S. and Kuwano, M.: A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation. *Cancer Res.*, **56**: 4124–4129 (1996).
5) Hinoshita, E., Uchiumi, T., Taguchi, K., Kinukawa, N., Tsuneyoshi, M., Maehara, Y., Sugimachi, K. and Kuwano, M.: Increased expression of an ATP-binding cassette superfamily transporter, multidrug resistance protein 2, in human colorectal carcinomas. *Clin. Cancer Res.*, **6**: 2401–2407 (2000).
6) Cui, Y., Konig, J., Buchholz, J.K., Spring, H., Leier, I. and Keppler, D.: Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. *Mol. Pharmacol.*, **55**: 929–937 (1999).
7) Wada, M., Toh, S., Taniguchi, K., Nakamura, T., Uchiumi, T., Kohno, K., Yoshida, I., Kimura, A., Sakisaka, S., Adachi, Y. and Kuwano, M.: Mutations in the canilicular multispecific organic anion transporter (cMOAT) gene, a novel ABC transporter, in patients with hyperbilirubinemia II/Dubin-Johnson syndrome. *Hum. Mol. Genet.*, **7**: 203–207 (1998).
8) Choi, J. H., Ahn, B. M., Yi, J., Lee, J. H., Lee, J. H., Nam, S. W., Chon, C. Y., Han, K. H., Ahn, S. H., Jang, I. J., Cho, J. Y., Suh, Y., Cho, M. O., Lee, J. E., Kim, K. H. and Lee, M. G..: MRP2 haplotypes confer differential susceptibility to toxic liver injury. *Pharmacogenet. Genomics*, **17**: 403–415 (2007).
9) Daly, A. K., Aithal, G. P., Leathart, J. B., Swainsbury, R. A., Dang, T. S. and Day, C. P.: Genetic susceptibility to diclofenac-induced hepatotoxicity: contribution of UGT2B7, CYP2C8, and ABCC2 genotypes. *Gastroenterology*, **132**: 272–281 (2007).
10) de Jong, F. A., Scott-Horton, T. J., Kroetz, D. L., McLeod, H. L., Friberg, L. E., Mathijssen, R. H., Verweij, J., Marsh, S. and Sparreboom, A.: Irinotecan-induced diarrhea: functional significance of the polymorphic ABCC2 transporter protein. *Clin. Pharmacol.Ther.*, **81**: 42–49 (2007).
11) Haenisch, S., Zimmermann, U., Dazert, E., Wruck, C. J., Dazert, P., Siegmund, S., Kroemer, H. K., Warzok, R. W. and Cascorbi, I.: Influence of polymorphisms of ABCB1 and ABCC2 on mRNA and protein expression in normal and cancerous kidney cortex. *Pharmacogenomics J.*, **7**: 56–65 (2007).
12) Han, J. Y., Lim, H. S., Yoo, Y. K., Shin, E. S., Park, Y. H., Lee, S. Y., Lee, J. E., Lee, D. H., Kim, H. T. and Lee, J. S.: Associations of ABCB1, ABCC2, and ABCG2 polymorphisms with irinotecan-pharmacokinetics and clinical outcome in patients with advanced non-small cell lung cancer. *Cancer*, **110**: 138–147 (2007).
13) Innocenti, F., Undevia, S. D., Chen, P. X., Das, S., Ramirez, J., Dolan, M. E., Relling, M. V., Kroetz, D. L. and Ratain, M. J.: Pharmacogenetic analysis of interindividual irinotecan (CPT-11) pharmacokinetic (PK) variability: Evidence for a functional variant of ABCC2. *2004 ASCO Annual Meeting Proceedings (Post-Meeting Edition).* Vol. 22, No. 14S (July 15 Supplement), 2004: Abstract No: 2010.
14) Naesens, M., Kuypers, D. R., Verbeke, K. and Vanrenterghem, Y.: Multidrug resistance protein 2 genetic polymorphisms influence mycophenolic acid exposure in renal allograft recipients. *Transplantation*, **82**: 1074–1084 (2006).
15) Rau, T., Erney, B., Gores, R., Eschenhagen, T., Beck, J. and Langer, T.: High-dose methotrexate in pediatric acute lymphoblastic leukemia: impact of ABCC2 polymorphisms on plasma concentrations. *Clin. Pharmacol. Ther.*, **80**: 468–476 (2006).
16) Zhou, Q., Sparreboom, A., Tan, E. H., Cheung, Y. B., Lee, A., Poon, D., Lee, E. J. and Chowbay, B.: Pharmacogenetic profiling across the irinotecan pathway in Asian patients with cancer. *Br. J. Clin. Pharmacol.*, **59**: 415–424 (2005).
17) Niemi, M, Arnold, K. A., Backman, J. T., Pasanen, M. K., Godtel-Armbrust, U., Wojnowski, L., Zanger, U. M., Neuvonen, P. J., Eichelbaum, M., Kivisto, K. T. and Lang, T.: Association of genetic polymorphism in ABCC2 with hepatic multidrug resistance-associated protein 2 expression and pravastatin pharmacokinetics. *Pharmacogenet. Genomics.*, **16**: 801–808 (2006).
18) Itoda, M., Saito, Y., Soyama, A., Saeki, M., Murayama, N., Ishida, S., Sai, K., Nagano, M., Suzuki, H., Sugiyama, Y., Ozawa, S. and Sawada, J.: Polymorphisms in the *ABCC2* (cMOAT/MRP2) gene found in 72 established cell lines derived from Japanese individuals: an association between single nucleotide polymorphisms in the 5′-untranslated region and exon 28. *Drug Metab. Dispos.*, **30**: 363–364 (2002).
19) Kitamura, Y., Moriguchi, M., Kaneko, H., Morisaki, H., Morisaki, T., Toyama, K. and Kamatani, N.: Determination of probability distribution of diplotype configuration (diplotype distribution) for each subject from genotypic data using the EM algorithm. *Ann. Hum. Genet.*, **66**: 183–193 (2002).
20) Ito, S., Ieiri, I., Tanabe, M., Suzuki, A., Higuchi, S. and Otsubo, K.: Polymorphism of the ABC transporter genes, MDR1, MRP1 and MRP2/cMOAT, in healthy Japanese subjects. *Pharmacogenetics*, **11**: 175–184 (2001).
21) Bosch, T. M., Doodeman, V. D., Smits, P. H., Meijerman, I., Schellens, J. H. and Beijnen, J. H.: Pharmacogenetic screening

for polymorphisms in drug-metabolizing enzymes and drug transporters in a Dutch population. *Mol. Diagn. Ther.*, **10**: 175–185 (2006).

22) Nebert, D. W.: Suggestions for the nomenclature of human alleles: relevance to ecogenetics, pharmacogenetics and molecular epidemiology. *Pharmacogenetics*, **10**: 279–290 (2000).

23) Hirouchi, M., Suzuki, H., Itoda, M., Ozawa, S., Sawada, J., Ieiri, I., Ohtsubo, K. and Sugiyama, Y.: Characterization of the cellular localization, expression level, and function of SNP variants of MRP2/ABCC2. *Pharm. Res.*, **21**: 742–748 (2004).

24) Meyer zu Schwabedissen, H. E., Jedlitschky, G., Gratz, M., Haenisch, S., Linnemann, K., Fusch, C., Cascorbi, I. and Kroemer, H. K.: Variable expression of MRP2 (ABCC2) in human placenta: influence of gestational age and cellular differentiation. *Drug Metab. Dispos.*, **33**: 896–904 (2005).

25) Izzedine, H., Hulot, J. S., Villard, E., Goyenvalle, C., Dominguez, S., Ghosn, J., Valantin, M. A., Lechat, P. and Deray, A. G.: Association between *ABCC2* gene haplotypes and tenofovir-induced proximal tubulopathy. *J. Infect. Dis.*, **194**: 1481–1491 (2006).

26) Saito, S., Iida, A., Sekine, A., Miura, Y., Ogawa, C., Kawauchi, S., Higuchi, S. and Nakamura, Y.: Identification of 779 genetic variations in eight genes encoding members of the ATP-binding cassette, subfamily C (ABCC/MRP/CFTR. *J. Hum. Genet.*, **47**: 147–171 (2002).

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Weekly Epoetin Beta Maintains Haemoglobin Levels and Improves Quality of Life in Patients with Non-Myeloid Malignancies Receiving Chemotherapy

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Objective: This study was aimed at investigating the effectiveness and safety of once-weekly epoetin beta for anaemic cancer patients receiving chemotherapy.
Methods: A total of 104 patients with a haemoglobin level of ≤11.0 g/dL were enrolled. Patients received a once-weekly subcutaneous dose of 36 000 IU epoetin beta for 12 weeks. If the increase in the haemoglobin level was <1.0 g/dL after 6 weeks, or a red blood cell transfusion was required between days 15 and 42, the dose of epoetin beta was increased to 54 000 IU from the subsequent week. The primary endpoint was the percentage of patients who achieved a haemoglobin increase of ≥2.0 g/dL; the haemoglobin response rate. Quality of life (QOL) was assessed using the Functional Assessment of Cancer Therapy-Anaemia (FACT-An) questionnaire.
Results: The haemoglobin response rate was 66.3% among the 98 patients (breast cancer: $n = 25$; malignant lymphoma: $n = 21$; ovarian cancer: $n = 20$; lung cancer: $n = 15$; other cancers: $n = 17$) assessable for a haemoglobin response. Thirty-nine patients (39.8%) required a dose escalation to 54 000 IU. At the end of the study, QOL assessable patients ($n = 96$) showed a mean improvement in the FACT-An total fatigue subscale score (FSS) of 0.3 points from baseline. Patients with a haemoglobin response had a mean change in the total FSS of +3.2, compared with −3.4 for patients without a haemoglobin response. No serious adverse event of epoetin beta was observed.
Conclusions: Epoetin beta administered at an initial dose of 36 000 IU once-weekly was well tolerated, with increased haemoglobin levels and improved QOL in anaemic cancer patients receiving myelosuppressive chemotherapy.

Key words: anaemia – erythropoietin – cancer – chemotherapy – quality of life

INTRODUCTION

Anaemia is a common complication of cancer patients undergoing chemotherapy. Symptoms of anaemia, including fatigue, palpitations, dizziness and dyspnea markedly reduce patient activity, resulting in impaired quality of life (QOL). In most cases, however, physicians hesitate to prescribe red blood cell (RBC) transfusions until the haemoglobin level is <8.0 g/dL, even if the patient has symptoms related to anaemia, such as fatigue. Although the safety of blood transfusion has improved in recent years, risks still remain, such as viral infections, graft versus host disease and haemolytic reactions.

In Europe and the United States, erythropoietin (EPO) agents have widely been used since the 1990s for the treatment of chemotherapy-induced anaemia. Although a three-times weekly dosing schedule was initially introduced (1–3), this schedule was inconvenient for outpatients. Several studies reported that once-weekly dosing of EPO increased the

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haemoglobin level and improved QOL in a manner comparable with those obtained by three-times weekly dosing (4,5).

Since EPO agents have not been approved for the treatment of chemotherapy-induced anaemia in Japan, we previously conducted a dose-finding study of weekly epoetin beta in patients with malignant lymphoma or lung cancer, resulting in a recommended weekly dose of 36 000 IU (6). In this prospective study, we investigated the haemoglobin response, the effects on QOL and the safety of once-weekly epoetin beta in anaemic patients with non-myeloid malignancies. We also investigated the effects of dose escalation to 54 000 IU in patients showing insufficient haemoglobin increase.

PATIENTS AND METHODS

Patient Eligibility

Inclusion criteria were as follows: (a) histological or cytological confirmation of non-myeloid malignancy diagnosis, (b) treatment with cyclic chemotherapy, (c) anaemia (haemoglobin level $\leq$11.0 g/dL) considered to be primarily chemotherapy-induced, (d) life expectancy of at least 4 months, (e) aged between 20 and 79 years, (f) Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 to 2, (g) eligibility for the QOL questionnaire and (h) adequate hepatic and renal function.

Exclusion criteria included: (a) iron deficiency (mean corpuscular volume $<80\ \mu m^3$ or iron saturation [{Fe/(Fe+unsaturated iron-binding capacity)} $\times$ 100] $<$15.0%); (b) surgery scheduled during the study period; (c) EPO therapy within 4 weeks prior to the study; (d) documented haemorrhagic lesions; (e) pregnancy, breastfeeding or non-use of adequate birth control measures; (f) history of myocardial, pulmonary, cerebral infarction, serious drug allergy, uncontrolled hypertension, hypersensitivity to any EPO agent or any serious complication; and (g) tumor in the central nervous system.

Study Design and Treatment Schedule

This multicentre, open-label study was conducted at 14 sites in Japan.

The protocol was approved by the institutional review board of the respective hospitals, and written informed consent was obtained from all patients who participated in the study.

The initial dose of epoetin beta (Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) was 36 000 IU, and a once-weekly treatment was administered subcutaneously for 12 weeks. If the patient's haemoglobin level did not increase by $\geq$1.0 g/dL from baseline after 6 weeks of treatment, or an RBC transfusion was required between days 15 and 42, the dose of epoetin beta was increased to 54 000 IU weekly from the subsequent week. If the haemoglobin level increased to $\geq$14.0 g/dL, epoetin beta was discontinued until the haemoglobin level decreased to $\leq$12.0 g/dL, and was then restarted at two-thirds (24 000 IU or 36 000 IU) of the previous dose (36 000 IU or 54 000 IU). RBC transfusion was allowed at the discretion of the investigator during the study. An oral daily dose of 100–200 mg elemental iron was recommended if the mean corpuscular volume was $<80\ \mu m^3$ or the iron saturation was $<$15.0%.

QOL was evaluated at baseline and week 12 using the Japanese Functional Assessment of Cancer Therapy-Anaemia (FACT-An) questionnaire (7,8), a well-validated instrument. In this study, the FACT-An total fatigue subscale, which consists of 13 fatigue related questions, was mainly analysed. The FACT-An total fatigue subscale scores (FSS) range from 0 to 52, with higher scores indicating less fatigue.

Evaluation of Efficacy and Safety

The American Society of Clinical Oncology/The American Society of Hematology guidelines (9) stipulate that the criteria for the haemopoietic effect should be an increase in haemoglobin level $\geq$1.0–2.0 g/dL in 6–8 weeks. Furthermore, there are reports (2,6), which showed that QOL is improved in patients with an increase in haemoglobin level of $\geq$2.0 g/dL.

The primary endpoint of the study was the percentage of patients achieving an increase in the haemoglobin level of $\geq$2.0 g/dL from the baseline between weeks 4 and 12, the haemoglobin response rate, excluding the data within 28 days after an RBC transfusion. The secondary endpoint was the change in FSS after 12 weeks of treatment. The percentage of patients receiving RBC transfusions between day 28 and the end of the study was also assessed. It was not expected that treatment with an EPO agent could influence transfusion requirements before day 28.

Adverse events (AEs) were assessed during the 12-week treatment period and during a 1-week observation period after the last dosing. Anti-erythropoietin antibodies were measured by the enzyme-linked immunosorbent assay and radio-immunoprecipitation (RIP) assay, and detection by either was judged as positive.

Statistical Analysis

We expected that 90 patients would need to be enrolled in the study to obtain a haemoglobin response rate of 70 $\pm$ 10% (95% confidence interval [CI]), as the primary endpoint.

Patients who received at least one dose of the study drug comprised the safety population. For efficacy analysis, the full analysis set (FAS) population was defined as eligible patients who received at least one dose of the study drug.

The changes in the haemoglobin level and FACT-An scores were calculated by subtracting each patient's baseline values from the last values. The rates of increase in haemoglobin before and after dose escalation were compared using a linear mixed-effects model. The potential factors influencing the change in FSS were examined by multiple

regression analysis. Pearson correlation coefficients were calculated to assess the association between changes in the haemoglobin level and FACT-An scores.

RESULTS

Demographics and Baseline Characteristics

A total of 104 patients were enrolled in the study between February and November 2004. Five patients discontinued the study before the first dosing for the following reasons: patient eligibility criteria violation, $n = 3$; patient denial, $n = 1$; and disease progression, $n = 1$. Thus, 99 patients were administered epoetin beta. One patient was excluded because of non-compliance with the eligibility criteria, leaving 98 patients as the FAS population. Eighty-seven patients (88.8%) completed all 12 weeks of the study. Eleven patients (11.2%) withdrew from the study. The primary reasons for withdrawal were progressive disease and AEs.

The demographics and baseline characteristics of the FAS population are listed in Table 1. Common types of cancer were breast ($n = 25$), malignant lymphoma ($n = 21$), ovarian ($n = 20$) and lung ($n = 15$). The mean age was 58.4 years (range: 23–78), and the mean body weight was 50.7 kg (range: 31.7–74.0). Most of the patients had an ECOG PS of 0 or 1 and a tumour stage of III or IV. The main chemotherapeutic agents used during the study were platinum for lung and other types of cancer, anthracycline for malignant lymphoma, taxane for breast cancer and platinum plus taxane for ovarian cancer. All patients met the criterion that they should not be iron-deficient at the time of enrollment.

Haemoglobin Response

The mean change in the haemoglobin level from baseline to the end of the study was 2.47 g/dL (standard deviation [SD]: 2.09; range: −2.8 to 6.0), as shown in Fig. 1. Figure 1 shows the mean changes in haemoglobin levels by tumour type. The pattern of changes in haemoglobin level was similar for the different tumour types. The mean increase in the haemoglobin level in patients with and without an initial EPO level of ≥100 mIU/mL were 1.76 g/dL (SD: 2.60) and 2.50 g/dL (SD: 1.85), respectively.

The haemoglobin response rates, defined as the percentage of patients achieving an increase in haemoglobin level of ≥2.0 g/dL from the baseline between weeks 4 and 12, are listed in Table 2. The overall haemoglobin response rate was 66.3% (65 of 98 patients). The median time to the haemoglobin response was 56 days from the first dosing, analysed by the Kaplan–Meier method. The percentage of patients with a haemoglobin level of ≥12.0 g/dL between weeks 4 and 12 was 59.2% (58 of 98 patients).

The percentage of patients who required dose escalation to 54 000 IU was 39.8% (39 of 98 patients). In these patients, the haemoglobin level increased after dose escalation, and the change in the haemoglobin level was 1.23 g/dL (SD: 2.19) at the end of the study. The haemoglobin response rate was 33.3% (13 of 39 patients) in patients who required dose escalation. The rate of haemoglobin increase before and after dose escalation was 0.023 g/dL/week (Weeks 0–6) and 0.266 g/dL/week (Weeks 7–12), respectively ($P = 0.0055$).

For three patients, the drug treatment was discontinued when the haemoglobin level exceeded 14.0 g/dL, and was restarted at a dose of 24 000 IU when the haemoglobin level decreased to ≤12.0 g/dL.

Quality of Life

Overall compliance in terms of the percentage of patients who completed the FACT-An was 100% at baseline and 97% (95 of 98 patients) at the end of the study. For three patients who dropped out due to progressive disease and were regarded as missing not at random, the scores at the end of the study were substituted with the minimum scores for all patients. Two patients were excluded from the evaluation of the change in the FSS because the responses to some items were missing.

The mean baseline FSS was 31.8 (SD: 11.4, $n = 98$) points. At the end of the study, the mean change from baseline was 0.3 (SD: 11.8, $n = 96$) points. The mean FSS change in the patients with progressive disease, as judged by each investigator, was −3.8 (SD: 16.7, $n = 15$) points (haemoglobin change: 2.4 g/dL). On the other hand, the mean change in patients without progressive disease was 1.9 (SD: 9.6, $n = 78$) points (haemoglobin change: 2.3 g/dL). These data indicated that progressive disease may be one of the independent variables affecting the change in FSS.

Relationship Between Haemoglobin Response and QOL Score

The results of a multiple regression analysis suggested that the change in the haemoglobin level ($P = 0.014$), the FSS at the initiation of dosing ($P < 0.0001$) and the PS at the end of the study ($P < 0.0001$) largely contributed to the change in the FSS. The correlation coefficient between the change in the FSS and the changes in the haemoglobin level was 0.280, indicating a significant correlation ($P = 0.006$, $n = 96$).

Patients who achieved an increase in the haemoglobin level of ≥2.0 g/dL experienced a 3.2-point mean change in FSS. On the other hand, patients who did not achieve an increase in haemoglobin level of ≥2.0 g/dL experienced a −3.4-point change (Fig. 2). There were no differences in the FSS at the initiation of dosing between patients with and without a change in haemoglobin level of ≥2.0 g/dL (32.0 versus 31.6). These data indicate that the change in FSS is dependent on the change in the haemoglobin level.

Concerning the relationship between the FSS at the initiation of dosing and the change in the FSS, patients with a baseline FSS of ≤36.0 reported greater improvement (mean ± SD: 1.6 ± 13.0) in the FSS at the end of the study (Table 3).

Table 1. Characteristics of the full analysis set population

	Characteristics	Total	Lung	Malignant Lymphoma	Breast	Ovarian	Other types
Sex	Male	27	11	10	0	0	6
	Female	71	4	11	25	20	11
Age (years)	Mean ± SD	58.4 ± 10.8	60.5 ± 10.5	56.5 ± 13.4	58.2 ± 9.0	54.4 ± 11.0	63.8 ± 8.0
	Range	23–78	41–78	23–74	39–77	30–75	40–76
ECOG performance status	0	48	1	9	14	13	11
	1	39	12	9	6	6	6
	2	11	2	3	5	1	0
Tumour stage	I	6	0	1	3	2	0
	II	17	1	4	7	4	1
	III	15	0	3	0	9	3
	IIIA	2	1	0	1	0	0
	IIIB	8	6	0	2	0	0
	IV	50	7	13	12	5	13
Treatment regimen	Platinum based	17	7	2	0	1	7
	Taxane based	28	5	0	19	3	1
	Anthracycline based	28	1	18	6	0	3
	Platinum, Anthracycline based	4	0	1	0	1	2
	Platinum,Taxane based	21	2	0	0	15	4
Weight (kg)	Mean ± SD	50.7 ± 8.2	53.8 ± 8.7	52.7 ± 9.9	47.9 ± 7.2	49.3 ± 6.6	50.9 ± 7.4
	Range	31.7–74.0	38.0–70.7	31.7–74.0	34.0–63.0	34.1–60.0	37.7–65.5
Haemoglobin (g/dL)	Mean ± SD	9.3 ± 1.4	9.6 ± 1.4	9.3 ± 1.4	9.4 ± 1.4	9.2 ± 1.6	9.1 ± 1.4
	Range	5.6–11.9	6.4–11.2	6.5–11.3	5.7–11.9	6.4–11.7	5.6–11.1
MCV (fL)	Mean ± SD	92.3 ± 6.5	89.0 ± 6.4	90.0 ± 5.4	91.9 ± 5.8	93.6 ± 7.5	95.7 ± 5.3
	Range	79.9–107.5	79.9–99.3	80–101	80.3–103.2	81.9–107.5	84–103.4
Reticulocyte (‰)	Mean ± SD	19.7 ± 16.4	20.8 ± 15.1	24.2 ± 24.1	18.0 ± 13.2	21.1 ± 15.6	14.1 ± 10.3
	Range	1–106	2–50	1–106	1–58	1–54	1.1–35.1
Transferrin saturation (%)	Mean ± SD	29.7 ± 22.3	22.4 ± 7.1	41.5 ± 30.6	21.9 ± 16.9	31.1 ± 24.3	30.5 ± 18.0
	Range	4.8–92.9	12.5–35.5	9.9–92.9	4.8–80.6	7.2–90.7	14.0–98.3
Serum endogenous erythropoietin (mIU/mL)	Mean ± SD	119.1 ± 310.5	64.3 ± 69.9	80.7 ± 108.0	88.4 ± 107.1	125.9 ± 144.8	252.0 ± 706.9
	Range	15.7–2970	15.7–254	17.3–399	16.7–472	23.2–578	20.4–2970
Baseline QOL:FACT-An							
Anemia subscale (0–80)	Mean ± SD	50.8 ± 14.5	47.0 ± 15.9	50.6 ± 11.7	47.1 ± 13.7	53.5 ± 11.1	56.7 ± 19.5
	Range	16–80	17–74	26–67	20–71	34–75	16–80
Fatigue subscale (0–52)	Mean ± SD	31.8 ± 11.4	29.6 ± 12.9	30.3 ± 10.6	29.7 ± 10.7	33.9 ± 8.7	36.5 ± 14.1
	Range	4–52	4–52	10–43	7–50	20–48	8–52

SD, standard deviation; ECOG, Eastern Cooperative Oncology Group; QOL, quality of life; FACT-An, Functional Assesment of Cancer Therapy-Anemia; MCV, mean corpuscular volume.

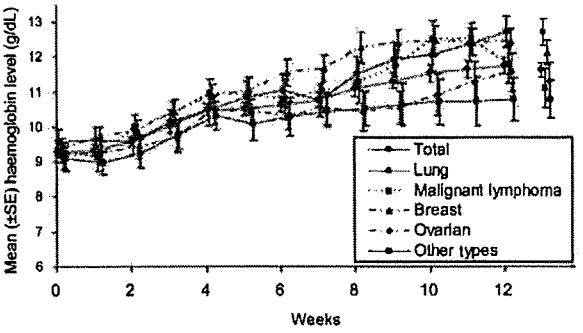

Figure 1. Change in haemoglobin level by tumor type. Mean weekly haemoglobin levels for the FAS population. Haemoglobin values within 28 days after RBC transfusion were excluded. FAS, full analysis set; RBC, red blood cell.

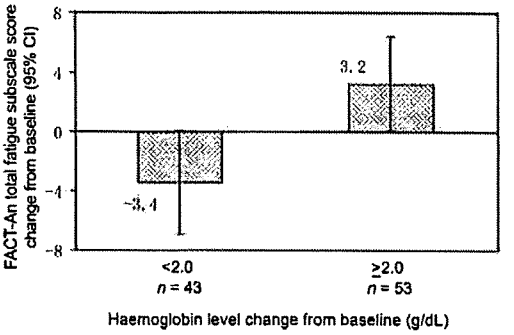

Figure 2. Changes in the FACT-An total fatigue subscale score by change in haemoglobin level. FACT-An, Functional Assessment of Cancer Therapy-Anaemia.

RBC Transfusion Requirement

The percentage of patients who received RBC transfusions between day 28 and the end of the study was only 6.1% (6 of 98 patients). The mean pretransfusion haemoglobin level at the time of the first transfusion was 6.2 g/dL (range: 5.4–7.3 g/dL). The percentage of patients whose haemoglobin level had decreased to <8.0 g/dL or who received an RBC transfusion between day 28 and the end of the study was 20.4% (20 of 98 patients).

Safety

AEs reported by at least 20% of the patients are summarised in Table 4. Death as a result of disease progression was not reported as an AE. Adverse drug reactions reported by at least 5% of patients are listed in Table 5. Among the 133 events in 48 patients (48.5%) that were considered related to the study drug, Grade III events were headache, hypertension, diarrhea, decreased serum potassium, impaired consciousness, anorexia and decreased serum phosphate. Three events (3.0%) of hypertension were reported as possibly related to epoetin beta treatment. An antihypertensive drug was administered after the onset of hypertension in one patient, who had hypertension as a comorbidity before the study. One patient (65-year-old female with malignant lymphoma) experienced a thrombovascular event, a lacunar infarction, at week 6. This event was evaluated as being unrelated to epoetin beta and was attributed to aging.

The incidence and type of AEs in patients who required dose escalation did not differ from those in patients who did not.

In two patients with ovarian and gastric cancer, anti-erythropoietin antibodies were detected only by RIP assay.

Table 2. Haemoglobin response rate by baseline haemoglobin, tumour type and dose escalation

	%	*n*
Response rate*	66.3	65/98
Response rate by baseline haemoglobin, g/dL		
<10.0	68.8	44/64
≥10.0	61.8	21/34
Response rate by tumour type		
Lung	80.0	12/15
Malignant lymphoma	66.7	14/21
Breast	76.0	19/25
Ovarian	65.0	13/20
Other types	41.2	7/17
Response rate by dose escalation		
Yes	33.3	13/39
No	88.1	52/59

*All patients, including those receiving transfusions.

Table 3. Changes in the FACT-An total fatigue subscale score by baseline FSS and final PS

Time period	Baseline		End of treatment		
	n	Mean score (SD)	*n*	Mean score (SD)	Mean change from baseline (SD)
Total	98	31.8 (11.4)	96*	31.8 (13.5)	0.3 (11.8)
Baseline FSS					
≤36.0	62	24.8 (7.9)	62	26.5 (12.0)	1.6 (13.0)
>36.0	36	43.9 (4.0)	34*	41.5 (10.3)	−2.2 (8.8)
Final PS					
0	58	35.5 (11.3)	56*	37.4 (10.3)	2.4 (10.2)
1	28	27.4 (9.1)	28	29.0 (11.5)	1.6 (12.2)
2	4	19.3 (9.4)	4	11.8 (11.4)	−7.5 (7.9)
3	3	29.7 (15.9)	3	21.0 (7.2)	−8.7 (13.8)
4	5	25.7 (7.3)	5	6.4 (7.1)	−19.3 (6.4)

*Two patients missing FSS. Collected but could not be calculated.
FSS, FACT-An total fatigue subscale score; PS, performance status.

Table 4. Frequencies of adverse events ($n = 99$)

Event	n	%	Grade*				
			I	II	III	IV	V
Neutropenia	83	83.8	3	11	24	45	0
Leukopenia	78	78.8	2	16	41	19	0
Nausea	57	57.6	38	11	8	0	0
Thrombocytopenia	55	55.6	21	9	23	2	0
Lymphopenia	52	52.5	0	18	34	0	0
Anorexia	46	46.5	22	13	10	1	0
Fatigue	39	39.4	22	14	3	0	0
Vomiting	36	36.4	18	16	2	0	0
Diarrhea	33	33.3	23	6	4	0	0
Increased lactate dehydrogenase	32	32.3	25	6	1	0	0
Peripheral neuropathy	26	26.3	21	5	0	0	0
Fever	26	26.3	17	7	2	0	0
Constipation	24	24.2	3	13	7	1	0
Increased alanine aminotransferase	24	24.2	15	6	3	0	0
Alopecia	22	22.2	7	15	0	0	0

*National cancer institutes common toxicity criteria, version 2.0.

Table 5. Frequencies of adverse drug reactions ($n = 99$)

Event	n	%	Grade*				
			I	II	III	IV	V
Increased lactate dehydrogenase	10	10.1	9	1	0	0	0
Headache	7	7.1	6	0	1	0	0
Nausea	7	7.1	5	2	0	0	0
Rash	5	5.1	3	2	0	0	0
Back pain	5	5.1	5	0	0	0	0

*National cancer institutes common toxicity criteria, version 2.0.

Neutralisation of EPO activity was detected in neither patient, and the haemoglobin level was elevated after dosing with the study drug. The investigators judged that the antibody did not cause pure red cell aplasia.

When re-examined six months after the last observation, one of these patients (ovarian cancer) was antibody negative, whereas the other (gastric cancer) could not be re-examined, having died of the underlying disease.

DISCUSSION

Several studies have been conducted to assess the effects of EPO agents in anaemic cancer patients, and increased haemoglobin levels and improvement in QOL that correlated with the increased haemoglobin level were reported (1,10).

The objectives of our study were to investigate the effects of an initial once-weekly 36 000 IU dose of epoetin beta on haemoglobin levels and QOL in patients with non-myeloid malignancy undergoing chemotherapy. The criterion for a haemoglobin response, an increase in the haemoglobin level of $\geq$2.0 g/dL, was based on a report that symptoms of anaemia assessed by the FACT-An are improved in patients with a change in the haemoglobin level of $\geq$2.0 g/dL (2,6). According to this index, the haemoglobin response rate in the present study was 66.3% (65 of 98 patients). The increases in haemoglobin levels that were observed were independent of the tumour type or the baseline haemoglobin level. None of the investigators performed a randomised comparison of a dose increase versus an unchanged dose in EPO low responders. In the present study, there was an increase in the rate of haemoglobin increase after dose escalation to 54 000 IU, and the haemoglobin response rate for patients who required a dose escalation was 33.3% (13 of 39 patients).

The secondary endpoint, the change in the FSS, showed an increase of 0.3 points; however, in patients who showed an increase in the haemoglobin level of $\geq$2.0 g/dL, the FSS was increased by 3.2 points, which was significantly higher than the $-$3.4-point change in patients whose haemoglobin level increased by $<$2.0 g/dL. A 3.2-point increase is comparable with the 3 points considered to be a clinically significant change in FSS (11). In addition, the mean change in FSS for patients with progressive diseases (PD) was $-$3.8 points (median: $-$6.5 points, range: $-$37 to 35 points) even though correction of anaemia was observed. In total, excluding PD cases, a 1.9- point improvement was observed.

Investigating the relationship between the FSS at the initiation of dosing and the change in the FSS showed that greater improvements in FSS were observed in patients with lower FSS. The FSS before treatment with epoetin beta was 31.8 $\pm$ 11.4 points, which is higher than the scores (FSS: 22.1–29.7 points, change in FSS: 1.6–5.2 points) in cancer patients with anaemia reported in several randomised trials (1,10,12–14). Nevertheless, the mean initial haemoglobin level (9.3 g/dL) in the present study was equal to the levels in the other trials (9.2–10.1 g/dL). Since it has been reported that the FSS after treatment with an EPO agent is aggravated in patients with an FSS exceeding 36.0 at the initiation of dosing (15), the scores were analysed after stratification at 36.0. This resulted in improved scores (1.6 $\pm$ 13.0 points) for those patients with a baseline score of $\leq$36.0, when compared with patients with a score $>$36.0 ($-$2.2 $\pm$ 8.8 points). The results of a multiple regression analysis of the change in the FSS demonstrated that the change in the haemoglobin level, the FSS at the initiation of dosing and the PS at the end of the study were factors that largely contributed to the change in the FSS. A positive and significant association was observed between

the degree of increase in the haemoglobin level and the degree of improvement in the FSS ($r = 0.280$, $P = 0.006$). It was comparable with the results ($r = 0.2879$, $P = 0.0002$; $r = 0.35$, $P = 0.001$ and $r = 0.2893$, $P < 0.0001$) of three other studies (1,10,16).

The RBC transfusion rate was only 6.1% (6 of 98 patients) between day 28 and the end of the study. As reported for once-weekly epoetin alfa administered to patients with various types of cancer (14), the transfusion rates between week 5 and the end of treatment were 14.5% (24 of 166 patients) for epoetin alfa and 29.3% (48 of 164 patients) for placebo. Furthermore, the mean pretransfusion haemoglobin levels for the first transfusion reported in the previous trial in the United States (7.9 and 7.8 g/dL, respectively) were higher than those (6.2 g/dL) in the present study in Japan. To evaluate the effect of EPO agents, the percentage of patients whose haemoglobin level had decreased to <8.0 g/dL or who received an RBC transfusion was considered to be a more objective index than the RBC transfusion rate in Japan, because RBC transfusion itself is prescribed at the discretion of the investigator and when the haemoglobin level is low.

Epoetin beta was well tolerated in the present study. Most of the AEs were consistent with the underlying disease or with the chemotherapy. Hypertension, which was judged to be related to epoetin beta was observed in three patients. It was alleviated either by no treatment or the administration of hypotensive agents. Lacunar infarction was also observed in one patient. A relationship to epoetin beta was ruled out, however, and this event was judged to be due to aging. Two recently published studies (17,18) targeting higher haemoglobin levels, in which survival was a primary endpoint, have raised concerns that EPO agents may have a negative impact on survival in cancer patients. A meta-analysis of 57 studies, including these two recent studies revealed an overall survival hazard ratio of 1.08 (95%CI: 0.99–1.18) and that uncertainties remain as to whether EPO agents affected survival (19). The FDA has provided new safety information on erythropoiesis-stimulating agents (ESAs), in which the target haemoglobin level is not to exceed 12 g/dL, because analyses of other studies in patients with cancer found a higher chance of serious and life-threatening adverse drug reactions or deaths with the use of ESAs (20). Although, in the present studies, there was no problem with safety when the haemoglobin level at which dosing was withheld was set at 14 g/dL, in consideration of FDA ALERTs, etc., we intend to investigate the use of lower values for target haemoglobin level and haemoglobin level at which dosing should be withheld.

In conclusion, once-weekly epoetin beta treatment increased the haemoglobin level and correspondingly improved the QOL in anaemic patients with non-myeloid malignancies receiving chemotherapy. Additionally, haemoglobin levels could be improved and controlled by once-weekly treatments at an initial dose of 36 000 IU followed by dose adjustment in the range of 24 000–54 000 IU.

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Conflict of interest statement

One of the authors, Hironobu Minami, receives honoraria from Chugai Pharmaceutical Co., Ltd. and Kirin Pharma Co., Ltd.

One of the authors, Yasuo Ohashi, consults on design and data analysis of clinical trials for Chugai Pharmaceutical Co., Ltd.

One of the authors, Nagahiro Saijo, holds stock option for Takeda Pharmaceutical Co., Ltd.

References

1. Littlewood TJ, Bajetta E, Nortier JW, Vercammen E, Rapoport B. Effects of epoetin alfa on hematologic parameters and quality of life in cancer patients receiving nonplatinum chemotherapy: results of a randomized, double-blind, placebo-controlled trial. *J Clin Oncol* 2001;19(11):2865–74.
2. Demetri GD, Kris M, Wade J, Degos L, Cella D. Quality-of-life benefit in chemotherapy patients treated with epoetin alfa is independent of disease response or tumor type: results from a prospective community oncology study. *J Clin Oncol* 1998;16(10):3412–25.
3. Glaspy J, Bukowski R, Steinberg D, Taylor C, Tchekmedyian S, Vadhan-Raj S. Impact of therapy with epoetin alfa on clinical outcomes in patients with nonmyeloid malignancies during cancer chemotherapy in community oncology practice. Procrit Study Group. *J Clin Oncol* 1997;15:1218–34.
4. Demetri GD, Gabrilove JL, Blasi MV, Hill RJ, Glaspy JJ. Benefits of epoetin alfa in anemic breast cancer patients receiving chemotherapy. *Clin Breast Cancer* 2002;3(1):45–51.
5. Gabrilove JL, Cleeland CS, Livingston RB, Sarokhan B, Winer E, Einhorn LH. Clinical evaluation of once-weekly dosing of epoetin alfa in chemotherapy patients: improvements in hemoglobin and quality of life are similar to three-times-weekly dosing. *J Clin Oncol* 2001;19(11):2875–82.
6. Morishima Y, Ogura M, Yoneda S, Sakai H, Tobinai K, Nishiwaki Y, et al. Qnce-weekly epoetin-beta improves hemoglobin levels in cancer patients with chemotherapy-induced anemia: a randomized, double-blind, dose-finding study. *Jpn J Clin Oncol* 2006;36(10):655–61.
7. Yoshimura A, Kobayashi K, Fumimoto H, Fujiki Y, Eremenco S, Kudoh S. Cross-cultural validation of the Japanese Functional Assessment of Cancer Therapy-Anemia (FACT-An). *J Nippon Med Sch* 2004;71(5):314–22.
8. Kurita M, Shimozuma K, Morita S, Fujiki Y, Ishizawa K, Eguchi H, et al. Clinical validity of the Japanese version of the Functional

Assessment of Cancer Therapy-Anemia Scale. *Support Care Cancer* 2007;15(1):1–6.
9. Rizzo JD, Lichtin AE, Woolf SH, Seidenfeld J, Bennett CL, Cella D, et al. Use of epoetin in patients with cancer: evidence-based clinical practice guidelines of the American Society of Clinical Oncology and the American Society of Hematology. *J Clin Oncol* 2002;20:4083–107.
10. Iconomou G, Koutras A, Rigopoulos A, Vagenakis AG, Kalofonos HP. Effect of recombinant human erythropoietin on quality of life in cancer patients receiving chemotherapy: results of a randomized, controlled trial. *J pain Symptom Manage* 2003;25(6):512–8.
11. Cella D, Eton DT, Lai JS, Peterman AH, Merkel DE. Combining anchor and distribution-based methods to derive minimal clinically important differences in the Functional Assessment of Cancer Therapy (FACT) anemia and fatigue Scales. *J Pain Symptom Manage* 2002;24:547–61.
12. Osterborg A, Brandberg Y, Molostova V, Iosava G, Abdulkadyrov K, Hedenus M, et al. Randomized, double-blind, placebo-controlled trial of recombinant human erythropoietin, epoetin beta, in hematologic malignancies. *J Clin Oncol* 2002;20(10):2486–94.
13. Cella D, Zagari MJ, Vandoros C, Gagnon DD, Hurtz HJ, Nortier JW. Epoetin alfa treatment results in clinically significant improvements in quality of life in anemic cancer patients when referenced to the general population. *J Clin Oncol* 2002;21(2):366–73.
14. Witzig TE, Silberstein PT, Loprinzi CL, Sloan JA, Novotny PJ, Mailliard JA, et al. Phase III, randomized, double-blind study of epoetin alfa compared with placebo in anemic patients receiving chemotherapy. *J Clin Oncol* 2005;23(12):2606–17.
15. Hedenus M, Adriansson M, San Miguel J, Kramer MH, Schipperus MR, Juvonen E, et al. Efficacy and safety of darbepoetin alfa in anaemic patients with lymphoproliferative malignancies: a randomized, double-blind, placebo-controlled study. *Br J Haematol* 2003;122(3):394–403.
16. Chang J, Couture F, Young S, McWatters KL, Lau CY. Weekly epoetin alfa maintains hemoglobin, improves quality of life, and reduces transfusion in breast cancer patients receiving chemotherapy. *J Clin Oncol* 2005;23(12):2597–605.
17. Henke M, Laszig R, Rübe C, Schäfer U, Haase KD, Schilcher B, et al. Erythropoietin to treat head and neck cancer patients with anaemia undergoing radiotherapy: randomised, double-blind, placebo-controlled trial. *Lancet* 2003;362:1255–60.
18. Leyland-Jones B, Semiglazov V, Pawlicki M, Pienkowski T, Tjulandin S, Manikhas G, et al. Maintaining normal hemoglobin levels with epoetin alfa in mainly nonanemic patiens with metastatic breast cancer receiving first-line chemotherapy: a survival study. *J Clin Oncol* 2005;23(25):5960–72.
19. Bohlius J, Wilson J, Seidenfeld J, Piper M, Schwarzer G, Sandercock J, et al. Recombinant human erythropoietins and cancer patients: updated meta-analysis of 57 studies including 9353 patients. *J Natl Cancer Inst* 2006;98(10):708–14.
20. FDA ALERT [Updated 2007 Mar 09; cited 2006 Nov 16] Information on Erythropoiesis Stimulating Agents (ESA), available at http://www.fda.gov/cder/drug/InfoSheets/HCP/RHE2007HCP.htm

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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.

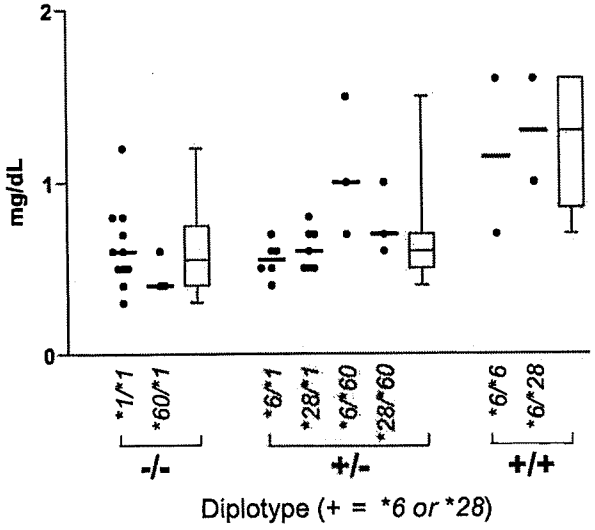

Fig. 1. Effects of *UGT1A1* genotypes on serum total bilirubin levels at baseline in Japanese cancer patients ($N = 37$). Each point represents a patient, and the median value of each diplotype is shown with a bar. All diplotypes are classified into −/−, +/−, and +/+ by the genetic marker, "*UGT1A1*6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

[1] J.G. Slatter, P. Su, J.P. Sams, L.J. Schaaf, L.C. Wienkers, Bioactivation of the anticancer agent CPT-11 to SN-38 by human hepatic microsomal carboxylesterases and the in vitro assessment of potential drug interactions, Drug Metab. Dispos. 25 (1997) 1157–1164.

[2] L. Iyer, C.D. King, P.F. Whitington, M.D. Green, S.K. Roy, T.R. Tephly, et al., Genetic predisposition to the metabolism of irinotecan (CPT-11). Role of uridine diphosphate glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes, J. Clin. Invest. 15 (1998) 847–854.

[3] M. Ciotti, N. Basu, M. Brangi, I.S. Owens, Glucuronidation of 7-ethyl-10-hydroxycamptothecin (SN-38) by the human UDP-glucuronosyltransferases encoded at the UGT1 locus, Biochem. Biophys. Res. Commun. 260 (1999) 199–202.

[4] J.F. Gagne, V. Montminy, P. Belanger, K. Journault, G. Gaucher, C. Guillemette, Common human UGT1A polymorphisms and the altered metabolism of irinotecan active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38), Mol. Pharmacol. 62 (2002) 608–617.

[5] T. Oguri, T. Takahashi, M. Miyazaki, T. Isobe, N. Kohno, P.I. Mackenzie, et al., UGT1A10 is responsible for SN-38 glucuronidation and its expression in human lung cancers, Anticancer Res. 24 (2004) 2893–2896.

[6] N. Hanioka, S. Ozawa, H. Jinno, M. Ando, Y. Saito, J. Sawada, Human liver UDP-glucuronosyltransferase isoforms involved in the glucuronidation of 7-ethyl-10-hydroxycamptothecin, Xenobiotica 31 (2001) 687–699.

[7] M. de Forni, R. Bugat, G.G. Chabot, S. Culine, J.M. Extra, A. Gouyette, et al., Phase I and pharmacokinetic study of the camptothecin derivative irinotecan, administered on a weekly schedule in cancer patients, Cancer Res. 54 (1994) 4347–4354.

[8] E. Gupta, T.M. Lestingi, R. Mick, J. Ramirez, E.E. Vokes, M.J. Ratain, Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea, Cancer Res. 54 (1994) 3723–3725.

[9] Y. Ando, H. Saka, M. Ando, T. Sawa, K. Muro, H. Ueoka, et al., Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis, Cancer Res. 60 (2000) 6921–6926.

[10] L. Iyer, S. Das, L. Janisch, M. Wen, J. Ramirez, T. Karrison, et al., UGT1A1*28 polymorphism as a determinant of irinotecan disposition and toxicity, Pharmacogenomics J. 2 (2002) 43–47.

[11] F. Innocenti, S.D. Undevia, L. Iyer, P.X. Chen, S. Das, M. Kocherginsky, et al., Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan, J. Clin. Oncol. 22 (2004) 1382–1388.

[12] E. Marcuello, A. Altes, A. Menoyo, E. Del Rio, M. Gomez-Pardo, M. Baiget, UGT1A1 gene variations and irinotecan treatment in patients with metastatic colorectal cancer, Br. J. Cancer 91 (2004) 678–682.

[13] E. Rouits, M. Boisdron-Celle, A. Dumont, O. Guerin, A. Morel, E. Gamelin, Relevance of different UGT1A1 polymorphisms in irinotecan-induced toxicity: a molecular and clinical study of 75 patients, Clin. Cancer Res. 10 (2004) 5151–5159.

[14] N. Kaniwa, K. Kurose, H. Jinno, T. Tanaka-Kagawa, Y. Saito, M. Saeki, et al., Racial variability in haplotype frequencies of UGT1A1 and glucuronidation activity of a novel single nucleotide polymorphism 686C> T (P229L) found in an African-American, Drug Metab. Dispos. 33 (2005) 458–465.