(unpublished data), and that the plasma level of SN-38 was largely influenced by *UGT1A1*6* and **28* [22]. Therefore, it is likely that the contribution of CYP3A4 to irinotecan clearance is rather small as compared with other genetic and non-genetic factors.

In accordance with the above observations, no significant associations were observed between the *CYP3A4* haplotypes and severe toxicities (grade 3 diarrhea and grade 3 or 4 neutropenia) in the patients with irinotecan monotherapy (Table 5). Similarly, we observed no significant effect of the *CYP3A4* haplotypes on incidence of the severe toxicities in the patients treated with both irinotecan and cisplatin (data not shown), although the numbers of patients bearing **16B* and **18B* were small. Taken together, the current study indicates that the influence of the *CYP3A4* genotypes on the activation pathway of irinotecan (generation of SN-38) might be small.

In conclusion, the current study suggested that *CYP3A4*16B* was associated with decreased metabolism of irinotecan to APC. However, impact of the *CYP3A4* haplotypes on total clearance of irinotecan and severe toxicities was not significant.

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Genetic variations and haplotypes of *ABCC2* encoding MRP2 in a Japanese population

Running title: *ABCC2* haplotypes in Japanese

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Footnotes

As of October 7, 2007, the novel variations reported here are not found in the database of Japanese

Single Nucleotide Polymorphisms (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB Database (http://www.pharmgkb.org/).

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Abstract

The multidrug resistance-associated protein 2 (MRP2) encoded by the *ABCC2* gene is expressed in the liver, intestine and kidneys and preferentially exports organic anions or conjugates with glucuronide or glutathione. In this study, all 32 exons and the 5'-flanking region of *ABCC2* in 236 Japanese were resequenced, and 61 genetic variations including 5 novel nonsynonymous ones were detected. A total of 64 haplotypes were determined/inferred and classified into five *1 haplotype groups (*1A, *1B, *1C, *1G, and *1H) without nonsynonymous substitutions and *2 to *9 groups with nonsynonymous variations. Frequencies of the major 4 haplotype groups *1A (-1774delG), *1B (no common SNP), *1C (-24C>T and 3972C>T), and *2 [1249G>A (Val417Ile)] were 0.331, 0.292, 0.172, and 0.093, respectively. This study revealed that haplotype *1A, which has lowered activity, is quite common in Japanese, and that the frequency of *1C, another functional haplotype, was comparable to frequencies in Asians and Caucasians. In contrast, the haplotypes harboring 3972C>T but not -24C>T (*1G group), which are reportedly common in Caucasians, were minor in Japanese. Moreover, the allele 1446C>T (Thr482Thr), which has increased activity, was not detected in our Japanese population. These findings imply possible differences in MRP2-mediated drug responses between Asians and Caucasians.

Key words: *ABCC2*, MRP2, genetic variation, haplotype, amino acid change

Introduction

The multidrug resistance-associated protein 2 (MRP2) or canalicular multispecific organic anion transporter (cMOAT) is a 190-200 kDa transmembrane glycoprotein comprised of 1545 amino acids and belongs to the superfamily C of ATP-binding cassette (ABC) transporters. This transporter is expressed on hepatic canalicular membranes, intestinal apical membranes, luminal membranes of renal proximal tubules, placental epithelial cells, and the blood brain barrier [1]. MRP2 exports endogenous and exogenous substances, preferentially organic anions or conjugates with glucuronide, glutathione and sulfate [1-3]. This protein originally identified in cisplatin-resistant tumor cells [4] is shown to confer drug resistance to other anti-cancer drugs, such as vincristine and doxorubicin [5, 6].

MRP2 is encoded by the *ABCC2* gene located on chromosome 10q24 and consists of 32 exons (31 coding exons) and spans 69 kb. Several *ABCC2* genetic variations have been detected in patients with Dubin-Johnson syndrome (DJS), an autosomal recessive disease characterized by hyperbilirubinemia with conjugated bilirubin or increased coproporphyrin excretion in urine [2,7]. Recent studies on *ABCC2* have identified common single nucleotide polymorphisms (SNPs) such as -24C>T and -3972C>T (Ile1324Ile) among several ethnic populations, and several studies have suggested their association with altered MRP2 expression or function [8-17]. In more recent studies on *ABCC2* haplotypes covering an extended 5'-flanking region, close linkages were found among -1549A>G in the 5'-flanking region and two common SNPs -24C>T and -3972C>T (Ile1324Ile) [8]. In addition, as possible functional SNPs, -1774delG in Koreans [8] and -1019A>G in Caucasians [10] were reported. However, there is little information on detailed haplotype structures throughout the gene, and comprehensive haplotype analysis in Japanese has not yet been conducted.

We previously analyzed *ABCC2* genetic variations within all 32 exons and the proximal 5'-flanking region (approximately 800 bp upstream of the translation initiation site) using established cell lines derived from Japanese cancer patients to obtain preliminary information on *ABCC2* SNPs in Japanese [18]. In this study, to reveal *ABCC2* haplotype structures in Japanese, we resequenced the *ABCC2* gene including the distal 5'-upstream region (approximately 1.9 kb upstream from the

translation initiation site) as well as all 32 exons in 236 Japanese subjects and conducted haplotype analysis using the detected genetic polymorphisms.

Materials and Methods

Human DNA samples

Genomic DNA samples were obtained from blood leukocytes of 177 Japanese cancer patients at two National Cancer Center Hospitals (Tokyo and Chiba, Japan) and Epstein-Barr virus-transformed lymphoblastoid cells prepared from 59 healthy Japanese volunteers at the Tokyo Women's Medical University under the auspices of the Pharma SNP consortium (Tokyo, Japan). Written informed consent was obtained from all subjects. Ethical review boards of all participating organizations approved this study.

PCR conditions for DNA sequencing

We sequenced all 32 exons of the *ABCC2* gene and approximately 800 bp upstream of the translation initiation codon (proximal 5'-flanking region) as described previously [18] and also extended the sequenced region to 1.9 kb upstream of the translation initiation site (distal 5'-flanking region). Briefly, for amplification of the proximal 5'-flanking region and 32 exons, 5 sets of multiplex PCR were performed from 200 ng of genomic DNA using 1.25 units of Z-taq (Takara Bio. Inc., Shiga, Japan) with 0.3 uM each of the mixed primers as shown in Table 1 [1st PCR]. The first PCR conditions consisted of 30 cycles of 98℃ for 5 sec, 55℃ for 5 sec, and 72℃ for 190 sec. Next, each exon was amplified separately using the 1st PCR product by Ex-Taq (0.625 units, Takara Bio. Inc.) with appropriate primers (0.3 uM) [Table 1, 2nd PCR]. The conditions for the second round PCR were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. For amplification of the distal 5'-flanking region, multiplex PCR was performed from 25 ng of genomic DNA using 1 unit of Ex-Taq (Takara Bio. Inc.) with 0.4 uM each of the 2 sets of primers as shown in Table 1 [PCR]. The PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min.

Following the PCR, products were treated with a PCR Product Pre-Sequencing Kit (USB Co.,

Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the sequencing primers listed in Table 1 (Sequencing). Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany), and the eluates were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All variations were confirmed by sequencing PCR products generated from new amplifications from genomic DNA. Genbank NT_030059.12 was used as the reference sequence.

Linkage disequilibrium (LD) and haplotype analyses

Hardy-Weinberg equilibrium and LD analyses were performed using SNPAlyze 3.1 software (Dynacom Co., Yokohama, Japan). Pairwise LDs were shown as rho square (r^2) and |D'| values in Figure 1. Diplotype configurations (haplotype combinations) were inferred by LDSUPPORT software, which determined the posterior probability distribution of diplotype configurations for each subject based on estimated haplotype frequencies [19].

Results and Discussion

In this study, sixty-one *ABCC2* genetic variations including 36 novel ones were detected in 236 Japanese subjects (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 Fig. 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 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 nonsysnonymous 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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Figure legends

Figure 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.

Figure 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".

Table 1. Primer sequences used in this study

Amplified or sequenced region	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplified region[a]
PCR (Ex-taq)			
5'-Flanking (for -1.9k to -1.7k)	CCACCAGTGCCAAGAGAAGTAT	CACAAGTCATCTGGAAAACACA	20289134-20289443
5'-Flanking (for -1.7k to -950)	ATGAGGTGGTATCTAACTGTGG	AAATGTTTTCTGTAGGGACGGG	20289392-20290182
1st PCR (Z-taq)			
5'-Flanking (for -1.2k) to exon 6	ATACTGCATGGGTGGTTATG	AACCTGCCTCCAAATTTTTC	20289942-20303347
Exons 7 to 11	GGAGAATCACTTTGAAGCCG	CTAGCAAGTGTGAGGGGTGT	20304874-20314079
Exons 12 to 19	TCTGTGAATGTGGCAAAACT	GGATCTACCAAGAATTTAGC	20315189-20328004
Exons 20 to 25	GATGAGCATTTTCAATTTAC	TCAGTTCACCCAGCACTTAT	20338211-20344941
Exons 26 to 32	GAGCAAGACCTTGTCTCATA	CCATGGATGAATCTCAGATA	20349821-20360334
2nd PCR (Ex-taq)			
5'-Flanking (for -880 to -130)	GGAAGATCGCTTGAACCCAT	TCATCCCAACCATTTAATCG	20290245-20290994
Exon 1	TTGTTGGCCAGCTCTGTTG	TTCTGGTTCTTGTTGGTGAC	20290810-20291254
Exon 2	GGGTAAGGCTGGATATGGAT	CTGGCTCTACCTGAGACAAT	20292767-20293194
Exon 3	CACCGGAAACCATTCTGTTC	TTTGCCTCACTATGGATCCC	20300442-20300773
Exon 4	GCCAGATTAGTCACGACAGT	CCAAAGGAAGTCTACATGGCC	20301708-20302134
Exon 5	CAGGTAAGGAAAAAAAGAGTGG	CCTTGTCATAAAATGGTCTG	20301966-20302418
Exon 6	TATGCCAGAAAATCTGATTA	AGGTGGAACATGAGCTTGAGT	20302499-20303070
Exon 7	GGTGGAGATAGCCTCTGACC	TGCACTGAGAAGTATGAAGTGC	20305320-20305728
Exon 8	CCTGTACAGAGAAGGCCACG	TGCGGTCTTCATGAACACAA	20307385-20307816
Exon 9	GGCTTTGGACAATTCTGGTC	TCCACCCATTGTCTGTGAAC	20308539-20309038
Exon 10	AGGCAAGAAGTCACAGTGCC	TTGCCCAAACTCCCATTAAG	20312158-20312650
Exon 11	ACAGTCAGGCAAGGGCTATG	GACAGGAGGACATGAAACAA	20313420-20313873
Exon 12	GATTTCTATTCCCCACATTT	GAGCTGGGGGTATGGTACAA	20315554-20315983
Exon 13	GTGACCTTGGAGAAGATATT	CTCTTGAAAGTTTACCAGCA	20316189-20316623
Exon 14	TTGCTCAAGGACTGAAATAG	CCTGCTTATCCTCAGAAGAG	20318223-20318732
Exon 15	GGTCTCATGGTCTCATTCTA	GGGTTTATCCTGCACTAGTA	20319650-20320025
Exon 16	AGAAGCACTTTGGGGTCTTGTA	GCTGAAATGGGAAGGAGAATC	20321144-20321581
Exon 17	GCTGAAAAACGATAGTCCAA	TCAACTAGATTACCCCTGTGT	20325354-20325863
Exons 18 and 19	TCACAGGGTGACAAGCAAC	TTGAATCTCTGGGTAGTTTG	20326820-20327678
Exon 20	GAAACCAGCAAGATCAGAGGA	TCACTCAGCTGGCATCAAAG	20338493-20338929
Exon 21	TGACTGTGACATCTGCTTGC	GGACAGAGGACATATTGCTCC	20338927-20339248
Exons 22 and 23	GCATTGTATTTCAGCATTGT	ACAGTGTTGTCTAGGGGGAC	20339701-20340506
Exon 24	GAACACACAGAATCCAACAGA	TCACTTCAGCTTCAGACAGT	20342562-20343001
Exon 25	TCTCATTGGTCTCCTCCTCG	AATTTCACACCACTAGCCAT	20344186-20344672
Exon 26	GAGGCATTGCCTAAGAGTGC	AAAGATGGAGCCAGGGTTTG	20350122-20350523
Exons 27 and 28	GGCAAGGATTGTCTTTCTTA	CGACAGCTGCGGTAAGTCTG	20351928-20352954
Exon 29	AGAGATGGAGTAGCCAGTCAC	CAGCCACAAATGCATATTACC	20353790-20354262
Exon 30	GAAGCTCAACCACAAACCAG	GCTCGACCAGTTTTCAAGAG	20355106-20355610
Exon 31	GCAAGGTACAGCTAGTTGAA	GCGTGATGTAAAATTTTGGC	20358730-20359248
Exon 32	GCTGTGGCTCATTGATTTTC	AAGGTGATAAAACAGAAATG	20359651-20360213