tumors irrespective of the p53 status and produces anti-tumor effects (29). Yang et al. reported that ONYX-015 killed MPM cells bearing the wild-type p53 gene yet lacking the $p14^{ARF}$ gene (26), which suggested a clinical possibility of ONYX-015 for MPM treatment.

MPM cells express anti-apoptotic genes such as Bcl-2 and Bcl-XL, which resulted in the resistant to radiation or anti-cancer agents. Mohiuddin et al. demonstrated that treatment of MPM cells with the Ad encoding pro-apoptotic molecules, BAK, BAX, together with sodium butyrate, a chemical known to down-regulate Bcl-2 and Bcl-XL expression, induced cell death (30). Melanoma differentiation-associated gene-7 (mda-7) is a cytokine (known as interleukin-24) with the ability to induce tumor cell apoptosis through multiple mechanisms and the MDA-7-mediated apoptosis was influenced by the BCL-2 expression in MPM cells (31). Thus, regulation of the expression balance between the anti-apoptotic and the pro-apoptotic genes is another strategy for MPM-targeted gene therapy. The major drawback of the replacement gene therapy is the limited efficacy, inducing cell death only in infected cells.

(2) Other candidates of gene therapy

Activation of host immune systems against tumor-specific or tumor-associated antigen(s) that exclusively or highly expressed in MPM cells is an approach for the prevention and the treatment. Recently, Hassan et al. demonstrated that mesothelin could be a target for immunotherapy (32). Mesothelin, a glycosylphosphatidyl inositol-linked membrane-bound and soluble protein, is a differentiation antigen expressed in normal mesothelial cells and non-MPM tumors such as ovarian and pancreatic cancers. Soluble mesothelin was found in 84 % of MPM patients but in only

2 % for non-MPM patients. Measurement of mesothelin protein in the blood or pleural effusion could be used for differential diagnosis between MPM and non-MPM tumors and for monitoring the clinical courses and the treatment efficacy (32, 33). Mesothelin will be a tumor-associated antigen, although not specific to MPM, and a target molecule for CTLs. Identification of the peptide sequences that bind to the class I and the class II molecules of the major histocompatibility complex enables us to develop a vaccine for the prevention and hopefully for the treatment. Antibody against mesothelin can be used for antibody-mediated cytotoxic activities (32).

Survivin can be a candidate for MPM gene therapy. Survivin is an anti-apoptotic protein that inhibits activation of the caspase cascade and the expression is linked with cell growth. Elevated expression of the survivin gene was demonstrated in many types of cancer including MPM. Xia et al. showed that down-regulated survivin expression in MPM cells with anti-sense oligonucleotides increased caspase 3 up-regulation and induced apoptosis (34). The transcriptional regulatory region of the survivin was also tested for activation the E1 genes of Ad and such replication-competent Ad modified with a surivin promoter region destroyed tumor cells with little toxicity to non-tumorous tissues (35).

Blocking angiogenesis for feeding vessels has been always a central aim for cancer therapy. MPM cells produce angiogenic factors such as vascular endothelial growth factor (VEGF) and also express its receptor VEGFR1, R2 and R3 (36). The level of VEGF is associated not only with angiogenesis but the cell proliferation rate and in fact increased VEGF expression levels were observed in serum and pleural effusion of the MPM patients. Li et al. showed the therapeutic effects of anti-VEGF antibody (Bevacizmab) in an orthotopically implanted animal model (37). Since epidermal

growth factor receptor (EGFR) is over-expressed in MPM, an inhibitor of EGFR tyrosine kinase can be beneficial. Although gefitinib, one of the inhibitors, failed to alter the patient's prognosis in a clinical trial (38), other possible EGFR inhibitors which are currently under development might produce anti-tumor effects with different clinical treatment protocols. Antibody-derived medicine and molecular-targeted chemicals, even if they are not effective alone, can be clinically applicable in the combination with gene therapy.

(3) Future directions

A number of clinical trials of gene therapy have been performed for cancer treatment but none of the gene medicine has yet approved in the Western society. Recently, two agents, Ad expressing the wild-type p53 gene and Ad defective of E1B 55 kDa gene (similar to ONYX-015) have become clinically available in China but these agents were not tested for MPM patients in a clinical setting. There are obviously several hurdles in current gene therapy to be overcome for better therapeutic effects. The most important issue is efficient gene delivery to tumors. Several polycation agents such as polybrene, poly-L-lysine, DEAE-dextaran and protamine improve Ad-mediated transduction in vivo (39). Expression levels of Ad receptors also play a crucial role in the transduction efficacy. The primary receptor is the coxsackievirus and adenovirus receptor (CAR) and the expression levels are often down-regulated in human tumors including MPM. Such tumors are resistant to Ad-mediated gene transfer and a large amount of Ad are required for the efficient transduction, increasing a risk for adverse reactions. Modification of the fiber-knob portion which binds to Ad receptors will improve the gene transfer. For example, insertion of RDG sequences in the H loop portion increases the

integrin-mediated Ad transduction and replacement of the fiber -knob portion with that of subtype B-derived Ad changes the Ad tropism and enables CD46-mediated Ad infection. Several lines of experiments showed that the expression level of CD46 was not down-regulated in human tumors in contrast to CAR.

Repeated Ad administration induces cell- and antibody-mediated immune responses to Ad, which hampers subsequent Ad-mediated gene transfer. Production of the neutralizing antibody occurred even when Ad was administered in the pleural cavity. Although intratumoral injection of Ad did not inhibit the gene transduction in the presence of such neutralizing antibody, the generation of CTLs for Ad cannot be beneficial to patients. Immunosuppressive agents could inhibit the host responses but are essentially unfavorable to cancer patients. It is thereby difficult to modulate the anti-Ad immunity.

Currently the efficacy of gene therapy is not optimal for a therapeutic modality and it is wise to use gene therapy in combination with a conventional therapy or as an adjuvant therapy. Since radiotherapy is not an option for MPM treatment, chemotherapy combined with gene therapy would be a choice. The typical example is the combination of forced expression of p53 gene, such as with Ad expressing p53 gene, and DNA-damaging anti-cancer agents since most of MPM cells have intact p53-mediated signal pathways but lack the p53-mediated functions. We also presume that the most promising strategy is a combination with immune responses since the clinical outcomes of gene therapy for MPM suggests immune responses could inhibit MPM extension and maybe recurrence. Local destruction of MPM cells with Ad-mediated gene transduction or replication-competent oncolytic Ad will subsequently releases putative tumor antigen(s) and enhanced presentation of the tumor antigen(s) by DCs facilitates

cell-mediated immunity against MPM. Inflammatory reactions induced by Ad rather favor maturation of DCs, which activate a differentiation process of naïve T cells into type I helper T cells. An efficient linkage between tumor cell destruction and activation of immune systems will be a modality to be investigated further. Fortification of the antigen-presentation by modulating the pathways, activation of DCs and facilitation of cytokines-induced proliferation/activation of tumor-specific $CD4^+$ and $CD8^+$ T cells are possible subjects that can be performed with gene transduction and could be associated with viruses-mediated tumor cell death. Another point to enhance the host immune responses is to suppress the functions of regulatory T cells, which inhibit cell-mediated responses by secreting a number of suppressive cytokines such as TGF-β. Depletion of the T cell population with chemotherapeutic agents or with siRNA technology would increase effects of such immune therapy. We presume that increased MPM patient numbers prompt further investigations on gene therapy as a possible therapeutic strategy.

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Table 1 Clinical trials of gene therapy for MPM

Phase Study	Vector (dose)	Gene expressed	Route of administration (Reference)	Patient numbers	Clinical outcomes
I	Ad	HSVtk	intrapleural		
	($5x10^{10}$ - $5x10^{12}$vp)		(11)	13	PD: 12 persons
					Alive (>113.5 mo): 1
	($1.5x10^{13}$ - $5x10^{13}$vp)			21	PD: 19
					Alive (>79.5 mo): 2
I	Ad	IFN-β	intrapleural	8	Judged by
	($9x10^{11}$ - $3x10^{12}$ vp)		(9)		day 60 CT responses.
					SD: 3, PD: 5
					Immune response: 2
I	Ad infected allogenic cells	HSV-tk	intrapleural	16	Injected cells:
			(7)		Adherent to
	($1x10^{8}$ – $1x10^{10}$ cells)				MPM lining the chest
Pilot	Vaccinia	IL-2	intratumoral	6	No response
			(8)		

Clinical outcomes, progressive disease (PD) and stable disease (SD), are judged by the

measurable tumor sizes.

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Genetic Variations and Haplotypes of *ABCC2* Encoding MRP2 in a Japanese Population

Kimie SAI[1,2,*], Yoshiro SAITO[1,2], Masaya ITODA[1], Hiromi FUKUSHIMA-UESAKA[1], Tomoko NISHIMAKI-MOGAMI[2], Shogo OZAWA[1,3,a], Keiko MAEKAWA[1,2], Kouichi KUROSE[1,4], Nahoko KANIWA[1,4], Manabu KAWAMOTO[5], Naoyuki KAMATANI[5], Kuniaki SHIRAO[6,b], Tetsuya HAMAGUCHI[6], Noboru YAMAMOTO[6], Hideo KUNITOH[6], Yuichiro OHE[6], Yasuhide YAMADA[6], Tomohide TAMURA[6], Teruhiko YOSHIDA[7], Hironobu MINAMI[8,c], Yasuhiro MATSUMURA[9], Atsushi OHTSU[10], Nagahiro SAIJO[11] and Jun-ichi SAWADA[1,2]

[1]*Project Team for Pharmacogenetics,* [2]*Division of Functional Biochemistry and Genomics,*
[3]*Division of Pharmacology,* [4]*Division of Medicinal Safety Science,*
National Institute of Health Sciences, Tokyo, Japan
[5]*Division of Genomic Medicine, Department of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan*
[6]*Division of Internal Medicine, National Cancer Center Hospital*
[7]*Genomics Division, National Cancer Center Research Institute, National Cancer Center, Tokyo, Japan*
[8]*Division of Oncology/Hematology,*
[9]*Investigative Treatment Division, Research Center for Innovative Oncology,*
[10]*Division of GI Oncology/Digestive Endoscopy,*
[11]*Deputy Director, National Cancer Center Hospital East, Chiba, Japan*

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SNP Communication

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[1]*Project Team for Pharmacogenetics,* [2]*Division of Functional Biochemistry and Genomics,*
[3]*Division of Pharmacology,* [4]*Division of Medicinal Safety Science,*
National Institute of Health Sciences, Tokyo, Japan
[5]*Division of Genomic Medicine, Department of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan*
[6]*Division of Internal Medicine, National Cancer Center Hospital*
[7]*Genomics Division, National Cancer Center Research Institute, National Cancer Center, Tokyo, Japan*
[8]*Division of Oncology/Hematology,*
[9]*Investigative Treatment Division, Research Center for Innovative Oncology,*
[10]*Division of GI Oncology/Digestive Endoscopy,*
[11]*Deputy Director, National Cancer Center Hospital East, Chiba, Japan*

Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: 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.

Keywords: ABCC2; MRP2; genetic variation; haplotype; amino acid change

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*To whom correspondence should be addressed: Kimie SAI, Ph.D., *Division of Functional Biochemistry and Genomics, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel: +81-3-3700-9478, Fax: +81-3-3707-6950, E-mail: sai@nihs.go.jp*

[a]Present address: Department of Pharmacodynamics and Molecular Genetics, Faculty of Pharmaceutical Sciences, Iwate Medical University, Iwate, Japan.

[b]Present address: Department of Medical Oncology, OITA University Faculty of Medicine, Yufu, Japan

[c]Present address: Medical Oncology, Department of Medicine, Kobe University Hospital and Graduate School of Medicine, Kobe, Japan.

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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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 (Ile 1324Ile) 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 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°C for 5 sec, 55°C for 5 sec, and 72 °C 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 DyeEx-96 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 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 −1.9 k to −1.7 k)	CCACCAGTGCCAAGAGAAGTAT	CACAAGTCATCTGGAAAACACA	20289134–20289443
5′-Flanking (for −1.7 k to −950)	ATGAGGTGGTATCTAACTGTGG	AAATGTTTTCTGTAGGGACGGG	20289392–20290182
1st PCR (Z-taq)			
5′-Flanking (for −1.2 k) 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
Sequencing			
5′-Flanking (for −1.7 k)	CCACCAGTGCCAAGAGAAGTAT	CACAAGTCATCTGGAAAACACA[b]	
(for −1.7 k to −1.3 k)	GGTATCTAACTGTGGTTTTG	GAAGGAAAGGAGTCAAAGGAAC	
(for −1.5 k to −950)	TCCCACACTGAATGCTGCCTTT	TAGGGACGGGGTCTCACTAT	
(for −880 to −400)	GGAAGATCGCTTGAACCCAT[b]	ATGTGCAGTTTCGCTTCTG	
(for −570 to −130)	CATATAGGCTCACACTGGAT	TCATCCCAACCATTTAATCG[b]	
Exon 1	TGGTTCCTTTTATGTATGGC	GTTCTTGTTGGTGACCACCC	
Exon 2	AAAGCAGTGGGATGTGCTG	TGTCTCTACTGTGCACCAAGG	
Exon 3	CACCGGAAACCATTCTGTTC[b]	TTTGCCTCACTATGGATCCC[b]	
Exon 4	CCTCCTTTCTTCCCATGTTC	CTCAACTTGATGCCATTTAC	
Exon 5	TGGGGCAACCTCTAACTCATA	TGAGACCCAGACATCTTAAA	
Exon 6	TTAGGGTCTCCAAATAAACA	ACTTTCAGAGGAGTGAGAGAGT	
Exon 7	GGTGGAGATAGCCTCTGACC[b]	TGCACTGAGAAGTATGAAGTGC[b]	
Exon 8	CCTGTACAGAGAAGGCCACG[b]	CACAATGCTGTAAGGTTAAG	
Exon 9	GGCTTTGGACAATTCTGGTC[b]	TCCACCCATTGTCTGTGAAC[b]	
Exon 10	GTGCCTTGGAGAAGCTGTGT	TTGCCCAAACTCCCATTAAG[b]	
Exon 11	TCACTGGGCACCTCAAGTTC	GGAATCCATCACCTCTACCA	
Exon 12	ACATTTTGGGGACTATATCT	ATGCCAGCTAGTCTATCAAA	
Exon 13	GGAGGCTGGATGATCCTTAAG	CTCTTGAAAGTTTACCAGCA[b]	
Exon 14	CATCTGTCTATGGTGGGATA	ATAGGCTCAAGACAAATCTC	
Exon 15	GATTTCATTCACCTCCTGTT	CATTTCCCCATGCATTCTAT	
Exon 16	CCAATCTTGAGGGGAAATCT	TCCAAGACCTCACCTACTAGC	

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.

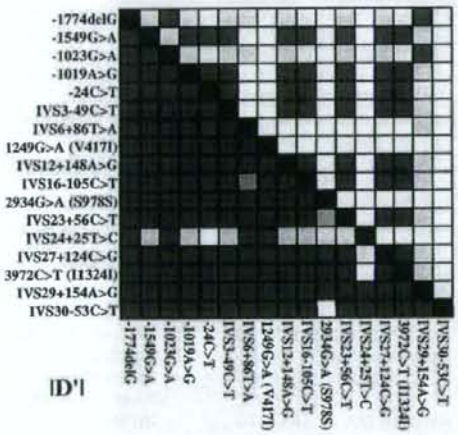

Fig. 1. Linkage disequilibrium (LD) analysis of *ABCC2*
Pairwise LD (r^2 values and |D′|) of polymorphisms detected in no less than 3% of allele frequencies is shown as a 10-graded blue color.

(**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*				5′-Flanking	20289538	−1590	ttaaatttgttaG/Atgtatgtttgct		0.002
MPJ6_AC 2079			8, 10, 17	5′-Flanking	20289579	−1549	tcottatagtatG/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*				5′-Flanking	20290395	−733	acagtttctagcG/Tactgatgccacc		0.004
MPJ6_AC 2029				5′-Flanking	20290395	−733	acagtttctagcG/Aactgatgccacc		0.002
MPJ6_AC 2030*				5′-Flanking	20290715	−413	ttgcagcagaagC/Tgaaactgcacat		0.002
MPJ6_AC 2003		ssj0000371	9, 12, 15–18, 20, 26	Exon 1	20291104	−24	tagaagagtcttcC/Tgttccagacgca		0.174
MPJ6_AC 2004			18	Exon 1	20291105	−23	agaagagtcttcG/Attccagacgcag		0.006
MPJ6_AC 2031		ssj0000386	17, 26	Intron 3	20301785	IVS3 −49	ctccccctcagtcC/Tccggttagtggc		0.203
MPJ6_AC 2032*				Intron 6	20302837	IVS6 +86	tatttttattatT/Attttttgagat		0.076
MPJ6_AC 2033*				Exon 7	20305479	732	caagtttgaaacG/Acacatgaagaga	Thr244Thr	0.002
MPJ6_AC 2066*				Intron 7	20307421	IVS7 −69	tcacaggctgacC/Gaccctggagctg		0.002
MPJ6_AC 2067*				Intron 7	20307423	IVS7 −67	acaggctgaccaC/Acctggagctgct		0.002
MPJ6_AC 2035*				Exon 9	20308814	1177	ggtgtaaaagtaC/Tggacagctatca	Arg393Trp	0.002
MPJ6_AC 2068*				Exon 9	20308839	1202	tggctctgtatA/Gtaagaaggtaag	Tyr401Cys	0.002
MPJ6_AC 2036*				Intron 9	20308859	IVS9 +13	gtaagcagaatcC/Tggcaggtatcac		0.002
MPJ6_AC 2037*				Exon 10	20312319	1227	gacccctatccaaC/Tttggccaggaag	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*				Intron 11	20315600	IVS11 −67	taaaacatgggtG/Agatcagatacac		0.002
MPJ6_AC 2038		ssj0000390	26	Intron 12	20315952	IVS12 +148	ccgcccccatgccA/Gttttcctcctctt		0.210
MPJ6_AC 2039*				Intron 13	20318344	IVS13 −73	tcatggactaacG/Aacaaagtcaaaa		0.002
MPJ6_AC 2070*				Intron 14	20318515	IVS14 +14	taaataaatttgG/Taagttgcttccc		0.002
MPJ6_AC 2040*				Intron 14	20318521	IVS14 +20	aatttggaagtt(del/ins)[a]cagcaaactga		0.002
MPJ6_AC 2071*				Intron 14	20318594	IVS14 +93	agcaaactgagaG/Tagagtgtggaga		0.002
MPJ6_AC 2041*				Intron 14	20319757	IVS14 −62	cggagagagacaC/Tgtgagggcagac		0.002
MPJ6_AC 2042*				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*				Intron 15	20321170	IVS15 −131	gtcttgtatatcC/Gaaggcaaatttt		0.004
MPJ6_AC 2045*				Intron 16	20325422	IVS16 −169	tgagtcctgagA/Tgtggaataacta		0.004
MPJ6_AC 2046		ssj0000396	17	Intron 16	20325486	IVS16 −105	tgcacagttattC/Taaatttaagctc		0.214
MPJ6_AC 2072*				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*				Intron 19	20327555	IVS19 +3	gaagccacaggtA/Gtgtaagaaggat		0.002
MPJ6_AC 2047*				Intron 19	20327645	IVS19 +93	agtatccagtgaA/Tctagatttggaa		0.002
MPJ6_AC 2048				Intron 20	20338745	IVS20 +29	gctggcagcccctC/Agtcagctctata		0.002
MPJ6_AC 2049*				Exon 21	20339052	2801	ccttgaaaactcG/Agaatgtgaatag	Arg934Gln	0.002
MPJ6_AC 2015		ssj0000398	8, 18, 26	Exon 22	20339944	2934	aggattgttttcG/Aatattcttcatc	Ser978Ser	0.040
MPJ6_AC 2050*				Exon 22	20340061	3051	cgactatccagcA/Gtctcagagggac	Ala1017Ala	0.002
MPJ6_AC 2051*				Exon 23	20340337	3181	cacaagcaacgC/Tgtgacaatatcc	Leu1061Leu	0.002
MPJ6_AC 2052		ssj0000399	17, 26	Intron 23	20340470	IVS23 +56	ggatctttctgaC/Taggaggaatta		0.222
MPJ6_AC 2074*				Exon 24	20342724	3320	ttacatgcttccT/Gggggataatcag	Leu1107Arg	0.002
MPJ6_AC 2053				Intron 24	20342843	IVS24 +25	atggctaagtcaT/Ccctcctctctc		0.030
MPJ6_AC 2075*				Intron 24	20342880	IVS24 +62	agcccagcctctT/Ctcctgagaatct		0.002
MPJ6_AC 2054				Intron 24	20342926	IVS24 +108	cactcactcctcC/Tcctcagcagctt		0.023
MPJ6_AC 2055*				Intron 24	20344318	IVS24 −56	agaaaggaggaaG/Aatgtggatgcc		0.002
MPJ6_AC 2056*				Intron 26	20352061	IVS26 −21	atgatgattttcA/Gtctctggttt		0.002
MPJ6_AC 2057*				Intron 27	20352227	IVS27 +44	ggcaaaaacaacA/Gtgcaactcctc		0.008
MPJ6_AC 2058		ssj0000404	17, 26	Intron 27	20352307	IVS27 +124	aaagttccttttC/Gctctaactccaa		0.222
MPJ6_AC 2076			26	Exon 28	20352688	3927	ccaagtgcggtaC/Tcgacctgagctg	Tyr1309Tyr	0.002
MPJ6_AC 2022		ssj0000407	8, 12, 13, 17, 18, 20, 26	Exon 28	20352733	3972	cacttgtgacatC/Tggtagcatggag	Ile1324Ile	0.216
MPJ6_AC 2059*				Intron 28	20352920	IVS28 +172	agggaaggtagC/Tagccagggatca		0.004
MPJ6_AC 2060*				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	ctttttcggcatG/Aagcccccaacagc		0.015
MPJ6_AC 2063*				Intron 30	20358793	IVS30 −92	gggggttttgaA/Gagtctgatctgg		0.008
MPJ6_AC 2064		IMS-JST185750		Intron 30	20358832	IVS30 −53	ccccctgccctgC/Tgtcttcctttgg		0.051
MPJ6_AC 2077*				3′-UTR	20359975	*61[b]	taatttattttT/Gtataaaatacag		0.002
MPJ6_AC 2065*				3′-Flanking	20360190	*193 + 83[b]	ttattcctttgcC/Gtttcatttctgt		0.002a8

*Novel genetic variation

[a]delGCTTCCCAAACTTATTCGCAGTACTGGTGCCAGAATTTTGATAATACAAGAGCTTAGTAGAinsTATTTACCT

[b]Numbered from the termination codon.

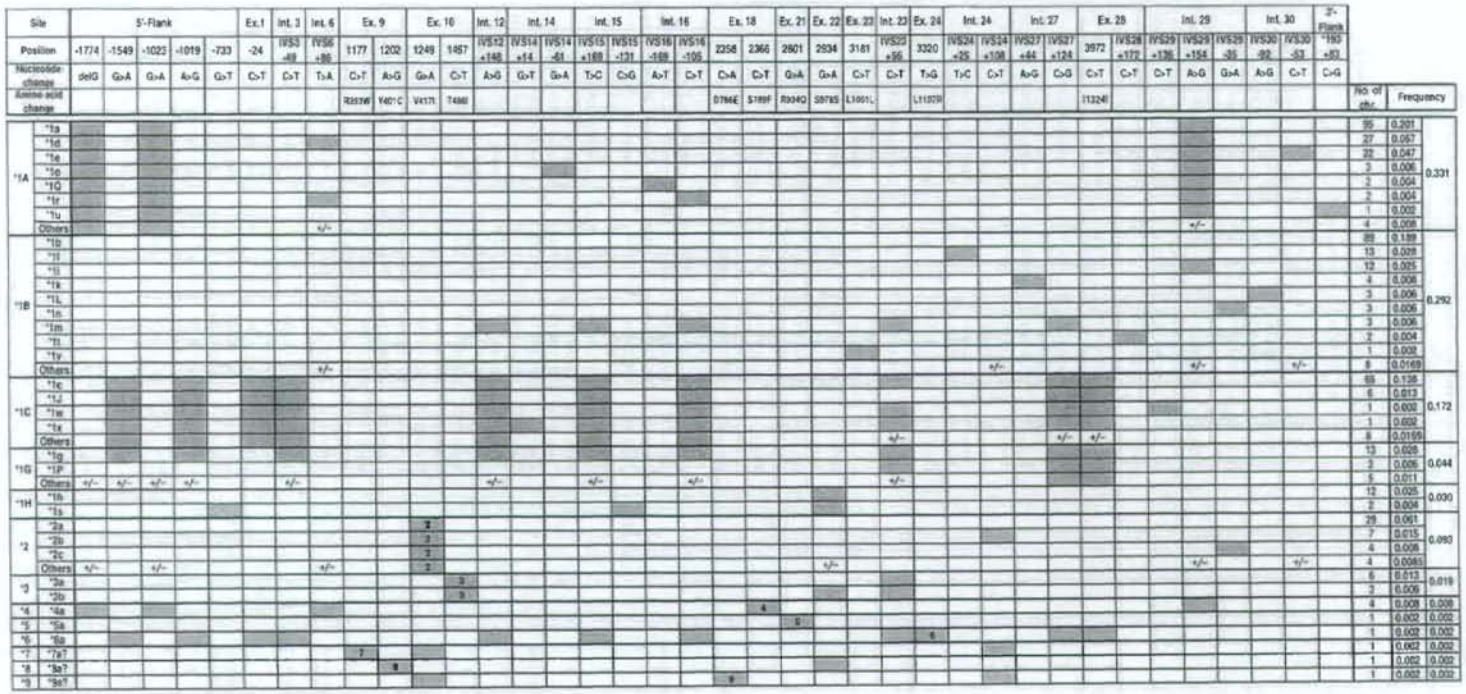

Fig. 2. *ABCC2* haplotypes in 236 Japanese subjects

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