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Haplotype-oriented genetic analysis and functional assessment of promoter variants in the *MDR1 (ABCB1)* gene

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Promoter variants and expression of human *MDR1* gene

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Abstract

Recently, a number of nucleotide variants have been described in the multidrug resistance 1 (*MDR1/ABCB1*) gene; however most studies have focused on the coding region. In the present study, we identified promoter variants of the *MDR1* gene, and evaluated their phenotypic consequences using a reporter gene assay and the real-time PCR method. Ten allelic variants were detected in the promoter region (approx. 2 kb), seven of which were newly identified. Certain mutations occurred simultaneously, and a total of ten haplotypes were observed. These promoter polymorphisms were found more frequently in Japanese than Caucasians. Some haplotypes were associated with changes in luciferase activity, and placental and hepatic mRNA levels. We also determined DNA methylation status in the proximal promoter region of the *MDR1* gene. The promoter region around potential binding sites for transcription factors was found to be hypomethylated, and thus likely to be independent of the gene expression. Nucleotide and/or haplotype variants not only in the coding region but also in the promoter region of the *MDR1* gene may be important for interindividual differences of P-glycoprotein expression.

Polymorphisms in the genes encoding membrane transporters have recently been reported to be associated with variations in the pharmacokinetic and pharmacological effects of clinically used drugs (Fromm, 2002; Kim et al., 2002; Takane et al., 2003). Among various drug transporters, P-glycoprotein, the multidrug resistance 1 (*MDR1/ABCB1*) gene product, is one of the best studied. P-glycoprotein is expressed in various human tissues such as the intestine, liver and kidney, and functions as a cellular efflux pump for foreign xenobiotics and endogenous substrates.

Although a number of nucleotide variants have been described in the *MDR1* gene, most studies in this area have focused on the association of single nucleotide polymorphisms (SNPs) in the coding region with the altered expression of P-glycoprotein or pharmacokinetics of clinically used drugs. Hoffmeyer et al. (1999) demonstrated that the synonymous C3435T polymorphism (Ile1145Ile) in exon 26 was associated with a low level of expression of P-glycoprotein in the duodenum, resulting in an increase in plasma concentrations after oral administration of digoxin, used as a probe for P-glycoprotein. In contrast, higher level of duodenum P-glycoprotein expression and lower level of serum digoxin after oral administration were observed in the subjects with this variant (Sakaeda et al., 2001; Nakamura et al., 2002). The association of the C3435T polymorphism with P-glycoprotein protein expression and function is controversial. Up to now, various investigators have reported that the variant

is associated with decreased or increased expression, or has no clearly discernible effect (Sparreboom et al., 2003; Ishikawa et al., 2004). In a recent report, an approach using gene-based haplotypes, which are specific combinations of SNPs located throughout the genome, proved superior to the use of individual SNPs for predicting the association between phenotypes and genomic variation (Judson et al., 2000). For example, Drysdale et al. (2000) reported that the bronchodilator response to β agonist is significantly related to haplotype pairs of the β_2-adrenergic receptor gene but not to individual SNPs. With regard to the *MDR1* gene, Johne et al. (2002) indicated that it was important to consider the variability in haplotype structure rather than in SNPs when characterizing the *MDR1* phenotype. However, the association of variants in the promoter region of the *MDR1* gene with the expression of P-glycoprotein has been not well investigated.

DNA methylation, referred to as the methylation of cytosine in a cytosine-guanosine pair (CpG), is the most common eukaryotic DNA modification and one of many epigenetic (an alteration in gene expression without a change in nucleotide sequence) phenomena (Singal and Ginder, 1999). Normally, both the core promoter and transcriptional start site are included within the CpG-rich region, and DNA methylation regulates gene expression by interfering with the binding of specific transcription factors to their recognition sites (Singal and Ginder, 1999; Jones and Takagi, 2001). Interestingly, the human *MDR1* gene has a CpG-rich promoter region. Hypomethylation

of *MDR1* during chemotherapy resulted in a high level of gene expression in recurrent tumors, and had important consequences for clinical outcome in acute myeloid leukemias (Nakayama et al., 1998) and bladder cancer (Toda et al., 2000). However, there is currently no data available on the role of DNA methylation in transcriptional regulation in normal tissues.

The aim of this study was to describe variants in the promoter region of *MDR1* in Japanese and Caucasian populations, and evaluate their functional significance with regard to transcriptional activity and mRNA expression in placentas and livers obtained from Japanese subjects. Furthermore, we determined the methylation status of the promoter region of *MDR1* and its association with the interindividual variability in gene expression in normal tissue.

Materials and methods

Isolation of genomic DNA and RNA.

Human full-term placentas (highly enriched placental trophoblast populations) and livers were obtained from 96 and 19 Japanese patients, respectively. These tissues were immediately frozen in liquid nitrogen and stored at -80 ℃ for the preparation of DNA and RNA. Blood samples were obtained from 96 healthy Caucasian volunteers. Genomic DNA from the samples was prepared using the Toyobo blood kit on a Toyobo HMX-2000 robot (Toyobo, Osaka, Japan). The isolation of genomic DNA from tissues was performed using a DNA preparation kit (QIAamp DNA Mini Kit, QIAGEN GmbH, Hilden, Germany). Total RNA was extracted using ISOGEN (Nippongene, Tokyo, Japan), and reverse transcription (RT) was performed with random hexamers (Promega, Madison, WI) and reverse transcriptase (Life Technologies, Rockville, MD). This study was approved by the Tottori University Ethics Committee, and informed consent was obtained from all individuals.

Identification of variants in the *MDR1* promoter region.

The genotypes of *MDR1* such as A-41aG, C -145 G, T -129 C and C3435T were identified by PCR-RFLP analysis as described previously (Tanabe et al., 2001). To identify unknown mutations in the *MDR1* promoter region, SSCP analysis was performed using the GenePhor system (Amersham Pharmacia Biotech AB, Uppsala,

Sweden) as described previously (Ieiri et al., 2000). PCR was performed in a total volume of 25 μl consisting of 50 ng of genomic DNA, 10 ×PCR buffer II, 1.5 mM $MgCl_2$, 1.25 U Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and 0.25 μM of each primer. The primer sets were designed to divide the promoter region (-1700a to Ex1/+88) of the *MDR1* gene (GenBank accession number; AC002457) into 5 fragments (~500 bp). After an initial denaturation at 94°C for 5 min, 45 cycles of 40 sec at 94°C, 45 sec at 50 to 59°C and 1 min at 72°C, as well as a final extension period of 5 min at 72°C, were performed.

Haplotype analysis.

A 2112 bp fragment including the promoter region of *MDR1* (-1700a to Ex1/+88) was amplified by using gene-specific primers (5'-GGAGCAAAGAAATGGA ATACAATA-3' and 5'-TTCTCCCGTGAAGACCAAGTTC-3'). The PCR mixture was essentially the same as for the identification of mutations except for the Taq polymerase (LA Taq; Takara, Shiga, Japan). After an initial denaturation at 94°C for 5 min, 45 cycles of 40 sec at 94°C, 15 sec at 58.3°C and 1 min at 72°C, as well as a final extension period of 5 min at 72°C, were performed. The PCR fragments were subcloned into pGEM-T easy vector (Promega, Madison, WI) and sequenced.

DNA sequence.

All PCR products were sequenced directly on an ABI 377 automatic sequencer (Applied Biosystems) using a Big-Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). Prior to the sequencing, reaction mixtures were purified with a DyeEx Spin kit (QIAGEN GmbH). The sequencing primers were those used in the PCR amplifications. The sequencing of both strands was performed for products from at least two independent PCR amplifications to ensure that the identified mutations identified were not PCR-induced artifacts.

Real-time quantitative PCR (TaqMan) analysis.

PCR was performed using a master mix based on the TaqMan® universal PCR master mix (Applied Biosystems) and run on the ABI PRISM 7000 sequence detection system (Applied Biosystems). The following primers and TaqMan probe were used for determining the MDR1 mRNA: forward primer, 5'-TATCAGCAGCCCACATCATCA T-3', reverse primer, 5'-CCAAATGTGACATTTCCTTCCA-3') and probe, 5'-TACAG CACGGAAGGCCTAATGCCGA-3'. The endogenous reference gene was determined using the commercially available human GAPDH TaqMan® PreDevelopped Assay Reagent (Applied Biosystems). Each primer set and TaqMan probe were used at final concentrations of 200 nM and 100 nM, respectively. The reactions were run in duplicate.

For all experimental samples, the amount of mRNA was determined from a standard curve (serial diluted samples from placental tissue expressed at higher levels of MDR1 and GAPDH mRNA). The mRNA level of MDR1 was expressed as a ratio to that of GAPDH.

Plasmid construction.

To obtain the first plasmid, a 2056 bp fragment (-1704a to Ex1/+28) of *MDR1* including the promoter region and exon 1, was initially amplified from genomic DNA with gene-specific primers incorporating 5'-*Kpn*I and 3'-*Nhe*I, for the 5'-end of inserts 5'- CGGGGTACCGGAGCAAAGAAATGGAATACA-3' and for the 3'-end of inserts 5'-CTAGCTAGCAGTAGCTCCCAGCTTTGCGTG-3'. The PCR fragment was subcloned into the pGEM-T easy vector, and then introduced into competent JM109 cells (Promega). The plasmids obtained were sequenced and digested with *Kpn*I and *Nhe*I. The digested fragment was ligated into the *Kpn*I/*Nhe*I site of the vector pGL3-enhancer (Promega). Manipulated DNA portions were sequenced again in their entirety.

Cell culture and transfection.

HepG2 human hepatoma cells were incubated at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. One

day before transfection, cells (5.5×10^5) were seed into culture plates (60 mm). The cells were washed 2 times with serum-free medium. The luciferase reporter gene constructs (5 μg) and the control reporter gene plasmid pRL-TK vector (0.5 μg)(Promega) were mixed with the Tfx-20 reagent (15 μl)(Promega), transferred to serum-free medium, and then incubated at room temperature for 15 min. The mixtures were added to the washed cells and incubated for 1 hr at 37°C under 5% CO_2. After the incubation, the cells were cultured in growth medium, and harvested after 48 hr.

Assay of luciferase activity.

Luciferase reporter gene activity was evaluated with the Dual luciferase reporter assay system (Promega). HepG2 cells were washed once with a phosphate-buffered saline solution and lysed in passive lysis buffer (400 μl). After incubation at 37°C for 15 min, lysates were mixed in a vortex blender for 15 sec and centrifuged at 4°C for 30 sec. Supernatants (20 μl) were mixed with the luciferase reagent (100 μl), and the luciferase activity was measured with a luminometer (Turner Designs, Sunnyvale, CA). After background correction (activity in untreated cells), results were expressed as the level of pGL3 activity divided by pRL activity. The total cellular protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

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Electrophoretic mobility shift assay (EMSA).

Nuclear extracts from HepG2 cells were prepared as reported previously (Takeuchi et al., 2000). Oligonucleotides for the *MDR1* gene including –1517a T (ACTGTTTAGGGAGGGTTTAAGGCCATTCAAA), -1517a C (ACTGTTTAGGGAGGGCTTAAGGCCATTCAAA), -1459a G (ATAAATGAAAGGTGAGATAAAGCAACAAAGC), -1459a A (ATAAATGAAAGGTGAAATAAAGCAACAAAGC), -1017a T (GAGGCAGGAGAATGGTGTGAACCCGGGAGGC), -1017a C (GAGGCAGGAGAATGGCGTGAACCCGGGAGGC), -145 C / –129 C (CTTTGCCACAGGAAGCCTGAGCTCATTCGAGTAGCGGCTCTTCCAAG), -145 G / -129 T (CTTTGCCACAGGAAGGCTGAGCTCATTCGAGTAGCGGCTCTTCCAAG) and -145 C / -129 C (CTTTGCCACAGGAAGCCTGAGCTCATTCGAGCAGCGGCTCTTCCAAG) were synthesized with both sense and antisense strands, the corresponding pairs of which were annealed and end-labeled with T4 polynucleotide kinase (Takara) and [γ-^{32}P]dATP (Amersham Pharmacia Biotech AB) according to standard methodology. The [γ-^{32}P]-labeled probe (1×10^4 cpm) was incubated for 30 min at 0°C with nuclear protein (5 μg) in binding buffer (10 μl) containing 25 mM Hepes (pH 7.9), 40 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 7.5% glycerol, 1 mM DTT, 0.5 mM PMSF, 0.1% NP-40, 5 μM ZnSO4, 0.2 μg poly (dI-dC) and 1 μg bovine albumin. Competitor oligonucleotides were added at 100-fold molar excesses. Reaction mixtures were electrophoresed on 5% polyacrylamide and 2.5% glycerol gel in a Tris-glycin-EDTA

buffer (250 mM Tris, 1.9 M glycin and 10 mM EDTA) at 4°C and visualized by a BAS-2500 Imaging Analyzer (Fuji Film, Tokyo, Japan).

Determination of methylation.

The methylation status of CpG sites within the proximal promoter region of *MDR1* was confirmed using the bisulfite sequencing method (Frommer et al., 1992). DNA was treated with sodium bisulfite using a CpGenome DNA modification kit (Intergen, Purchase, NY) according to the manufacturer's instructions. PCR (578 bp, -214a to Ex1/+40) was performed in a total volume of 25 µl consisting of 50 ng of bisulfite modified genomic DNA, 0.625 to 2.5 U of Amplitaq Gold DNA polymerase and 0.25 µM of each primer; 5'-AAGGTGTTAGGAAGTAGAAAGGT-3' and 5'-AAA CTATCCCATAATAACTCCCAA-3'. After an initial denaturation at 95°C for 5 min, 35 cycles of 45 sec at 95°C, 45 sec at 55°C and 1 min at 72°C, as well as a final extension period of 5 min at 72°C, were performed. The PCR product was cloned into the pGEM-T easy vector according to the manufacturer's instructions. The CpG methylation status of individual DNA strands was determined based on a comparison with the sequence obtained from the genomic DNA without the addition of bisulfite modifications. The number of methylated CpGs at a specific site was divided by the number of clones analyzed (N > 15) to yield percent methylation for each site.

Statistical analysis

The 95% confidence interval was calculated to compare the differences in genotype or haplotype frequencies between Japanese and Caucasians. Results of MDR1 mRNA expression and mutation (C3435T) were analyzed with a Kruskal-Wallis test. Comparisons between two groups were performed using a Mann-Whiteney U-test. A 5% level of probability was considered to be significant.

Results

Identification of variants in the promoter region of the *MDR1* gene

Ten variants were detected in the promoter region of the human *MDR1* gene by PCR-SSCP analysis using DNAs obtained from unrelated Japanese and Caucasian subjects (Fig. 1 and Table 1). Seven variants, at positions -1517a, -1459a, -1423a, -1132a, -1017a, -824a and -755a, were newly identified in this study. The most common mutation in Japanese was G-1459aA (allelic frequency: 0.250), whereas A-41aG (0.106), T-1517aC (0.080), T-1017aC (0.080), T-129C (0.080) were found at low frequency. The 5-base deletion at position -1132a to -1128a, C-145G and T-824aC were detected at extremely low frequency (0.037, 0.032 and 0.005, respectively). The frequencies of T-1017aC and T-129C were significantly lower in Caucasians than Japanese ($P < 0.05$). The 2-base deletion at position -1423a to -1422a and A-755aG were identified only in Caucasian subjects, but at frequencies below 0.010. In contrast, T-1517aC, G-1459aA, a 5-base deletion at position -1132a to -1128a, T-824aC, A-41aG and C-145C were not detected in Caucasian subjects. These results indicate that genotypic frequencies of variants in the promoter region of the *MDR1* gene appeared to be dependent on race.

Identification of *MDR1* promoter haplotypes

On the basis of a haplotype analysis using subcloning and direct sequencing, 10 haplotypes derived from all identified promoter variants were found to be present in

both populations (Table 2). In Japanese, 7 haplotypes were identified with a frequency ranging from 0.005 to 0.665. Unlike in Caucasians, three variants at -1517a, -1017a and -129 occurred simultaneously in Japanese. In total, 13 different haplotype pairs were found in the subjects examined (Table 3). In Caucasians, the most common haplotype pair was 1/1 (0.923). In contrast, hetero- or homogenous combinations of haplotypes with one or more variant sites were found at a relatively high frequency in Japanese compared with Caucasians (0.553 versus 0.077).

Association of *MDR1* promoter haplotypes with mRNA expression in placenta and liver

Prior to investigating the influence of *MDR1* promoter haplotype combinations on mRNA expression in placental and hepatic tissues, we determined whether the C3435T variant influences MDR1 mRNA expression. As shown in Fig. 2, the synonymous C3435T polymorphism in exon 26 was associated with a low level of placental MDR1 expression ($P < 0.05$; Kruskal-Wallis test). Next, we compared *MDR1* promoter haplotype pairs (haplotypes 1/1, 1/2, 1/3, 1/4 and 4/4) with corresponding placental and hepatic MDR1 levels in 29 and 11 samples with the 3435 C/C and C/T genotype, respectively (Fig. 2). The MDR1 expression in placental tissue with haplotype 1/2 or 1/3 tended to increase compared with that in 1/1 samples ($P = 0.091$; Mann-Whitney U-test; Fig. 2). However, mean mRNA levels in hetero- and

homogygous samples for haplotype 4 was comparable with those in 1/1 samples. Also, the MDR1 expression in hepatic tissue of haplotype 1/2 or 1/3 tended to increase when compared with that in 1/1 samples (P = 0.07; Mann-Whitney U-test; Fig. 2).

Luciferase reporter gene assay

To investigate the influence of promoter haplotypes on the potential for transcriptional regulation, 10 reporter plasmids containing *MDR1* promoter sequences were transiently transfected in HepG2 cells, and then luciferase activities were measured. As shown in Fig. 3, haplotypes 2, 3 and 9 increased the luciferase activity by 41, 32 and 30%, respectively, when compared with that by haplotype 1. In contrast, the haplotype 6 construct resulted in a 28 % reduction in activity. Other haplotypes did not appear to influence the activity.

Binding of nuclear proteins to the promoter variant sites

To determine whether the variants in the promoter region altered binding for transcription factors, we performed EMSA using nuclear extracts prepared from HepG2 cells. By competition assays using an excess of unlabeled probe, allele-specific binding of nuclear proteins was observed when the nuclear extracts were incubated with probes including -1517a C (complex I) and -1459a G (Complex II) (Figs. 4A, B). Also, strong nuclear protein-DNA binding (complex III) was observed with the probe containing the

-1017a T allele when compared with the -1017a C allele (Fig. 4C). The higher binding completely disappeared under an excess of unlabeled -1017a T probe, and weaker inhibition of the binding was observed with the -1017a C probe. With the -145/-129 C/T and G/T probes, nuclear protein-DNA binding (complex VI) was detected, but with the C/C probe it was not detected or was much weaker (Fig. 4D). The protein-DNA binding was completely inhibited by an excess of unlabeled C/T and G/T probes, and slightly competed with by an excess of unlabeled C/C probe. No nuclear protein-DNA binding was observed when the probes containing the -41a A/G and -145 G/C alleles were incubated with nuclear extracts.

Correlation between placental MDR1 mRNA expression and methylation status at CpG sites in the *MDR1* promoter region

The proximal region of the human *MDR1* is rich in CpG (Fig. 5A). This region including the Y box and GC box elements is required for activation of the *MDR1* promoter. We focused on the CpG-rich proximal promoter region in the *MDR1* gene, and determined the relationship between methylation status at each CpG site and MDR1 mRNA expression using placenta with promoter haplotype 1/1 and 3435 C/C genotypes. Results of a bisulfite sequencing analysis of 26 CpG sites in 7 subjects, whose MDR1 levels varied considerably, are shown in Figure 5B. In all samples, methylated CpG sites were found upstream of the promoter region. Moreover, an interindividual difference in

methylation status was observed; however, no clear association was observed. In addition, methylation was not observed around either the Y box or GC box element in most samples.

Discussion

Despite evidence supporting an association of coding SNPs with pharmacokinetics and pharmacodynamics, little is known about the presence or functional relevance of allelic variants in the promoter region of *MDR1*. We described ten polymorphic variants in the *MDR1* promoter in Japanese and Caucasian populations. A-41aG, C-145G and T-129C have been detected in the proximal promoter region of the *MDR1* gene as low frequency variants (Horinouchi et al., 2002; Tang et al., 2002; Kroetz et al., 2003). Here, we identified another seven variants. Their presence and frequency varied according to race. For example, C-145G was identified in Asian-Americans but not Caucasians (Kroetz et al., 2003). In the present study, this variant was observed only in Japanese. In addition, the new G-1459aA variant was the most frequent variant in Japanese (25.0 %), but was not found in Caucasians.

The promoter region of the *MDR1* gene has been isolated and sequenced (Ueda et al., 1987; Madden et al., 1993). The promoter has an initiator sequence at the transcriptional start site, without a TATA box (van Groenigen et al., 1993). Numerous studies have shown that the Y box (inverted CCAAT box) and GC box, recognized by the transcription factors NF-Y and Sp1, respectively, are required for efficient transcriptional regulation of the *MDR1* promoter (Goldsmith et al., 1993; Sundseth et al., 1997). Several studies suggest that a region upstream of the Y box negatively regulates

the *MDR1* promoter activity (Ogura et al., 1992; Cornwell and Smith, 1993), although the exact positions of the negative element differ. However, our variants are not located within those *cis*-elements.

We identified ten different *MDR1* promoter haplotypes using subcloning and direct sequencing methods. A comparison of *MDR1* haplotype pairs with placental and hepatic expression showed that haplotypes 1/2 and 1/3 were associated with increased mRNA expression, independent of the C3435T mutation in the coding region. Interestingly, haplotypes 2 and 3, in which T-1517a C, T-1017aC and T-129C mutations occurred simultaneously in both populations, were associated with an increase in transcriptional activity in human hepatoma cell line. Moreover, we showed that the T-1517aC, T-1017aC and T-129C variants affected putative transcriptional protein-DNA binding. Heterozygosity for the -129 C allele is associated with a high level of transport activity of P-glycoprotein in hematopoietic stem cells (Calado et al., 2002). The tacrolimus oral dose requirement is higher in renal transplant recipients with the T/C allele than T/T allele at position -129 (Anglicheau et al., 2003). Although these findings are not significant because the T-129C variant was rare, the C allele at position -129 may be associated with high level of expression of P-glycoprotein, resulting in an increase in transport activity and then a decrease in tacrolimus bioavailability after oral administration. In contrast to these haplotypes, haplotype 6 (G-1459aA and C-145G

variant) was associated with a low level of transcriptional activity in hepatoma cells. We found that the G-1459aA variant inhibits the binding of unknown transcriptional factor to DNA. Therefore, allelic variants not only in the coding region but also in the promoter region of the *MDR1* gene, may be important to the interindividual difference in P-glycoprotein expression.

We determined the interindividual difference in methylation status in the proximal promoter region of the *MDR1* gene using 7 placentas whose mRNA levels varied considerably. Extensive methylation of the *MDR1* gene assembled into chromatin enriched with methylated CpG binding protein interfered with the binding of transcription factors to their elements, resulting in transcriptional repression of the gene (Jin and Scotto, 1998; El-Osta et al., 2002). In this study, the region upstream of the promoter region was relatively well methylated, but individual differences in methylation status were unlikely to cause large variation in MDR1 mRNA expression. Nakayama et al. (1998) reported that methylation at CpG sites near the Y box was important for *MDR1* gene expression, and was associated closely with clinical outcome in acute myeloid leukemia. However, we did not find any methylation around either the Y box or GC box element. Similar results for methylation status in the promoter region have been reported in CD8-positive cells (Fryxell et al., 1999). The element Sp1 protects the promoter from *de novo* methylation (Brandeis et al., 1994). Macleod et al.

(1994, 1998) have suggested that the presence of a functional element within the GC-rich domain maintains the methylation-free status. Accordingly, although the proximal promoter of the *MDR1* gene is important for regulating basal gene expression in normal human tissues, epigenetic status appears not to be associated with variability in the transcriptional activity of the *MDR1* gene.

In conclusion, we identified various variants in the promoter of the *MDR1* gene, and investigated their effects on transcriptional activity and mRNA expression in the placenta. Among these variants, the promoter haplotypes containing T-1517aC, T-1017aC and T-129C were particularly associated with high level of transcriptional activity and mRNA expression, but independently of the coding SNP C3435T. Since these promoter haplotypes or SNPs are found at a relatively low frequency in Japanese and Caucasian populations, further study is needed to establish the impact of their allelic variants on drug disposition and responses in clinical situations. Interestingly, one study has shown a possibility of regulation by tissue-specific factors for the *MDR1* gene expression (Kohno et al., 1990). Also, Sundseth et al. (1997) have reported that an element just upstream of the Y box had opposite effects in different human carcinoma cell lines. Their results suggest that allelic variants in the promoter region of the *MDR1* gene contribute to the polymorphic expression of P-glycoprotein in a tissue-specific manner. Future studies may need to estimate the influence of *MDR1* promoter variants

on transcriptional activity in various P-glycoprotein-expressing tissues.

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Footnotes;

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Legends for figures

Fig. 1 Nucleotide sequence of the human *MDR1* gene promoter. Positions of nucleotide variants are in bold and are underlined. The exons are boxed. The location of variants in the coding region is relative to the initiation site for translation, which is defined as +1 based on the cDNA nucleotide sequence. The position in the promoter region is relative to the nucleotide sequence immediately preceding exon 1, which is defined as -1a.

Fig. 2 Influence of *MDR1* promoter haplotypes and SNPs in the coding region on placental and hepatic MDR1 mRNA levels in Japanese. Statistical significance between the two genotypes was analyzed with the Mann-Whiteney U-test.

Fig. 3 *MDR1* promoter region reporter gene constructs and their relative luciferase activity levels. The value for the haplotype 1 construct was set at 100 %. Each value is the mean ± S.D. of relative luciferase activity from 4 - 5 experiments.

Fig. 4 EMSA analysis with oligonucleotides corresponding to variants (A: T-1517aC; B: G-1459aA; C: T-1017aT; and D: C-145G and T-129C) in the *MDR1* gene promoter. The nuclear extracts (NE; 5 μg protein) from HepG2 cells were incubated with

^{32}P-labeled oligonucleotides. Specificity of nuclear protein binding was demonstrated by a 100-fold molar excess of unlabeled oligonucleotide. Data are representative of three similar experiments.

Fig. 5 A) Location of CpG sites in the *MDR1* promoter region. The CpG sites are represented by short vertical bars. B) Methylation status of individual sites of the *MDR1* promoter region and mRNA expression in placental tissues with the promoter haplotype 1/1 and 3435 C/C genotypes.

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Table 1 Variants in promoter region of the *MDR1* gene in Japanese (n = 94) and Caucasian (n = 96) subjects

Location	Position	Allele	Nucleotide sequence	Allele frequency		Genotype	Frequency	
				Japanese	Caucasians		Japanese	Caucasians
Promoter	-1517a[1)]	T [3)]	agggTttaa	0.920	1.000	T/T	0.840	1.000
				(0.881-0.959)[4)]		T/C	0.160	0.000
		C	agggCttaa	0.080	0.000	C/C	0.000	0.000
				(0.041-0.119)				
	-1459a[1)]	G [3)]	gtgaGataa	0.750	1.000	G/G	0.553	1.000
				(0.688-0.812)		G/A	0.394	0.000
		A	gtgaAataa	0.250	0.000	A/A	0.053	0.000
				(0.188-0.312)				
	-1423a[1)]	GA [3)]	cagaGAtcat	1.000	0.995	GA/GA	1.000	0.990
					(0.985-1.005)	GA/ -	0.000	0.010
		deletion	caga_tcat	0.000	0.005	- / -	0.000	0.000
					(-0.027-0.015)			
	-1132a[1)]	CCATC [3)]	aagaCCATCctgg	0.963	1.000	CCATC/CCATC	0.926	1.000
				(0.936-0.990)		CCATC/ -	0.074	0.000
		deletion	aaga_ctgg	0.037	0.000	- / -	0.000	0.000
				(0.01-0.064)				
	-1017a[1)]	T [3)]	atggTgtga	0.920	0.984	T/T	0.840	0.969
		C	atggCgtga	(0.881-0.959)	(0.966-1.002)	T/C	0.160	0.031
				0.08	0.016	C/C	0.000	0.000
				(0.041-0.119)	(-0.002-0.034)			
	-824a[1)]	T [3)]	attaTggct	0.995	1.000	T/T	0.989	1.000
				(0.985-1.005)		T/C	0.011	0.000
		C	attaCggct	0.005	0.000	C/C	0.000	0.000
				(-0.027-0.015)				
	-755a[1)]	A [3)]	agtgAtttt	1.000	0.995	A/A	1.000	0.990
					(0.985-1.005)	A/G	0.000	0.010
		G	agtgGtttt	0.000	0.005	G/G	0.000	0.000
					(-0.027-0.015)			
	-41a[1)]	A [3)]	cccaAtgat	0.894	1.000	A/A	0.798	1.000
				(0.850-0.938)		A/G	0.191	0.000
		G	cccaGtgat	0.106	0.000	G/G	0.011	0.000
				(0.062-0.150)				
	-145[2)]	C [3)]	gaagCctga	0.968	1.000	C/C	0.936	1.000

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				(0.943-0.993)		C/G	0.064	0.000
		G	gaagGctga	0.032	0.000	G/G	0.000	0.000
				(0.007-0.057)				
Exon 1	-129[2)]	T [3)]	cgagTagcg	0.920	0.984	T/T	0.840	0.969
		C	cgagCagcg	(0.881-0.959)	(0.966-1.002)	T/C	0.160	0.031
				0.080	0.016	C/C	0.000	0.000
				(0.041-0.119)	(-0.002-0.034)			

1) Position is relative to the initiation site of exon 1a, which is defined as +1a.

2) Position is relative to the initiation site of translation, which is defined as +1.

3) Reference sequence GenBank accession number AC002457.

4) The 95% confidence intervals are given in parentheses

Table 2. Localization of variants and identification of the *MDR1* promoter haplotypes in Japanese and Caucasian subjects

Nucleotide	-1517a	-1459a	-1423a	-1132a	-1017a	-824a	-755a	-41a	-145	-129	Frequency	
	T/C	G/A	GA/ delete	CCATC/ delete	T/C	T/C	A/G	A/G	C/G	T/C	Japanese	Caucasians
Haplotype											*n = 94*	*n = 96*
1	T	G	GA	CCATC	T	T	A	A	C	T	0.665 (0.598-0.732)*	0.964 (0.937-0.991)
2	**C**	G	GA	CCATC	**C**	T	A	**G**	C	**C**	0.043 (0.014-0.072)	0.000
3	**C**	G	GA	**delete**	**C**	T	A	**G**	C	**C**	0.037 (0.010-0.064)	0.000
4	T	**A**	GA	CCATC	T	T	A	A	C	T	0.191 (0.135-0.247)	0.000
5	T	**A**	GA	CCATC	T	T	A	**G**	C	T	0.027 (0.004-0.050)	0.000
6	T	**A**	GA	CCATC	T	T	A	A	**G**	T	0.032 (0.007-0.057)	0.000
7	T	G	GA	CCATC	T	**C**	A	A	C	T	0.005 (-0.005-0.015)	0.000
8	T	G	**delete**	CCATC	T	T	A	A	C	T	0.000	0.010
9	T	G	GA	CCATC	**C**	T	A	A	C	**C**	0.000	0.016
10	T	G	GA	CCATC	T	T	**G**	A	C	T	0.000	0.010

* The 95% confidence intervals are given in parentheses

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Table 3. Haplotype configurations in promoter region of the *MDR1* gene in Japanese and Caucasian subjects

Promoter haplotype pair	Frequency	
	Japanense (n = 94)	Caucasians (n = 96)
1/1	0.447 (0.376-0.518)*	0.923 (0.885-0.961)
1/2	0.043 (0.014-0.072)	0.000
1/3	0.053 (0.021-0.085)	0.000
1/4	0.234 (0.173-0.295)	0.000
1/5	0.043 (0.014-0.072)	0.000
1/6	0.064 (0.029-0.099)	0.000
1/8	0.000	0.038 (0.011-0.065)
1/9	0.000	0.038 (0.011-0.065)
2/4	0.021 (0.001-0.0041)	0.000
2/5	0.011 (-0.004-0.026)	0.000
2/7	0.011 (-0.004-0.026)	0.000
3/4	0.021 (0.001-0.0041)	0.000
4/4	0.053 (0.021-0.085)	0.000

* The 95% confidence intervals are given in parentheses

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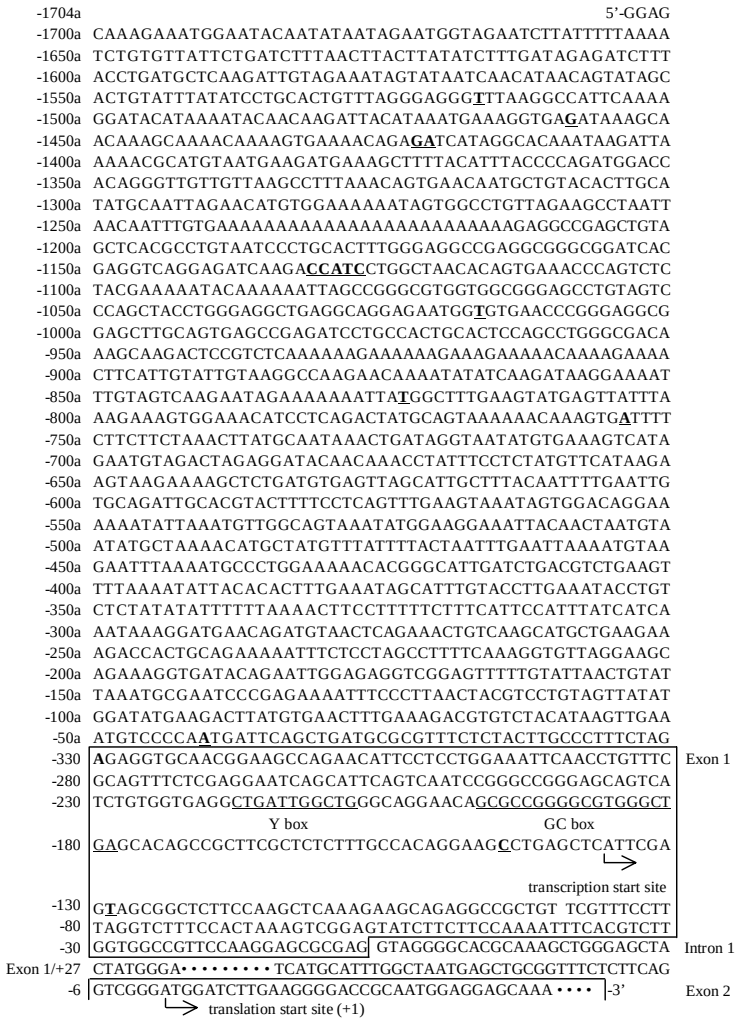

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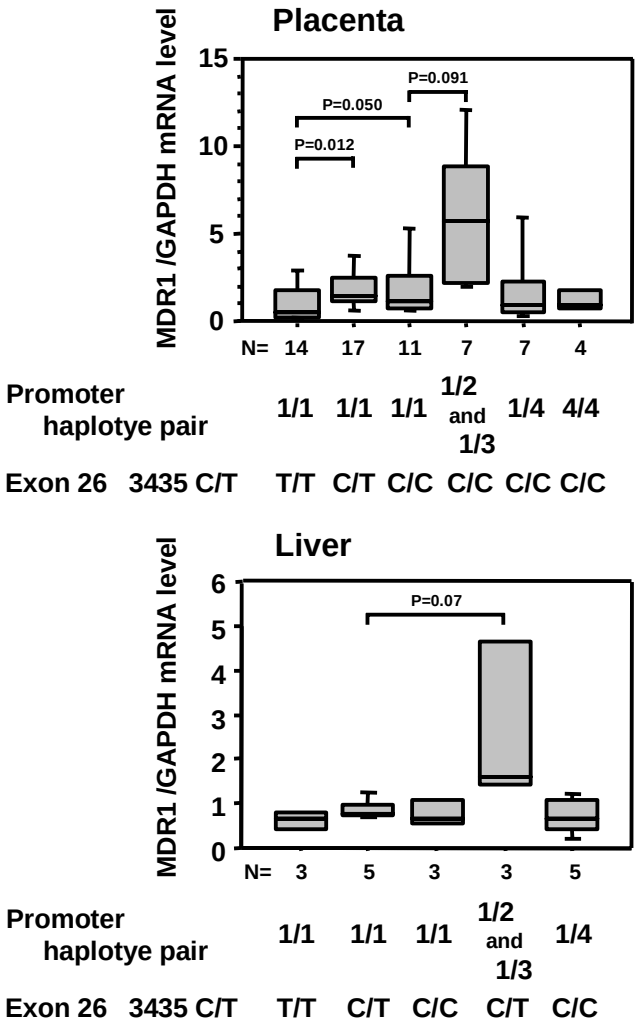

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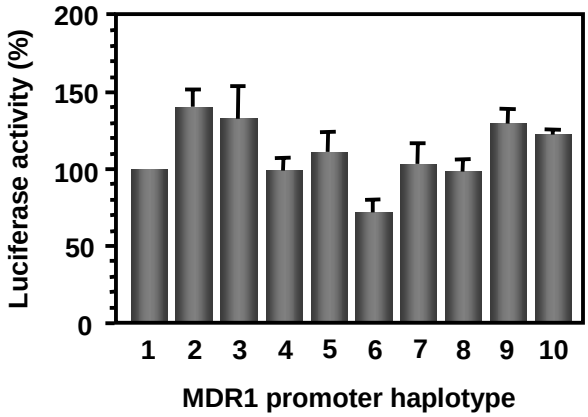

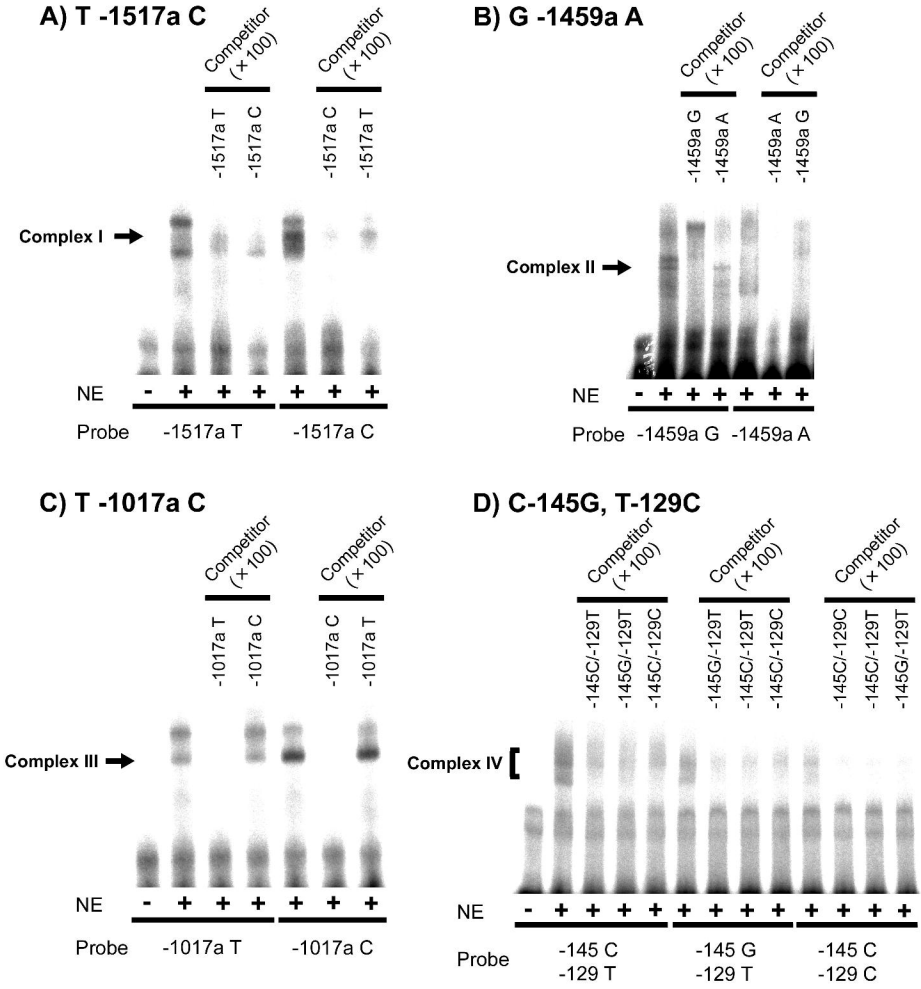
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B) G -1459a A
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Complex II
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C) T -1017a C
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-145C/-129T
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