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Differential effect of *ABCB1* haplotypes on promoter activity

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Abstract

Objective—Promoter single nucleotide polymorphisms (SNPs) of the *ABCB1* gene, encoding the placental efflux transporter P-glycoprotein, can affect its expression and alter xenobiotic transfer from the maternal to the fetal circulation. Because SNPs are arranged in specific combinations as defined haplotypes, the aims of this study were to: 1) determine the placental haplotype structure of the *ABCB1* promoter and 2) determine the differential effect of these haplotypes on placental *ABCB1* promoter activity.

Methods—DNA samples from 100 healthy placentas were PCR-amplified and sequenced to identify existing SNPs in the proximal *ABCB1* promoter. The haplotype structure encompassing these SNPs was inferred by PHASE analysis. Luciferase-reporter constructs representing these haplotypes were generated and transfected into human placental 3A cells and their effect on *ABCB1* promoter activity was determined using a dual-luciferase assay.

Results—We identified 12 *ABCB1* promoter SNPs. These SNPs were predicted by PHASE to segregate into 28 haplotypes with frequencies ranging between 0.019 to 0.88. We found 12 of these haplotypes in our population in addition to 2 haplotypes not predicted by PHASE. We also generated 2 haplotypes to determine individual SNP effects for a total of 16 studied. Compared with the ancestral haplotype, 4 haplotypes significantly up-regulated (107% to 266% increase; $P < 0.05$), 1 significantly down-regulated (95.4% decrease; $P < 0.01$), and 11 had no statistically significant effect on *ABCB1* promoter activity.

Discussion and Conclusion—Our data show that the effect of SNPs on promoter activity depends on their presence in a specific haplotype. This indicates that haplotypes, rather than individual SNPs, could play a significant role in regulating placental P-glycoprotein expression and affect placental transfer and fetal exposure to xenobiotics.

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Conflict of Interest

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Keywords

ABCB1; P-glycoprotein; Promoter Haplotypes; Single Nucleotide Polymorphisms

Introduction

The growing use of medications during pregnancy [1] emphasizes the need for maximizing therapy for the mother while minimizing risks to her fetus. Further understanding of the mechanisms regulating drug transfer across the placenta is an important step toward this goal. Placental efflux transporters regulate drug transfer from the maternal to the fetal circulation. P-glycoprotein (P-gp), encoded by the *ABCB1* (*MDR1*) gene, is an important placental efflux transporter highly expressed in trophoblasts [2] that interacts with many compounds [3]. It actively extrudes its substrates from the trophoblasts back into the maternal circulation, thus limiting fetal exposure [4]. Therefore, variability in placental P-gp expression/activity poses a challenge to physicians treating pregnant women since it can significantly influence maternal and fetal exposure to prescribed medications.

We [5] and others [6,7] demonstrated interindividual variability in placental P-gp expression/activity. Though the underlying mechanisms are not well understood, such variability could largely be due to single nucleotide polymorphisms (SNPs) of the *ABCB1* gene, which could affect P-gp expression/function. A few *ABCB1* coding region SNPs (C1236T, rs1128503; C3435T, rs1045642, and G2677T/A, rs2032582) have been evaluated for their effect on P-gp expression/function. However, there is considerable discrepancy in the results, where increased, decreased, and non-significant effects have been reported [6–14]. Besides coding SNPs, other SNPs in the *ABCB1* promoter could alter P-gp expression by affecting *ABCB1* transcription. A few studies evaluated some of these SNPs individually but conflicting data were also reported [7,15–19].

The controversial results are not surprising since SNPs are not arrayed individually, but rather form defined combinations or haplotypes with varying degrees of linkage disequilibrium. Thus, several SNPs, creating specific haplotypes, act in concert to provide the observed variability in response to an exposure. Recently, we reported the phenotypic effect of a SNP is not always consistent and varies depending on its presence within a specific haplotype [20,21]. Therefore, genotyping of a single or a few individual SNPs may fail to capture true functionality of genetic variants.

The haplotype structure of the *ABCB1* promoter is currently unknown, and their functional and biological significance remain elusive. We therefore comprehensively identified SNPs of the *ABCB1* promoter and determined the haplotype structure encompassing them. We then determined the effect of the haplotypes on *ABCB1* promoter activity. Our hypothesis is that promoter haplotypes, rather than individual SNPs, differentially alter *ABCB1* promoter activity and thus affect placental P-gp expression.

Materials and Methods

Placenta collection

Term placentas (38–41 weeks) were collected from 100 volunteers upon delivery in the Ob/Gyn Department at UTMB by research nurses according to a protocol approved by the UTMB Institutional Review Board (IRB #02-106). Volunteers were recruited without regard to age or race/ethnicity and signed a written informed consent form. Placentas collected were transported to our laboratory along with a de-identified data sheet with information on maternal age, self-reported race/ethnicity, health conditions (if any), current medications, gestational age and type of delivery. Placentas were excluded if there were pregnancy complications, pre-term delivery, documented drug abuse during pregnancy, intake of medications that are known P-gp substrates, or infection with HIV or hepatitis. DNA was isolated for *ABCB1* promoter amplification via PCR from a 100mg tissue section obtained from the fetal side of each placenta using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Inc., Valencia, CA).

ABCB1 promoter PCR amplification and SNP determination

The proximal *ABCB1* promoter was PCR-amplified using primers encompassing a 2050bp sequence upstream and 300bp downstream of the transcription start site, respectively [22]. PCR conditions were 95°C (20 seconds), annealing at 63°C (30 seconds), and extension at 68°C (50 seconds). Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Four sets of primers were designed (See Supplemental Table S1) for DNA sequencing on an ABI Prism™ 3130XL sequencer at the UTMB Molecular Genomics Core Facility. Sequences were assembled into contigs using DNA Baser version 4.16 (Heracle Biosoft S.R.L, Romania) as previously described [20]. Variants were determined using mixed-base calling with the Mutation Detection module of the DNA Baser software. The detection settings were: SNP scanning: 50, peak-area ratios: 30% and peak overlap: 50%. All called SNPs were manually verified, and complete sequences were manually verified for any non-called SNPs.

Haplotype inference

SNPs were identified by comparing the generated sequences to the reference *ABCB1* promoter sequence (Entrez GeneID: [5243](#), GenBank accession [NT_007933](#)) using DNA Baser. Composite genotypes were used to infer haplotypes by Bayesian statistics implemented in PHASE (Phylogenetic and Sequence Evolution; <http://c4c.uwc4c.com/expresslicensetechnologies/phase>). Haplotype pairs were assigned to each placenta and their frequencies were determined.

ABCB1 promoter haplotypes luciferase reporter construct generation

To investigate the differential haplotype effects on *ABCB1* promoter activity, 16 reporter constructs were generated. These represented 14 haplotypes that were found in our population (see Results) in addition to 2 constructs we generated to assist in evaluating the effect of genetic variants on promoter activity.

To generate vectors containing the different haplotypes, PCR-amplified *ABCB1* promoter haplotypes were double digested with the restriction enzymes KpnI-HF and NheI-HF (New England Biolabs, Ipswich, MA) and ligated into a NanoLuc™ Luciferase pNL1.1 vector (Promega, Madison, WI). Then, 5α *E. coli* (New England Biolabs, Ipswich, MA) were transformed with the generated reporters and plated on 100 µg/ml ampicillin LB agar plates. Colonies were grown in LB broth with 100µg/ml ampicillin for 18–24 hours. Plasmids were isolated using the endotoxin-free ZR Plasmid Miniprep™ - Classic kit (Zymo Research Corp, Irvine, CA) and quantified at 260nm using a DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE). Plasmids were then sequenced to verify the haplotype. Isolated plasmids were stored at –20°C until transfection into human 3A trophoblast cells (CRL-1584, ATCC, Manassas, VA). The effect of the haplotypes on *ABCB1* promoter activity in 3A cells was determined using the manufacturer's protocol for the Nano-Glo® Dual-Luciferase® Assay System (Promega, Madison, WI).

Site-directed mutagenesis

To elucidate the effects of specific SNPs found in *ABCB1* haplotypes expressing increased and decreased activity, we utilized the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Site-Directed mutagenesis primers were designed according to the manufacturer's protocol, and utilizing the QuikChange Primer Design Tool (www.genomics.agilent.com). Mutant synthesis was performed with 100ng double-stranded promoter DNA in the pNL1.1 plasmid with an extension time of 3 minutes. Plasmids were then restriction-digested with DpnI for 30 minutes to remove template DNA. Then, 5α *E. coli* were transformed with the DNA as described above. Mutant colonies were confirmed by DNA sequence analysis.

Cell culture and haplotype constructs transfection

Human 3A placental cells were used as the host for haplotype construct transfection. Cells were maintained in 75cm² flasks with Minimal Essential Medium with Earle's Salts and L-Glutamine (Gibco Cat. 11095-080, Grand Island, NY) supplemented with 10% FBS in 5% CO₂ at 37°C. Cells were passaged at 85% confluency and sub-cultured at a 3:1 ratio. A solution of Trypsin-EDTA was used to detach the cells for sub-culture or transfer to 6-well plates for transfection. Transfections were performed between passages 6–8 with cells at low confluency (40%) with 2µL Fugene 6 (Promega, Madison, WI), 0.6µg promoter haplotype plasmid DNA, and 0.06µg of the constitutively active firefly luciferase plasmid pGL4.53 PGK (Promega, Madison, WI), which was used for in-well normalization of transfection efficiency. Transfection efficiency for 3A cells was determined using a green fluorescent protein plasmid (AcGFP1-C1) under these same transfection conditions. Cells were allowed to recover for 36–48h before harvest.

NanoGlo® Dual-Luciferase® Assay for determination of the effect of the different haplotypes on *ABCB1* promoter activity

The Nano-Glo Dual-Luciferase Reporter Assay (Promega, Madison, WI) was performed according to the manufacturer's protocol. Briefly, 3A cells were harvested using Passive Lysis Buffer 36–48 hours after transfection. Luciferase activity was determined by chemiluminescence in triplicate using a Tecan GenIOS Pro (Tecan, Durham, NC). To

account for variability in transfection efficiency, Nanoluciferase luminescence was normalized in-well against a co-transfected firefly luciferase. Each experiment was repeated at least three times.

***In silico* analysis of the *ABCB1* promoter region**

The putative transcription factor binding site predictor PROMO (ALGGEN Research Software <http://algggen.lsi.upc.edu>)[23,24] was used for *in silico* confirmation of reported cis-binding elements [15] and to predict potential transcription factor binding sites within the *ABCB1* promoter region. A map of the proximal promoter sequence with the identified SNPs with known and predicted binding sites was generated (see Results).

Statistical analysis

Genotype frequencies for each SNP were estimated from our 100 placenta samples and tested for deviation from Hardy-Weinberg equilibrium (HWE) by Chi-square and permutation tests, both implemented in the Linkage Disequilibrium Analyzer version 1.0 program[25]. Deviations from HWE were considered significant at $P < 0.05$ for either test. None of the SNPs identified deviated from HWE. Frequencies of haplotypes inferred from the PHASE analysis were used to determine haplotype frequencies. A Kruskal-Wallis One Way ANOVA was used to compare luminescence values corresponding to the different haplotypes to determine their effect on *ABCB1* promoter activity. Post-Hoc analysis using Dunn's Method was used to compare the luminescence values for the individual haplotypes against the ancestral haplotype. P-values < 0.05 were considered significant.

Results

***ABCB1* promoter SNPs and haplotype Inference**

In our study population, we identified a total of 12 out of 23 SNPs reported by the 1000 genome project [26] (Table 1). These SNPs had minor allele frequencies (MAF) ranging from 5% for the most frequent SNP (T-1017aC) to 0.5% for the least frequent SNPs. Some SNPs occurred at frequencies comparable to the 1000 genome project (A-1572aT, G-1459aA, T-1017aC, T-129C and A-43G), others were observed at frequencies 2 to 8 times higher (A-684aT, G-274aA and G-240A) or 2 to 4 times lower (T-1517aC, G-1157aA, A-41aG and C133A).

Bayesian statistics implemented in PHASE were then used to infer the haplotypes encompassing these 12 SNPs. PHASE analysis inferred 28 potential haplotypes of which 12 were found in our population with frequencies ranging from 0.5% to 88% (Table 2). Two additional haplotypes that were not inferred by PHASE (Haplotype 29, 30; Table 2) were also found in the study population. The ancestral haplotype 1 (-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C) was the most common (frequency of 88%) and was considered the reference haplotype for subsequent promoter activity comparisons. Within the study population, there were 6 haplotypes which were found only one time with a MAF of 0.5%. The 14 haplotypes identified formed 12 paired haplotype combinations. We evaluated the effect of the 14 haplotypes found in our population on promoter activity, as well as 2 additional haplotypes

that we generated (M1 and M4) to determine effects of individual SNPs found in specific haplotypes.

These SNPs were found in 2 haplotypes: one in haplotype 30 conferring decreased expression and the other in haplotype 29 conferring increased expression. Haplotype 30 is composed of two SNPs (G-1156A; rs28381797 and T-1017aC; rs28746504). To determine the effect of each SNP individually on promoter activity, two constructs would have to be available, each containing one of these 2 SNPs. SNP T-1017aC is found individually as haplotype 9. To determine the effect of G-1156A individually, we used site-directed mutagenesis to generate haplotype M4. Similarly, haplotype 29 is composed of two SNPs (G-1459aA; rs12720464 and T-129C; rs3213619). SNP G-1459aA is found individually as haplotype 16. To determine the effect of SNP T-129C individually, we created construct M1 using site-directed mutagenesis.

Effect of different *ABCB1* haplotypes on promoter activity

Each of the 16 haplotype sequences (14 observed in our population and 2 generated by site-directed mutagenesis) were ligated into pNL1.1 luciferase reporter plasmids. The luciferase reporter gene is driven by the inserted *ABCB1* haplotype promoter, allowing the promoter activity to be determined from luciferase chemiluminescence. Promoter haplotype NanoLuc plasmids were co-transfected into placental 3A trophoblast cells with a firefly luciferase containing plasmid to control for transfection efficiency.

Using this assay system, we found significant haplotype-dependent variations in luciferase activity. As shown in Figure 1, compared to the ancestral haplotype (haplotype 1), haplotype 30 showed a 94.4% decrease in promoter activity ($p < 0.01$), while haplotypes M4, 29 and 4 produced significant increases ($p < 0.05$) in promoter activity with 107.4, 247.4 and 290.2% increases, respectively. Other haplotypes evaluated showed differential effects on promoter activity, however the differences were not statistically significant.

Differential effects of SNPs in the context of haplotypes

Our data indicate the effects from individual SNPs on promoter activity depends on their presence in a specific haplotype. For example, the T-1017aC SNP is found in several haplotypes. When this SNP is found with the variant G-1157aA (haplotype 30), the promoter activity was dramatically reduced (94% reduction; $P < 0.01$). However, when this variant is found individually (haplotype 9), an increase in promoter activity of 48.8% relative to haplotype 1 was observed (Figure 1). When the effect of the G-1157aA SNP alone was tested (haplotype M4), the promoter activity was significantly increased to 107.4% of haplotype 1 ($p < 0.05$).

The only haplotype containing the G-240A variant is haplotype 4. This haplotype exhibited a significant increase in activity (300%; $P < 0.001$). Thus, this increase could be attributed to this specific variant. Haplotype 29, associated with a significant 250% increase in promoter activity, is composed of the G-1459A and T-129C SNPs. When T-129C was found in conjunction with another SNP (as in haplotype 10), the promoter activity was not significantly affected (reduced by 9% compared to haplotype 1). However, when the T-129C SNP was tested alone using haplotype M1 generated by site-directed mutagenesis, only a

slight non-significant increase in activity (13% compared to haplotype 1) was observed. It seemed plausible to assume that the G-1459A variant is responsible for that dramatic increase observed with haplotype 29 based on the T-129C data. However, when G-1459A is present alone (haplotype 16), a non-significant increase (~12%) in activity was observed compared to haplotype 1.

Discussion

We determined the haplotype structure encompassing the SNPs present in the *ABCB1* promoter region and investigated their effects on promoter activity. *ABCB1* promoter haplotype structures have been previously described for a Japanese (n=115) and a Caucasian (n=96) population [16]. In this study, Takane et al. described 10 SNPs/deletions in the *ABCB1* promoter, identifying 8 SNPs in Japanese and 2 additional SNPs in Caucasians. They reported the SNPs segregating into 10 haplotypes varying in frequency between Japanese and Caucasians.

In our investigation, we identified 12 SNPs but none of the reported insertions/deletions [16]. Moreover, we found 14 haplotypes of which 3 were reported by Takane et al. [16]: haplotype 1 and our haplotypes 10 and 16. However, the frequencies of these haplotypes varied considerably. In our study, the frequency of haplotype 1 was 88.1% compared to 66.5% and 96.4% for Japanese and Caucasians [16], respectively. Similarly, haplotype 10 was not found in the Japanese, but was found at a frequency of 1.6% in Caucasians [16] compared to 2.5% in our population. Haplotype 16 was found at a frequency of 19.1% in Japanese but was not found in Caucasians [16] and was 3.0% in our population. The discrepancy between our study and Takane et al. could be attributed to the different racial/ethnic backgrounds and the number of subjects evaluated within each population (Supplemental Table S2). In our study, PHASE predicted 28 possible haplotypes in our population, however we only observed 12 in addition to 2 that were not predicted. This could be attributed to the relatively small number of subjects we evaluated (n=100).

Consistent with our previous studies [20,27], our data indicate that evaluation of SNPs in the context of haplotypes is more accurate to determine their functional and biological effects. A case in point is the G-1157aA, which when found in haplotype 30, it produced a significant decrease in promoter activity, however, when G-1157aA is found alone (haplotype M4), the activity was significantly increased, indicating that single SNPs are not always the phenotype drivers. This corroborates our earlier observations involving the O-6-methylguanine DNA methyltransferase (*MGMT*) gene [20,21]. In these studies, we found the *MGMT* promoter SNP C575A (rs113813075) may have no effect in one haplotype but can have dramatic effects on *MGMT* expression when found in another haplotype. Our data, therefore, provide explanations for discrepant results of studies evaluating effects of the same SNP. For example, when considering the pharmacogenomic effects of the coding *ABCB1* SNP C3435T in the context of total body digoxin exposure, this SNP was reported to be associated with increased, decreased, and also with no effect on digoxin AUC [Reviewed in 28]. The differences observed could be due to the lack of consideration of *ABCB1* promoter haplotypes in these studies which could potentially increase or decrease P-gp expression, thus masking the effect of the coding SNP evaluated.

While there was some concordance between our study and Takane et al. [16] with respect to haplotypes structures, there were discrepancies in the biological effects observed. For example, while Takane et al. reported a 30% increase with haplotype 10, we found no significant change in promoter activity. This could be attributed to several factors including experimental differences and host cells used for the expression assays. In their studies, Takane et al. used HepG2 hepatoma cells for promoter expression assays while we used 3A placental cells. In fact, when we tested our same *ABCB1* haplotype constructs in U87MG glioblastoma cells, we observed differences in the activity of some promoter haplotypes compared to those observed with the 3A trophoblasts (data not shown). Wang et al. [29] reported differences in promoter expression when evaluating *ABCB1* promoter SNPs depending on cell lines used. Differences in expression in varying cell lines is not surprising and could be due to differences in transcription factors (TF) known to exist between cells [30].

The mechanisms underlying the differential *ABCB1* haplotype effects on promoter activity remains elusive. One mechanism may involve alterations in TF binding sites, resulting in differential TF binding and/or modification of cofactor recruitment[31–33]. *In silico* analysis of the *ABCB1* promoter predicted many putative binding sites for more than 80 TFs. Several putative sites are depicted in Figure 2. All SNPs identified in this study, with the exception of T-129C, were predicted to form or eliminate one or more putative TF binding site(s). Therefore, it is plausible to hypothesize that the observed expression changes are driven by SNPs-induced sequence alterations in close proximity or within TF binding motifs. Changes in these motifs may have different effects when SNPs are found together in specific haplotypes. Among the TFs binding sites predicted to be altered are GR- α , GR- β , C/EBP β , AhR, GATA-1, NF-1, and several others (Supplemental Table S3). Consistent with this hypothesis, changes in *ABCB1* promoter activity was observed after introducing mutations in promoter Sp1 binding sites [34]. Importantly, one Sp1 binding domain was found to have a repressive role and alteration of this site led to higher promoter activity [34]. This region contains the G-240A SNP found in haplotype 4, which could affect the repressive Sp1 regulation, possibly explaining our observed significant increase in activity with this haplotype. Alternatively, the G-240A variant could generate a higher affinity binding site for a transcription activator or alter the secondary structure of the DNA. Additional support to this hypothesis is provided by our previous work with *MGMT*[20,21]. In these studies, we observed haplotype-dependent TF binding profiles that significantly correlated with *MGMT* promoter activity [21].

Other possible mechanisms for the observed haplotypes-dependent promoter activity could involve non-cis-acting mechanisms. For example, it has been reported that SNPs can alter methylation patterns within gene promoters [35]. Therefore, haplotypes could alter methylation patterns, especially when SNPs occur in CpG rich regions. For *ABCB1*, there are 2–3 CpG islands [36,37] and variable interindividual levels of methylations have been observed [38]. Takane et al. demonstrated that methylation pattern variability within the *ABCB1* promoter led to differential expression of the gene [16]. However, the effects of haplotypes on the *ABCB1* promoter methylation pattern needs further investigation.

Another potential mechanism is allelic imbalance, which is a deviation from the expected equal expression from each individual allele. In studies addressing multiple genes, it was found that this ratio can deviate from the expected 1:1 up to 4.3:1 expression [39]. In studies specifically involving *ABCB1*, this phenomenon appears to favor the expression of an allele containing C3435T if present in the gene [40]. However, these studies evaluated only two SNPs in the distal promoter region and six tagging SNPs within the introns and exons but did not address the more commonly studied proximal *ABCB1* promoter.

In summary, we provided a detailed haplotype structure for the *ABCB1* promoter in a mixed ethnic/racial population. We demonstrated the effect of an individual SNP is not always consistent and differs in a haplotype-specific manner, indicating that *ABCB1* haplotypes, rather than individual SNPs, affect its expression and could thus play a significant role in the expression of placental P-gp. Our study underscores the need for future mechanistic investigations elucidating the multiple cis- and trans-acting effects of promoter haplotypes to better understand how they affect promoter activity and subsequent protein expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Mitchell AA, Gilboa SM, Werler MM, Kelley KE, Louik C, Hernández-Díaz S. Medication use during pregnancy, with particular focus on prescription drugs: 1976–2008. *Am J Obstet Gynecol*. 2011; 205:51.e1–51.e8. [PubMed: 21514558]
2. Young AM, Allen CE, Audus KL. Efflux transporters of the human placenta. *Adv Drug Deliv Rev*. 2003; 55:125–132. [PubMed: 12535577]
3. Ceckova-Novotna M, Pavek P, Staud F. P-glycoprotein in the placenta: Expression, localization, regulation and function. *Reprod Toxicol*. 2006; 22:400–410. [PubMed: 16563694]
4. Nakamura Y, Ikeda S, Furukawa T, Sumizawa T, Tani a, Akiyama S, et al. Function of P-glycoprotein expressed in placenta and mole. *Biochem Biophys Res Commun*. 1997; 235:849–53. [PubMed: 9207250]
5. Hemaier SJ, Nanovskaya TN, Abdel-Rahman SZ, Patrikeeva SL, Hankins GDV, Ahmed MS. Modulation of human placental P-glycoprotein expression and activity by MDR1 gene polymorphisms. *Biochem Pharmacol*. 2010; 79:921–5. [PubMed: 19896927]
6. Hitzl M, Schaeffeler E, Hoher B, Slowinski T, Halle H, Eichelbaum M, et al. Variable expression of P-glycoprotein in the human placenta and its association with mutations of the multidrug resistance 1 gene (MDR1, ABCB1). *Pharmacogenetics*. 2004; 14:309–318. [PubMed: 15115917]
7. Tanabe M, Ieiri I, Nagata N, Inoue K, Ito S, Kanamori Y, et al. Expression of P-glycoprotein in human placenta: relation to genetic polymorphism of the multidrug resistance (MDR)-1 gene. *J Pharmacol Exp Ther*. 2001; 297:1137–43. [PubMed: 11356939]
8. Hoffmeyer S, Burk O, von Richter O, Arnold HP, Brockmöller J, John A, et al. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and

correlation of one allele with P-glycoprotein expression and activity in vivo. *Proc Natl Acad Sci U S A*. 2000; 97:3473–8. [PubMed: 10716719]

9. Hitzl M, Drescher S, van der Kuip H, Schäffeler E, Fischer J, Schwab M, et al. The C3435T mutation in the human MDR1 gene is associated with altered efflux of the P-glycoprotein substrate rhodamine 123 from CD56+ natural killer cells. *Pharmacogenetics*. 2001; 11:293–298. [PubMed: 11434506]
10. Mölsä M, Heikkinen T, Hakkola J, Hakala K, Wallerman O, Wadelius M, et al. Functional role of P-glycoprotein in the human blood-placental barrier. *Clin Pharmacol Ther*. 2005; 78:123–131. [PubMed: 16084847]
11. Kim RB, Leake BF, Choo EF, Dresser GK, Kubba SV, Schwarz UI, et al. Identification of functionally variant MDR1 alleles among European Americans and African Americans. *Clin Pharmacol Ther*. 2001; 70:189–199. [PubMed: 11503014]
12. Salama NN, Yang Z, Bui T, Ho RJY. MDR1 haplotypes significantly minimize intracellular uptake and transcellular P-gp substrate transport in recombinant LLC-PK1 cells. *J Pharm Sci*. 2006; 95:2293–2308. [PubMed: 16883550]
13. Taniguchi S, Mochida Y, Uchiumi T. Genetic polymorphism at the 5' regulatory region of multidrug resistance 1 (MDR1) and its association with interindividual variation of expression level in the colon. *Mol Cancer Ther*. 2003; 1:1351–1359.
14. Kroetz DL, Pauli-Magnus C, Hodges LM, Huang CC, Kawamoto M, Johns SJ, et al. Sequence diversity and haplotype structure in the human ABCB1 (MDR1, multidrug resistance transporter) gene. *Pharmacogenetics*. 2003; 13:481–94. [PubMed: 12893986]
15. Lourenço JJ, Maia RC, Scheiner MaM, Vasconcelos FC, Moreira MaM. Genomic variation at the MDR1 promoter and P-glycoprotein expression and activity in AML patients. *Leuk Res*. 2008; 32:976–9. [PubMed: 18001833]
16. Takane H, Kobayashi D, Hirota T. Haplotype-oriented genetic analysis and functional assessment of promoter variants in the MDR1 (ABCB1) gene. *J Pharmacol Exp Ther*. 2004; 311:1179–1187. [PubMed: 15280437]
17. Sai K, Saito Y, Maekawa K, Kim S-R, Kaniwa N, Nishimaki-Mogami T, et al. Additive effects of drug transporter genetic polymorphisms on irinotecan pharmacokinetics/pharmacodynamics in Japanese cancer patients. *Cancer Chemother Pharmacol*. 2010; 66:95–105. [PubMed: 19771428]
18. Sai K, Itoda M, Saito Y, Kurose K, Katori N, Kaniwa N, et al. Genetic variations and haplotype structures of the ABCB1 gene in a Japanese population: an expanded haplotype block covering the distal promoter region, and associated ethnic differences. *Ann Hum Genet*. 2006; 70:605–22. [PubMed: 16907707]
19. Ito S, Ieiri I, Tanabe M, Suzuki A, Higuchi S, Otsubo K. Polymorphism of the ABC transporter genes, MDR1, MRP1 and MRP2/cMOAT, in healthy Japanese subjects. *Pharmacogenetics*. 2001; 11:175–84. [PubMed: 11266082]
20. Xu M, Nekhayeva I, Cross CE, Rondelli CM, Wickliffe JK, Abdel-Rahman SZ. Influence of promoter/enhancer region haplotypes on MGMT transcriptional regulation: A potential biomarker for human sensitivity to alkylating agents. *Carcinogenesis*. 2014; 35:564–571. [PubMed: 24163400]
21. Xu M, Cross CE, Speidel JT, Abdel-Rahman SZ. MGMT DNA repair gene promoter/enhancer haplotypes alter transcription factor binding and gene expression. *Cell Oncol*. 2016; 39:435–447.
22. Chen C, Clark D, Ueda K, Pastan I, Gottesman MM, Roninson IB. Genomic organization of the human multidrug resistance (MDR1) gene and origin of P-glycoproteins. *J Biol Chem*. 1990; 265:506–514. [PubMed: 1967175]
23. Nec Farré D, Roset R, Huerta M, Adsua JE, Roselló L, Albà MM, et al. Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. *Nucleic Acids Res*. 2003; 31:3651–3653. [PubMed: 12824386]
24. Messeguer X, Escudero R, Farre D, Nunez O, Martinez J, Alba MM. PROMO: detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics*. 2002; 18:333–334. [PubMed: 11847087]
25. Ding K, Zhou K, He F, Shen Y. LDA--a java-based linkage disequilibrium analyzer. *Bioinformatics*. 2003; 19:2147–2148. [PubMed: 14594722]

26. Auton A, Abecasis GR, Altshuler DM, Durbin RM, Abecasis GR, Bentley DR, et al. A global reference for human genetic variation. *Nature*. 2015; 526:68–74. [PubMed: 26432245]
27. Rondelli CM, El-Zein Ra, Wickliffe JK, Etzel CJ, Abdel-Rahman SZ. A comprehensive haplotype analysis of the XPC genomic sequence reveals a cluster of genetic variants associated with sensitivity to tobacco-smoke mutagens. *Toxicol Sci*. 2010; 115:41–50. [PubMed: 20106949]
28. Pauli-Magnus C, Kroetz DL. Functional implications of genetic polymorphisms in the multidrug resistance gene MDR1 (ABCB1). *Pharm Res*. 2004; 21:904–913. [PubMed: 15212152]
29. Wang B, Ngoi S-M, Wang J, Chong SS, Lee CGL. The promoter region of the MDR1 gene is largely invariant, but different single nucleotide polymorphism haplotypes affect MDR1 promoter activity differently in different. *Mol Pharmacol*. 2006; 70:267–276. [PubMed: 16608921]
30. Wilhelm M, Schlegl J, Hahne H, Moghaddas Gholami A, Lieberenz M, Savitski MM, et al. Mass-spectrometry-based draft of the human proteome. *Nature*. 2014; 509:582–7. [PubMed: 24870543]
31. Roeder RG. Role of General and Gene-specific Cofactors in the Regulation of Eukaryotic Transcription. 1998; LXIII:201–218.
32. Lee TI, Young Ra. Transcription of Eukaryotic Protein- Coding Genes. *Annu Rev Genet*. 2000;77–137. [PubMed: 11092823]
33. Orphanides G. A Unified Theory of Gene Expression. *Cell*. 2002; 108:439–451. [PubMed: 11909516]
34. Cornwell MM, Smith DE. SP1 activates the MDR1 promoter through one of two distinct G-rich regions that modulate promoter activity. *J Biol Chem*. 1993; 268:19505–11. [PubMed: 8103518]
35. Kerkel K, Spadola A, Yuan E, Kosek J, Jiang L, Hod E, et al. Genomic surveys by methylation-sensitive SNP analysis identify sequence-dependent allele-specific DNA methylation. *Nat Genet*. 2008; 40:904–908. [PubMed: 18568024]
36. Takai D, Jones PA. The CpG island searcher: a new WWW resource. *In Silico Biol*. 2003; 3:235–40. [PubMed: 12954087]
37. Li W, Cowley A, Uludag M, Gur T, McWilliam H, Squizzato S, et al. The EMBL-EBI bioinformatics web and programmatic tools framework. *Nucleic Acids Res*. 2015; 43:W580–W584. [PubMed: 25845596]
38. Wu LX, Wen CJ, Li Y, Zhang X, Shao YY, Yang Z, et al. Interindividual epigenetic variation in ABCB1 promoter and its relationship with ABCB1 expression and function in healthy Chinese subjects. *Br J Clin Pharmacol*. 2015; 80:1109–1121. [PubMed: 25940551]
39. Vogelstein B, Kinzler KW, Yan H, Yuan W, Velculescu VE. Allelic Variation in Human Gene Expression. *Science*. 2002; 297:21218.
40. Loeuillet C, Weale M, Deutsch S, Rotger M, Soranzo N, Wyniger J, et al. Promoter polymorphisms and allelic imbalance in ABCB1 expression. *Pharmacogenet Genomics*. 2007; 17:951–9. [PubMed: 18075465]

Figure 1. *ABCB1* Promoter Haplotype Luciferase Activity

ABCB1 promoter haplotype Nanoluciferase activity was measured and normalized in-well with firefly luciferase for each of the 14 haplotypes observed in the population as well as for the 2 haplotypes generated by site-directed mutagenesis. The resulting activity was normalized to the activity of the ancestral promoter haplotype (Haplotype 1) for comparison. Data presented as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The map shows the 2350bp *ABCB1* proximal promoter region sequenced in this study. The grey nucleotides represent the 12 SNPs identified in this study. Boxes represent some of the PROMO predicted transcription factor binding sites near SNPs. Underlined nucleotides represent CpG islands predicted by CpG Island Searcher[36], while the italicized bases are CpG islands predicted by EMBL-EBI[37]. For SNP variants: R = A or G, Y = C or T, W = A or T, M = A or C.

Table 1

SNPs found in the promoter of *ABCB1* in the 1000 genome project.

SNP variants found in the promoter of *ABCB1* locations, reference SNP (rs) identification, frequency of SNP in 1000 genome project and the frequency of the variants within our study population.

SNP ^a	rs number	Database ^b Reported MAF (Allele, Count)	Our MAF (Allele, Count)
A-1572aT	rs28381795	0.0134 (T,67)	0.015 (T,3)
T-1517aC	rs28381796	0.0116 (C,58)	0.005 (C,1)
A-1503aG	rs149353773	0.0006 (G,3)	ND
G-1459aA	rs12720464	0.0581 (A,291)	0.035 (A,7)
C-1360aT	rs181041839	0.0004 (T,2)	ND
C-1167aT	rs191072244	0.0002 (T,1)	ND
G-1157aA	rs28381797	0.0236 (A,118)	0.01 (A,2)
C-1073aA	rs185837535	0.0008 (A,4)	ND
T-1017aC	rs28746504	0.0543 (C,272)	0.05 (C,10)
T-824aC	rs182495914	0.0006 (C,3)	ND
A-684aT	rs28381798	0.001 (T,5)	0.005 (T,1)
G-496aA	rs138442576	0.004 (A,2)	ND
A-297aG	rs140872872	0.0004 (G,2)	ND
G-274aA	rs35462624	0.0022 (A,11)	0.005 (A,1)
C-223aT	rs34762047	0.0012 (T,6)	ND
A-131aG	rs148824712	0.0008 (C,4)	ND
G-68aA	rs145617883	0.0006 (A,3)	ND
A-60aT	rs28381800	0.0078 (T,39)	ND
A-41aG	rs2188524	0.0214 (G,107)	0.005 (G,1)
G-240A	rs35265821	0.0006 (A,3)	0.005 (A,1)
T-129C	rs3213619	0.0543 (C,272)	0.04 (C,8)
A-43G	rs28381801	0.0048 (G,24)	0.005 (G,1)
C133A	rs28381802	0.0156 (A,78)	0.005 (A,1)

^aSmall "a" nomenclature represents nucleotide before the non-transcribed Exon 1. The (–) without the "a" are within or after Exon 1 before the transcription start site. Derived from Takane et al., 2004 (Takane et al., 2004).

^bFrequency of SNP in 1000 genome project

Table 2

Haplotypes and frequencies inferred by PHASE.

Haplotype #	ABCB1 Promoter Haplotype Structure	PHASE Frequency	Sample Frequency	Pairs	Number of Subjects (Frequency)
1	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.8817	0.880	1,1	80
2	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133A	0.0004	ND	1,3	1
3	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43G/133C	0.0050	0.005	1,4	1
4	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240A/-129T/-43A/133C	0.0050	0.005	1,7	1
5	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aG/-240G/-129T/-43A/133C	0.0004	ND	1,8	1
6	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aG/-240G/-129T/-43A/133A	0.0004	ND	1,9	2
7	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aA/-129T/-43A/133C	0.0050	0.005	1,10	2
8	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.0050	0.005	1,15	1
9	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.0150	0.010	1,16	6
10	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129C/-43A/133C	0.0210	0.025	10,27	3
11	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129C/-43A/133A	0.0004	ND	13,19	1
12	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aG/-240G/-129C/-43A/133C	0.0004	ND	29,30	1
13	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aG/-240G/-129C/-43A/133A	0.0004	0.005		
14	-1572aA/-1517aT/-1459aG/-1157aA/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.0144	ND		
15	-1572aA/-1517aT/-1459aG/-1157aA/-1017aT/-684aA/-274aG/-41aA/-240G/-129C/-43A/133C	0.0004	0.005		
16	-1572aA/-1517aT/-1459aA/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.0293	0.030		
17	-1572aA/-1517aT/-1459aA/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129C/-43A/133C	0.0002	ND		
18	-1572aA/-1517aT/-1459aA/-1157aA/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.0005	ND		
19	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.0004	0.005		
20	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133A	0.0004	ND		
21	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aG/-240G/-129T/-43A/133C	0.0004	ND		
22	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aG/-240G/-129T/-43A/133A	0.0013	ND		
23	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129C/-43A/133C	0.0004	ND		
24	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129C/-43A/133A	0.0004	ND		
25	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aG/-240G/-129C/-43A/133C	0.0004	ND		
26	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aG/-240G/-129C/-43A/133A	0.0012	ND		
27	-1572aT/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.0003	0.015		

Haplotype #	ABCB1 Promoter Haplotype Structure	PHASE Frequency	Sample Frequency	Pairs	Number of Subjects (Frequency)
28	-1572aT/-1517aT/-1459aG/-1157aG/-1017aC/-684aA/-274aG/-41aA/-240G/-129C/-43A/133C	0.0097	ND		
29*	-1572aA/-1517aT/-1459aA/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129C/-43A/133C	ND	0.005		
30*	-1572aA/-1517aT/-1459aG/-1157aA/-1017aC/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	ND	0.005		

PHASE inferred haplotype structure with PHASE predicted frequency, observed haplotype frequency and pairing in sample population.

* Haplotype found in population not predicted by PHASE.