Promoter polymorphisms and allelic imbalance in *ABCB1* expression

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Objective **The *ABCB1* (*MDR1*) gene, encoding the transporter P-glycoprotein, is known to act on a broad range of prescription medicines. For this reason a large number of studies have assessed the functional consequences of variation in this gene. Particular attention has focused on the *ABCB1*_3435C>T polymorphism, an exonic variant resulting in a synonymous change. This variant has been associated with mRNA, protein and serum levels, and with responses to a number of medicines. The results of association studies have, however, been variable and it is not currently clear whether this polymorphism is functional or is in linkage disequilibrium with functionally distinct alleles.**

Results **To identify functional variation in the *ABCB1* gene we assessed allelic imbalance by pyrosequencing cDNA from 80 lymphoblastoid B cell lines from the Centre d'Etude du Polymorphisme Humain (CEPH) collection, including 74 individuals heterozygous for 3435C>T. We found that the degree of *ABCB1* allelic imbalance differed among B-cell lines. In an effort to fine-map variants that influence the proportion of the two allelic mRNA species we genotyped representative common variations near the 3435C>T polymorphism by using a tagging single nucleotide polymorphism (SNP) approach. In one approach, we assessed in segregating families the impact of *cis*-acting variants on mRNA levels by using allelic imbalance as the phenotype in a regression analysis that distinguishes the coupling arrangements (phase) among alleles. In a second approach, we assessed allelic imbalance levels in lymphoblastoid B-cell lines from unrelated HapMap individuals, and performed an association using tagSNPs in a 5-Mb region surrounding the gene. Two potential *cis*-acting variants, a promoter rs28656907/rs28373093 dinucleotide polymorphism ($P=0.007$) and the rs10245483 SNP ($P=0.0003$) located 2 Mb upstream from the gene, were predictors of *ABCB1* expression.**

Conclusions **The study outlines a general experimental approach for fine mapping gene variants that influence mRNA expression by using cultured cell lines.**
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Introduction

Uptake, distribution and excretion of endogenous and exogenous compounds is controlled by polyspecific membrane transporters expressed in intestine, liver, kidney, placenta, testis, blood cells and the endothelial cell lining of brain capillary where they constitute the blood–brain barrier. The best investigated transporter is P-glycoprotein, encoded by the *ABCB1* (*MDR1*) gene. The association of polymorphisms in *ABCB1* with changes in transport function spurred a significant amount of research [1–8]. At present, there is still controversy regarding the validity of these associations.

Particular interest is seen on *ABCB1*_3435C > T (referred herein as 3435C > T), an exonic variant resulting in a synonymous amino acid change that has been associated with differences in mRNA expression, in protein levels, in transport function and possibly in pharmacogenetic response [2,8–10]. Efforts at identifying a causal variant included the analysis of the region of high linkage disequilibrium around this single nucleotide polymorphism (SNP) [11], and a comprehensive investigation of the haplotypic structure of *ABCB1* [10]. More work is, however, needed to describe the mechanisms determining *ABCB1* expression: for instance, scanning for genomic

regions modulating expression, including undiscovered *cis* and *trans*-acting regulatory elements [12,13].

The difficulty of unequivocally linking the 3435C > T site to a specific biological effect may stem, at least in part, from the effect of other polymorphisms in *ABCB1*, both known and unknown. This controversy illustrates a more general problem – how to progress from gene-specific to allele-specific effects, and in particular, how to define a framework to assess possible differences in relative expression levels of different allelic transcripts, and identify causality effects for specific genetic polymorphisms. We therefore characterized the allelic expression associated with the variant 3435C > T with the goal of evaluating *cis*-regulatory influences leading to *ABCB1* allelic imbalance. For this, we investigated the *ABCB1* allelic expression in cell lines from multigeneration families consisting of Epstein–Barr virus-immortalized lymphoblastoid B-cell lines (LBLs) [14,15]. In addition, we assayed LBL cells from unrelated Centre d'Etude du Polymorphisme Humain (CEPH) individuals that have been genotyped at a high density in the frame of the HapMap project [16]. Information from families and from unrelated individuals served to (i) define the pattern of allelic expression linked to 3435C > T, (ii) map *cis*-acting variants putatively influencing allelic expression in *ABCB1* and (iii) assess the relevance of candidate *cis*-acting SNPs on drug pharmacokinetics for nelfinavir, a substrate of the transporter [17,18].

Materials and methods

Cells

The CEPH family pedigrees [14,15] consist of multigenerational Caucasian families. Epstein–Barr virus transformed B-cell lines from these individuals are available through the Coriell Institute for Medical Research (*http://locus.umdnj.edu/nigms/ceph/ceph.html*). Additional information about these samples may be found through the Fondation Jean Dausset – CEPH (*http://www.cephb.fr/*). B-cell lines were obtained from CEPH Caucasian pedigrees (numbers 102/Venezuelan, 884/Amish, 1331/Utah, 1332/Utah and 1341/Utah), and from unrelated Caucasian individuals included in the human HapMap [16]. Cells were cultivated in RPMI 1640/Glutamax-I medium (Invitrogen, Carlsbad, California, USA) supplemented with 15% FCS (Inotech, Dietikon, Switzerland) and maintained by replacing half medium twice a week. For pyrosequencing analysis, we selected 42 heterozygous 3435C > T individuals from families, 32 heterozygous HapMap individuals, plus six control individuals including three 3435C, and three 3435T homozygous.

Genotyping

To represent genetic variation in *ABCB1* in families, we considered both common genetic variation and polymorphisms identified through resequencing of ~3.5 kb of the promoter region. Common variation was represented by six tagging SNPs (tSNPs) described in Soranzo *et al.* [11], which include the 3435C > T site and five additional tagging SNPs (tSNPs): Tag1 (rs3789243), Tag5 (rs1128503), Tag6 (rs2235046), Tag11 (rs1186746), and Tag12 (rs1186745) [10,11]. For accurate haplotype inference, the tSNPs, and newly identified promoter variants were genotyped in 32 additional individuals from the five pedigrees. This allowed all phases to be resolved. Primers information is given in supplementary Table S1.

For the HapMap-based association study we downloaded all SNPs from the HapMap project (*http://www.hapmap.org/*) spanning a region of 5 Mb centered on the *ABCB1* gene. This region included 4200 SNPs with a minimum allele frequency greater than 0.05. We calculated tag SNPs using haploview [19] with a minimum r^2 of 0.8, resulting in 643 tag SNPs that were used for the analysis.

Allele quantification by pyrosequencing

For each individual, DNA and RNA were extracted from 10^6 cells from a single cell culture suspension. DNA extraction was performed using MagnaPure (Roche Diagnostics, Basel, Switzerland). Total RNA was extracted using the RNeasy Mini kit (Qiagen, Qiagen AG, Hombrechtikon, Switzerland) and quality was controlled with the RNA 6000 Nano assay (Agilent Bioanalyzer, Agilent Technologies, Waldbronn, Germany). RNA was reverse transcribed into cDNA with the use of random hexamers, and PCR was performed with standard protocols. For pyrosequencing, we amplified 191 and 157 base pairs of *ABCB1* mRNA (cDNA) and DNA, respectively, spanning the 3435C > T SNP, from all studied lymphoblastoid cell lines. Pyrosequencing reactions performed as previously described [20,21], and in accordance with the manufacturer's instructions (*http://www.biotage.com*), were used to measure the ratio of the two alleles in genomic DNA and in cDNA samples, respectively. Pyrosequencing software reports a peak height directly proportional to the number of molecules incorporated into the growing DNA chain. cDNA ratios were normalized to genomic DNA measurements. As both alleles are extracted and measured in a single sample, this method is insensitive to differences in extraction efficiency and eliminates the need for 'control' genes or quantification of total RNA recovery. All measurements of allelic imbalance at position 3435C > T were performed on two different cDNAs in triplicate.

Global *ABCB1* mRNA quantitation

LBL from unrelated individuals from the HapMap CEPH Utah residents with ancestry from northern and western Europe (CEU) set and the grandparents from the CEPH

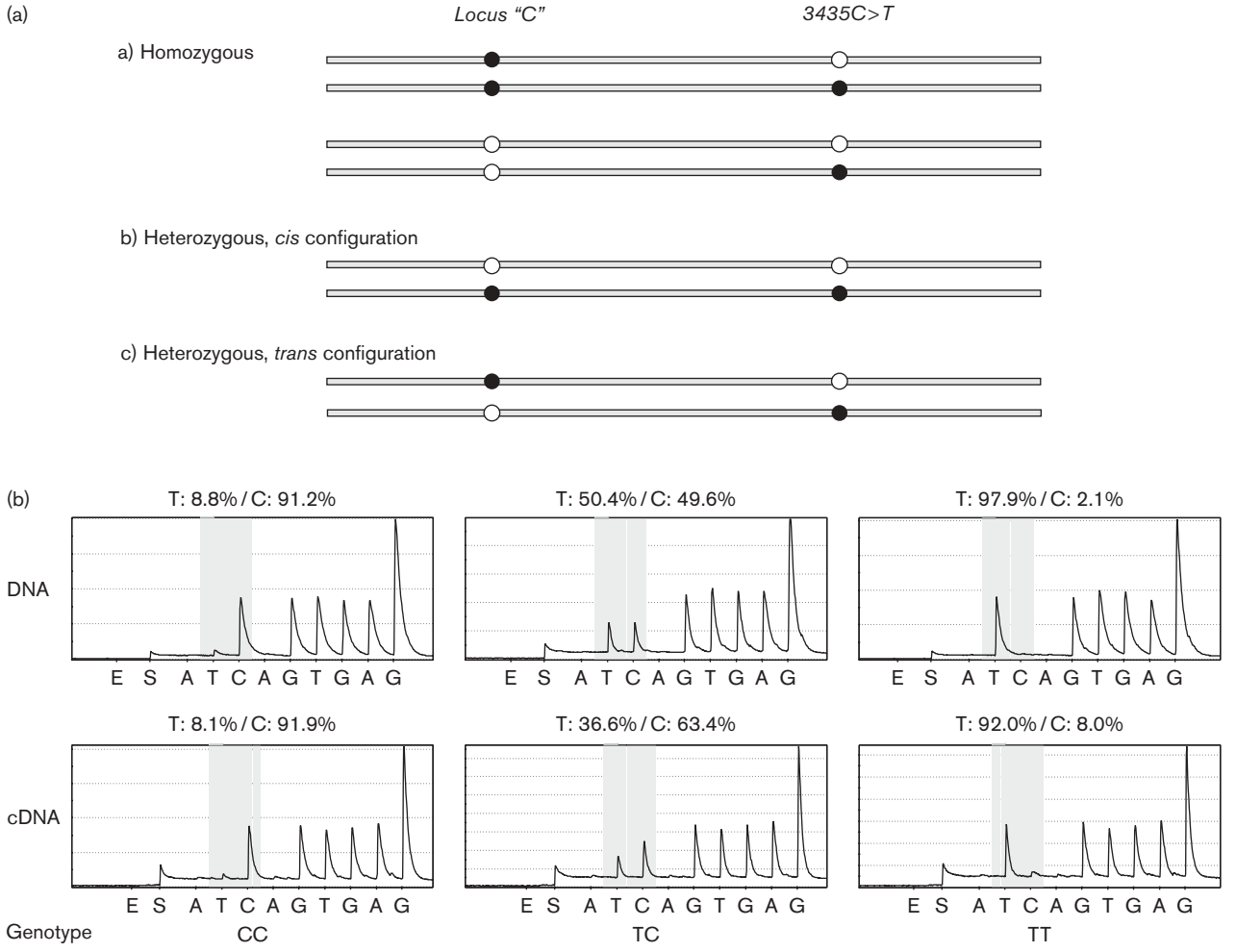
families were used for the analysis of global *ABCB1* expression by TaqMan gene expression assay (Applied Biosystems, Foster City, California, USA) using β-actin as internal control gene (Table S1).

Statistical analysis

To investigate the presence of *cis* variants influencing *ABCB1* in CEPH families, we determined the phase between each genetic variant and the 3435C > T site from pedigree data, using the software Merlin (*http://www.sph.umich.edu/csg/abecasis/Merlin/*) [22]. All haplotypes could be assigned unambiguously. We then restricted analysis to the 42 cell lines that were heterozygous at 3435C > T. For each of the SNPs examined, we used phase information to categorize each cell line as (i) homozygote; (ii) *cis*-heterozygote; or (iii) trans-heterozygote as described in Fig. 1a.

As an alternative method we performed an association study using unrelated individuals from the HapMap CEU set. For this, we selected the 34 individuals who were heterozygous for the 3435C > T SNP. All heterozygous individuals were then classified using the results of the allelic quantification as either (i) balanced (average difference between the two alleles was less than 14% corresponding to 57 : 43) or (ii) imbalanced (average difference between the two alleles was greater than 14%), with the threshold being determined based on the

Fig. 1



Detecting the genetic influence of *cis*-acting polymorphisms in Centre d'Etude du Polymorphisme Humain (CEPH families). (a) The allelic imbalance associated with the 3435C>T polymorphism suggests an influence of *cis*-acting genetic variation on *ABCB1* expression. This effect could be due to the 3435C>T polymorphism itself, or to other uncharacterized variants in phase with 3435C>T. Such genetic variants could in principle be anywhere in the noncoding as well as coding regions of ABCB1. The relative-rates model considers a single locus C linked to 3435C>T. Allelic imbalance is, by definition, detected only in individuals heterozygous at 3435C>T, and so we restrict analysis only to such individuals. Four possible haplotypic configurations of the C locus with 3435C>T are present, which can be classified in three classes as illustrated in this figure.
(b) Representative pyrosequencing output for homozygous (3435CC, 3435TT) and heterozygous (3435CT) individuals. The percentage of expression for each allele is shown.

average error. To perform the association study we used the following rationale: if a *cis* regulatory SNP (rSNP) is a major contributor to determining the allelic transcript levels (*cis* eQTL), one would expect that individuals showing an expression imbalance at the marker SNP position (in this case 3435C > T) should be heterozygous for the rSNP regardless of the phase. Likewise, individuals in whom no allelic imbalance is observed would be expected to be homozygous for the rSNP. Using this principle we performed a 1 degree of freedom χ^2 test (Aa vs. AA + aa), to identify SNPs with significant differences in the levels of heterozygosity between the two groups. We performed a simulation study with the same dataset, but randomizing the phenotype (balanced vs. imbalanced) to determine the empirical significance threshold given the number of tests performed.

Substrate analysis

Candidate SNPs associated with allelic imbalance were tested for association with differences in substrate (nelfinavir) pharmacokinetics and transport *in vivo*. For this, we genotyped 151 individuals contributing plasma drug level data. These individuals were recruited in the frame of the genetic project of the Swiss HIV Cohort Study (*www.shcs.ch*) after providing informed consent [10]. Plasma samples were analysed by high-pressure liquid chromatography with ultraviolet detector technique; limit of quantitation, 200 ng/ml. Plasma concentration values, along with sampling time and usual dosage, were used to estimate individual drug exposure (area under the curve) through Bayesian calculations based on pharmacokinetic studies of the previous population.

Results

Allele-specific expression

To understand the role of the two *ABCB1* alleles, defined by the exonic SNP 3435C > T, we assessed their allele-specific rates of expression. For this, we used pyrosequencing quantification of *ABCB1* mRNA, using the 3435T site as a marker. RNA was obtained from the 42 CEPH cell lines heterozygous at the tested allele, and from the six selected CEPH cell lines from individuals homozygous 3435CC or TT. Representative pyrograms are shown (Fig. 1b). Pyrosequencing experiments were repeated on two sets of cDNA in triplicate. Similar analysis was done for the 32 HapMap individuals heterozygous 3435C > T. The evaluation of interday, intertest reproducibility gave a significant correlation despite the multiplicity of experimental steps (thawing, cell growth, RNA extraction, cDNA synthesis, amplification and pyrosequencing). Thus, expression of the T/C alleles appeared stable over time and characteristic of the individual cell line, with a correlation coefficient of $r^2 = 0.55$, $P < 0.0001$. Results for each individual were averaged for subsequent analysis.

In homozygous individuals, a single allele is identified in the DNA and expressed in the corresponding cDNA sample (Fig. 2). In heterozygous individuals, both alleles are detected at DNA level, with a minor bias in favor of the probe 3435T (Fig. 2a and c). The average (± SEM) $\%_{3435T}$ was 50.5 ± 2.9 and 49.34 ± 1.86%, in families and unrelated HapMap individuals, respectively. This value reflects a considerable heterogeneity in allelic imbalance, with individual cell lines exhibiting from 25.3 to 72.5% expression of 3435T alleles. Overall, analysis of cDNA from heterozygous individuals detected significant allelic imbalance (defined by 57 : 43 based on the average error) in 19 of 42 (45%) and in 15 of 32 (46%) of families and unrelated HapMap individuals, respectively (Fig. 2b and d).

We found significant interfamily differences in the overall 3435C > T allelic expression, with $P = 0.007$ for an analysis of variance analysis comparing logit-transformed $\%_{3435T}$ against pedigree (Fig. 3). This could be due to a *cis*-acting variation associated with the different pedigrees. The contribution of differential environmental factors is unlikely, as cells are grown under common standardized experimental conditions *in vitro* and analysis was performed twice starting from frozen cells 4 months apart.

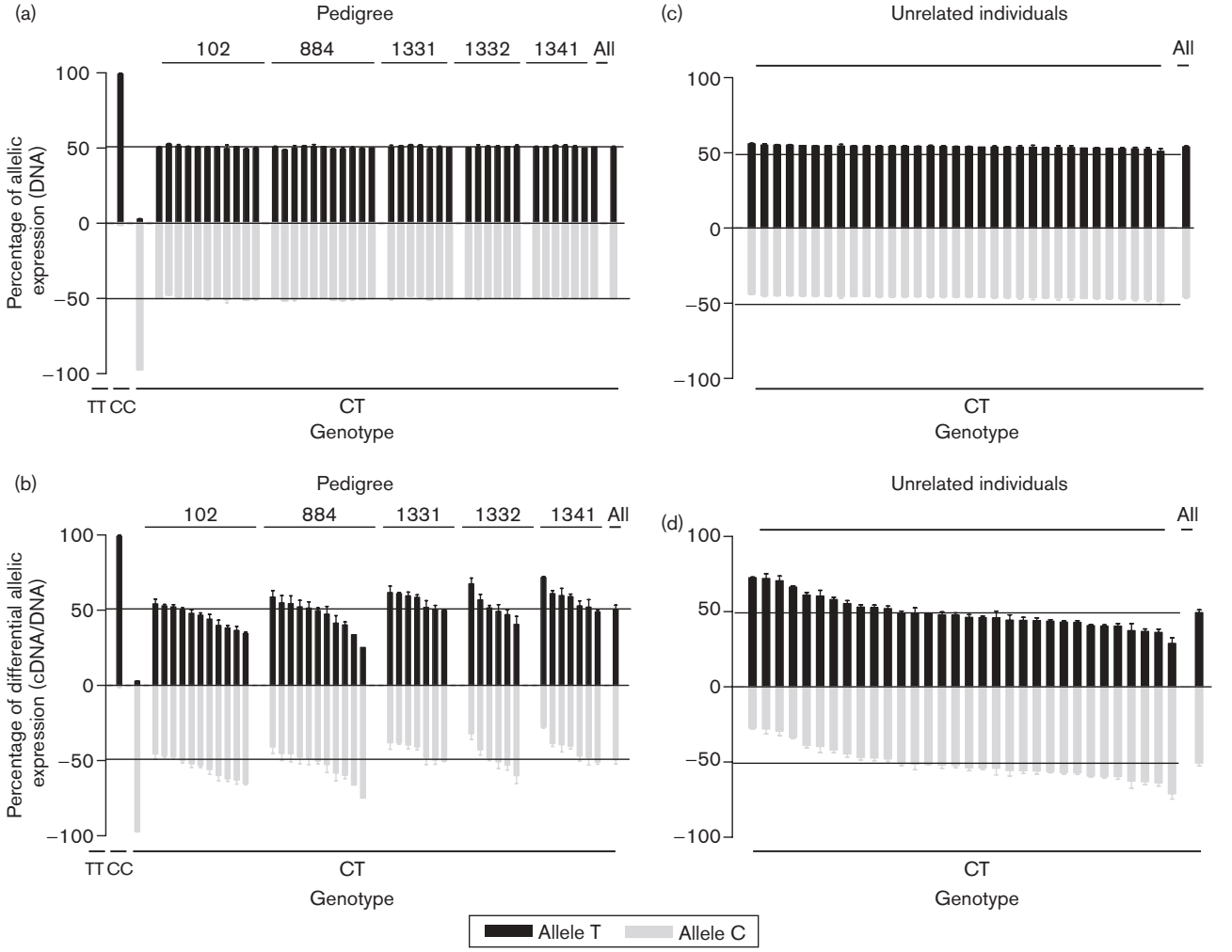
ABCB1 promoter analysis

Promoter analysis was undertaken with the goal of better characterizing *cis*-acting variants associated with the pattern of allelic imbalance. For this, we resequenced ~3.5 kb of the *ABCB1* promoter from 14 of the CEPH cell lines, representing the range of allelic expression. We found a frequent ($f = 0.4$) dinucleotide polymorphism mapping within 2 kb of mRNA transcript, rs28656907/rs28373093, resulting in two possible configurations: AC or GG. For simplicity, we will refer to these two variables jointly as promoter dinucleotide variant. The proximal promoter region is largely invariable, as previously reported [23].

Analysis of genetic variants affecting allelic expression in families

We hypothesize that the interindividual and interfamily patterns of allelic imbalance may be due to one or more *cis*-acting regulatory polymorphism(s). We considered common genetic variation, represented by tagging SNPs (tSNPs), in association with the promoter variant described above (Fig. 4). As a first step, we inferred haplotype phase of each SNP with 3435C > T using the pedigree data. We then categorized the 42 cell lines based on the genotype of each SNP as homozygote, *cis*-heterozygote (het-*cis*) or *trans*-heterozygote (het-*trans)* with the 3435C > T polymorphism as explained in box 1. We then applied a logistic regression to test the association of each of these two-locus configurations with logit-transformed $\%_{3435T}$ allele expression. We found

Fig. 2



ABCB1 differential allelic expression. Allelic expression in DNA (a, c), and the corresponding mRNA (cDNA) expression (b, d) in families and in HapMap individuals, respectively. Analysis corresponds to measures in triplicate by pyrosequencing. (a and b) Two controls of 3435 homozygosity (CC, TT) and 42 heterozygous (CT) samples from five Centre d'Etude du Polymorphisme Humain (CEPH) cell lines pedigrees. The last column corresponds to the average allelic expression (± SEM) for all heterozygous cell lines; 50.6 ± 0.6%T and 49.4 ± 0.6%C for DNA; 50.5 ± 2.9%$_{3435T}$ and 49.4 ± 2.9%C for cDNA measurements.

evidence of an effect of genotype category on %$_{3435T}$ expression in particular for the promoter variant ($P = 0.007$) and for Tag12 ($P = 0.022$). These may represent SNPs having a *cis*-acting influence on *ABCB1* allelic expression. Twelve distinct haplotypes defined by the tags and promoter variants were present. No association was detected between these haplotypes and the *ABCB1* allelic expression ($P = 0.19$).

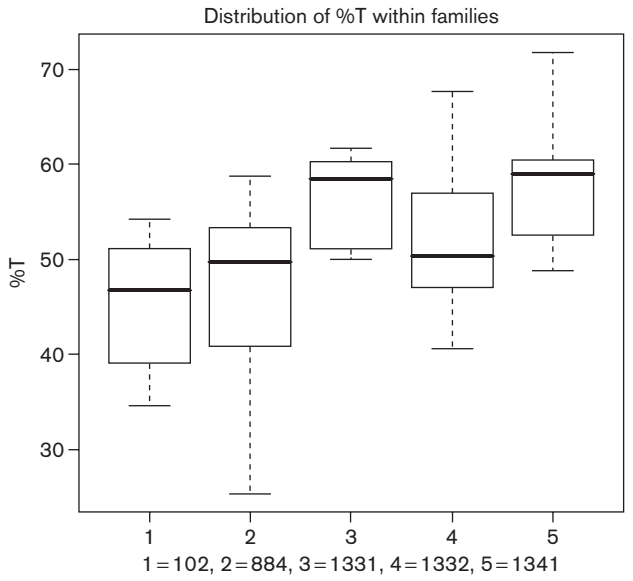
Analysis of genetic variants affecting allelic expression in HapMap individuals

As an alternative approach we performed an association study in unrelated HapMap individuals. Thirty-two individuals who were heterozygous at the 3435C > T position were used for the analysis. Individuals were split into two phenotypic groups (balanced and imbalanced) according to the results of the allelic quantification experiment. In principle we expect imbalanced individuals to be heterozygous for the regulatory SNP and vice versa (see Materials and methods), so we performed a heterozygosity test for 643 tag SNPs on a 5 Mb region centered on the *ABCB1* gene to identify putative regulatory variants. We identified a single SNP with significant excess of heterozygosity in imbalanced individuals ($P = 3.0\text{E-}4$). To determine whether this finding is significant given the number of tests performed, we ran a simulation study ($n = 500$), which showed that the empirical significance threshold is below the result we obtained (Fig. 5).

Effect of candidate *cis*-acting variants on global *ABCB1* mRNA expression

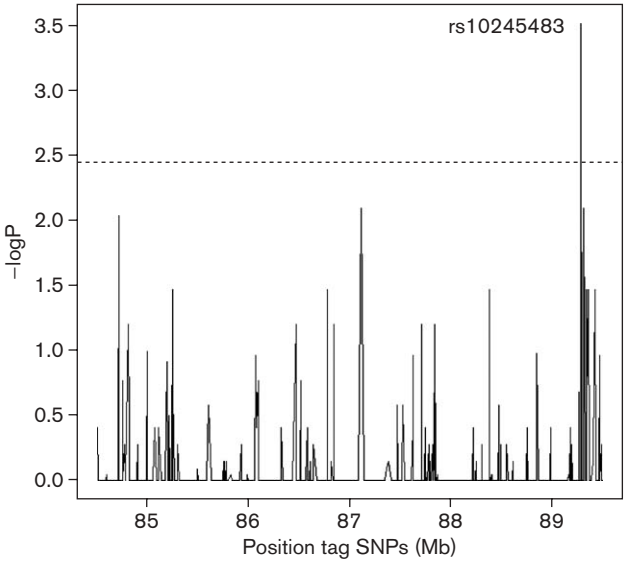
As a result of the large distance from the two candidate loci to the ATG, we did not use a reporter assay for the assessment of the SNPs. The promoter dinucleotide is 1454 bp from the exon 1; however, it is 1 124 524 bp away from the ATG for the open reading frame coding P-gp

Fig. 3



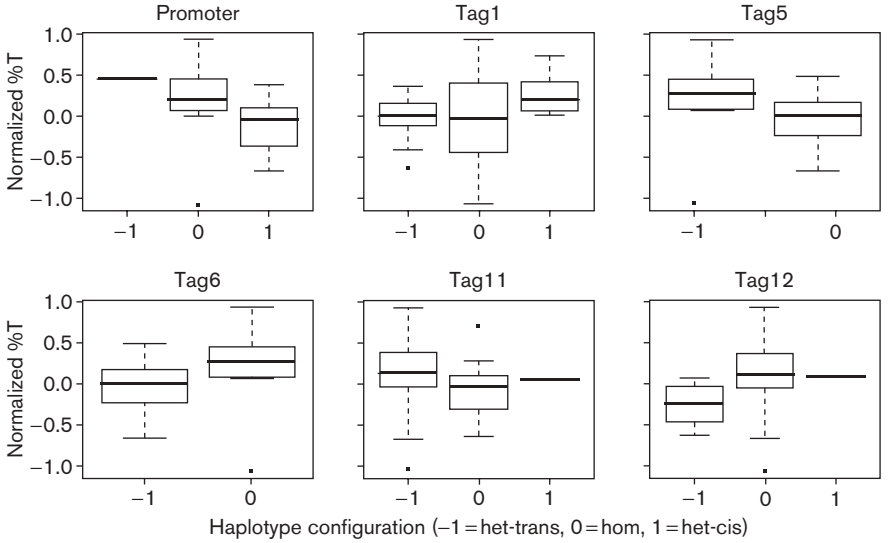
Interfamily differences in allelic expression. The distribution of 3435C>T allelic expression values expressed as %T are shown. Significant inter-family differences are seen in the overall %T, with *P* value = 0.007 for an analysis of variance analysis comparing %T against pedigree.

Fig. 5



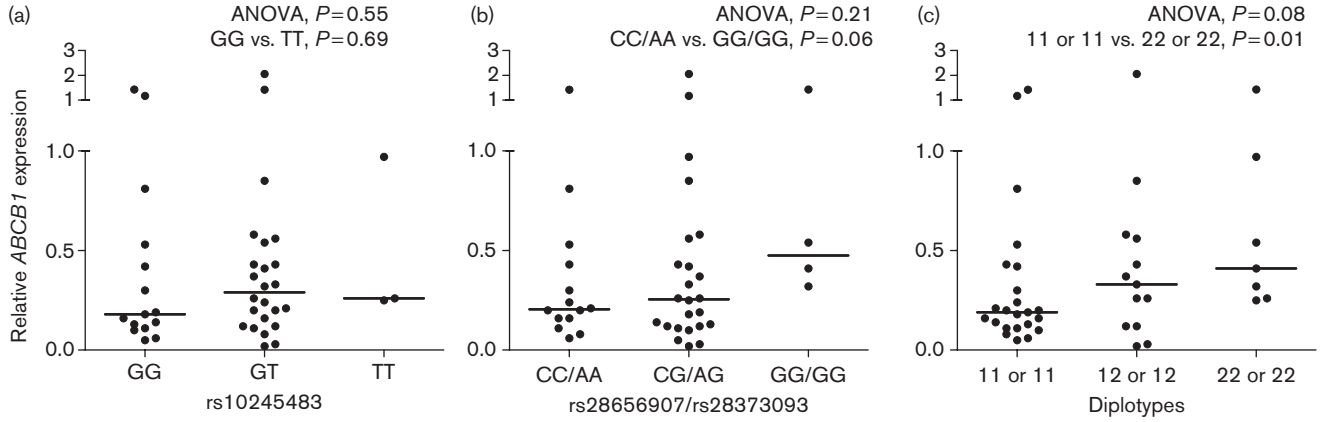
HapMap-based association study. Analysis used 643 tag SNPs downloaded from the HapMap project (*http://www.hapmap.org/*) spanning a region of 5 Mb centered on the *ABCB1* gene. SNP rs10245483, located 2 Mb upstream from the gene, was found to be an independent predictor of *ABCB1* expression (*P* = 0.0003). The empirical significance threshold (dashed line) is below the result obtained for the marker. SNP, single nucleotide polymorphism.

Fig. 4



Two-loci analysis. Box plots for each tag SNP/3435C>T two-loci haplotype configuration (−1 = het-*trans*, 0 = homozygous, +1 = het-*cis*). The distribution of 3435C>T allelic expression values expressed as %T are shown. A statistically significant association was observed between genotype category on %T expression in particular for the promoter variant (*P* = 0.007) and for Tag12 (*P* = 0.022). SNP, single nucleotide polymorphism.

Fig. 6



Association of candidate *cis*-acting variants on global *ABCB1* mRNA expression. Analysis used lymphoblastoid B-cell lines (LBL) from unrelated individuals from the HapMap CEU set and grandparents from the Centre d'Etude du Polymorphisme Humain (CEPH) families. *ABCB1* expression was assessed by quantitative RT-PCR, and results were plotted in relation to the rs10245483 genotype (a), the rs28656907/rs28373093 locus (b), or according to the main diplotypes (c). Diplotype '11 or 11' includes cells homozygous for the common allele for one or both loci (11/11, 11/12, 12/11); diplotype '12 or 12' includes cells heterozygous at both loci; diplotype '22 or 22' includes cells homozygous for the minor allele for one or both loci (effective: 12/22, 22/11, 22/12). Alternative grouping of data did not change the results. The upper *P* value, represents analysis of variance analysis; the lower *P* value represents Mann–Whitney test of extreme genotypes.

(on *ABCB1* exon 3). The second candidate, identified through HapMap, is located 2.4 mb from the gene, and thus cannot be assessed by using reporter approach. Thus, to explore whether the variants identified as influencing allelic expression could represent candidate *cis*-acting SNPs, we assessed global *ABCB1* mRNA expression in LBLs from unrelated HapMap individuals and CEPH grandparents ($n = 42$). Although the promoter rs28656907/rs28373093 dinucleotide polymorphism, and the rs10245483 SNP had no definitive one-locus association with gene expression, the resulting diplotypes were associated with differences in mRNA expression (Fig. 6). Diplotypes consisting of the common alleles at both loci had a median interquartile range (IQR) relative to mRNA expression of 0.19 (IQR = 0.11–0.42), whereas diplotypes including homozygous; minor allele frequency alleles had median (IQR) values of 0.41 (IQR = 0.26–0.97), $P = 0.016$.

Effect of candidate single nucleotide polymorphism on Nelfinavir concentration

We classified HIV-infected patients receiving the antiretroviral treatment according to their genotype and assessed the association of candidate SNPs influencing allelic expression with plasma and intracellular concentrations of a recognized substrate of the transporter – the protease inhibitor Nelfinavir [10]. We tested the dinucleotide variant identified in the analysis of allelic imbalance in families, and the variant identified in the association analysis in HapMap unrelated individuals. These SNPs were not associated with a significant effect on plasma nelfinavir levels in families (both $P > 0.3$), or in unrelated individuals (both $P > 0.6$).

Discussion

Differential allelic expression, described as allelic imbalance, is a subject of increasing interest [24–28], it appears to be more common than previously thought [29], and could account for phenotypic differences relevant to health. Although many sources of expression variation such as environmental or genetic influences may act in *trans* and affect both alleles, the effect of *cis*-acting genetic variation can be measured precisely by assessing deviation of expression from the expected equimolar ratio in heterozygous individuals for a given allele [20,24,27,29]. This study examines an experimental paradigm for fine mapping gene variants that influence mRNA expression. The main points of our analysis were the following: (i) by using cultured cell lines we diminish confounding factors owing to variable environmental conditions, (ii) using cells from three generation families allowed for phase to be reconstituted, and the relative effects of various SNPs or haplotypes to be compared across block sizes spanned by usual population linkage disequilibrium, (iii) performing an association study for allelic imbalance in unrelated individuals by looking for excess heterozygosity. Earlier, Pastinen *et al.* [30] described a systematic effort to map common *cis*-acting variants in 64 genes, using association methods in HapMap samples.

Thus, we performed a comprehensive analysis of *ABCB1* allelic expression, in the context of its genetic variation and haplotype structure. Allelic imbalance was a characteristic of the cell lines of an individual. We could not, however, associate in a systematic fashion the allele 3435T with reduced mRNA expression, which would

suggest that the 3435 SNP itself is not responsible for the differences in allelic expression in lymphoblastoid cell lines. This contrasts with the recent analysis by Wang *et al.* [31] that assessed allelic ratios in 18 human liver autopsy samples heterozygous for 3435C > T. They described less $\%_{3435T}$ and attributed allelic imbalance to lesser stability of 3435T transcripts, as measured in CHO cells 16 h after transfection. Differences between the two studies may relate to the analytic technique used and the sample size. In particular, the measurement of allelic imbalance in the study of Wang *et al.* used, SnaPshot (Applied Biosystems). This technique resulted in biased fluorophore incorporation for T and C ddNTP in primer extension. In contrast, pyrosequencing leads to negligible bias, thus imposing minimal correction of the results. Differences may also reflect the type of tissue, and differences in the number of heterozygous individuals tested in these two studies.

As many known regulatory polymorphisms are located in gene promoter regions we resequenced ~3.5 kb of the *ABCB1* promoter. We found a common dinucleotide polymorphism, which was not described previously. We detected a significant association between haplotype configuration at this locus and allelic imbalance, suggesting that this locus may exert *cis*-acting influences on expression. In addition, in a separate approach, using the comprehensive HapMap resource and association analysis we identified an additional SNP as candidate marker for *cis* influences on *ABCB1* allelic expression.

We assessed the role of these variants on global *ABCB1* mRNA expression. Diplotypes carrying homozygous minor allele at one or both loci were associated with two-fold increase in *ABCB1* expression. We did not assess the role of the promoter variants on P-gp expression levels. Several reports have signaled a lack of correlation between *ABCB1* mRNA and P-gp levels due to a mechanism of translational regulation [32]. Functional assessment by analysis of pharmacokinetic and intracellular data for nelfinavir did not identify a significant association of the candidate SNPs and the study phenotype. Although nelfinavir is a recognized substrate of the multidrug transporter encoded by *ABCB1* [17,18], the contribution of other transporters in drug disposition [33] may confound the study.

The absence of a defined association of ABCB1_3435C > T with levels of expression of *ABCB1* transcript is consistent with recent data of Kimchi-Sarfaty *et al.* [34] that identify a role of this silent variant in the timing of cotranslational folding of the P-gp. This novel mechanism, that results in changes in substrate specificity, explains to a large extent the controversial results published until date, and the lack of association of this variant, directly or through linkage disequilibrium, with causal variants or with changes in mRNA expression.

The results from this study contribute both to defining an approach to the characterization of allelic imbalance, and to shed light on the controversy surrounding the effect of 3435C > T. The fact that 3435C > T is used as a marker relates exclusively to its high frequency of this SNP in the population. Whereas the study led to information that concerns 3435C > T, the overall approach is of general value and does not require known or presumed functionality of the common SNP that is used as marker for the purpose of mapping *cis* influences on allele-specific expression.

Supplementary data

Supplementary data are available at *The FPC journal* Online (*www.pharmacogeneticsandgenomics.com*).

Acknowledgements

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