Polymorphisms in the methotrexate transport pathway: a new tool for MTX plasma levels prediction in pediatric acute lymphoblastic leukemia

Running head

SNPs in MTX transport genes and MTX toxicity

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Abstract

Objectives:

Methotrexate (MTX) is an important component of therapy for pediatric acute lymphoblastic leukemia (ALL). Treatment with MTX often causes toxicity, which can necessitate dose reduction or treatment cessation. Interindividual differences in adverse reactions can be due to different factors, including polymorphisms in key genes

Recently, we confirmed the association between SLCO1B1 rs11045879 polymorphism and toxicity previously proposed by Treviño and collaborators. As SLCO1B1 is a transporter involved in MTX elimination, other polymorphisms in genes from this pathway could also have a role in MTX toxicity.

The aim of the present study was to analyze in depth the role of polymorphisms in the genes of MTX transport pathway as putative toxicity predictors in pediatric ALL.

Methods:

We analyzed 384 SNPs in 12 transporter genes (SLCO1B1, SLCO1B3, SLCO1A2, ABCB1, ABCG2, ABCC1, ABCC2, ABCC3, ABCC4, SLC19A1, SLC22A6 and SLC22A8) and their correlation with different toxicity parameters in 151 pediatric ALL patients treated with the LAL/SHOP protocol.

Results:

Significant association with MTX plasma levels was found for 21 polymorphisms from 7 genes and 15 haplotypes. After correction, rs9516519 in ABCC4, rs3740065 in ABCC2 and haplotype GCGGG in ABCC2 remained significantly associated.

Conclusions:

Our results suggest that polymorphisms in ABCC4 and ABCC2 could be novel markers for methotrexate toxicity in pediatric ALL.

Keywords

Methotrexate, polymorphism, toxicity, acute lymphoblastic leukemia

Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer, accounting for 30% of all pediatric malignancies [1-2]. During the last 20 years, survival rates for ALL have improved dramatically due to advances in chemotherapy for childhood ALL, with expected cure rates higher than 80% [3].

An important component of ALL therapy is methotrexate (MTX). Despite its clinical success, treatment with high-dose MTX often causes toxicity, requiring a dose reduction or cessation of treatment. Therefore, it would be useful to identify a predictor of the adverse effects of MTX [4]. Interindividual differences in adverse reactions can be due to a number of factors, including polymorphisms in key genes. In the last years, several studies have investigated the relationship between genetic variation and MTX-related toxicity [5-15].

The results of the associations between polymorphisms and MTX toxicity are usually conflicting. Frequently, this lack of replication could be due to small or non-homogeneous populations, differences in treatment protocols among studies, or the use of different toxicity criteria.

Recently, in our group, we demonstrated the association between MTX plasma levels 72h after infusion and renal toxicity (p=0.005) and other less clinically relevant parameters such as vomiting and overall toxicity, and a tendency towards increased hepatic toxicity and hyperbilirubinemia. For that reason, we have considered it as a good and objective toxicity marker. Using this parameter, we carried out a pharmacogenetic study of genes involved in MTX pathway, confirming the association between the *SLCO1B1* rs11045879 polymorphism and toxicity [16], previously proposed by Treviño and collaborators in a GWAS study [17]. As

SLCO1B1 is a hepatic transporter involved in MTX elimination, other polymorphisms in this gene and in transporter genes from the same pathway could also have a role in MTX toxicity.

In the present study, we have analyzed polymorphisms in the 12 most important genes involved in MTX transport in order to select markers that may predict MTX toxicity in pediatric B-ALL patients (Figure 1). We have performed an exhaustive selection of SNPs to deeply cover the genetic variability of each gene. The study has been carried out in a large and homogeneous population of 151 Spanish pediatric B-ALL patients, all of whom were treated according to the standardized LAL/SHOP protocol. MTX plasma concentration was used as an objective and quantifiable measure of toxicity, as confirmed in our previous study [16].

Methods

Patients

This is a retrospective study based on the systematic appraisal of chart data regarding treatment protocols. The patients included in this study were 151 Spanish children, mainly Caucasian, all diagnosed with B-ALL from 2000 to 2011 at the Pediatric Oncology Units of 4 reference hospitals (Hospital Cruces; Hospital Donostia; Hospital Vall d'Hebrón and Hospital La Paz). Informed consent was obtained from all patients or their parents before sample collection. The study was approved by the ethic committee of the University of the Basque Country (UPV/EHU).

Treatment and toxicity evaluation

All the patients included in the study were homogeneously treated with the LAL-SHOP 94/99 and 2005 protocols, which included the same consolidation therapy for all the risk groups: three doses of methotrexate (each dose consisted of 3 g/m^2 or 5 g/m^2 of MTX), 6-

mercaptopurine (30 mg/m^2/day for 6 weeks), four doses of cytarabine (1 g/m^2) and four doses of triple intrathecal therapy. Each of the 3 MTX doses was given in 24 h infusion with homogeneous folinic acid rescue. After each dose, MTX plasma concentration was monitored by a fluorescence polarization immunoassay on a TDx system (Abbott Laboratories, Abbott Park, IL). Measurements were performed daily until the concentration was below 0.2 μM.

Toxicity data were collected objectively, blinded to genotypes, from the patients' medical files. Toxicity was graded according to the Spanish Society of Pediatric Hematology and Oncology (SEHOP) standards, adapted from the WHO criteria (grades 0-4). The highest grade of toxicity observed for each patient during the consolidation therapy period was recorded. Data collected included: hepatic toxicity (AST/ALT), hyperbilirubinemia, vomiting, diarrhea, mucositis and renal toxicity (creatinine), as well as MTX concentrations 48 h, 72 h and 96 h after infusion. MTX levels were considered high if the concentration was over 1 μM at 48h or over 0.2 μM at 72 h or 96 h after at least one of the 3 doses. Comparable distributions were observed for the 3 measurements and we used the 72 h time point for its critical interest for MTX clearance in the LAL/SHOP protocol. Other clinical data including age, sex, and risk group were systematically recorded from the clinical records.

Gene and polymorphism selection

A total of 12 candidate genes reported to be involved in the methotrexate transport and elimination pathway were selected based on the information available in the Pharmacogenomics Knowledge database, PharmGKB (www.pharmgkb.com). These genes encode the following transporter proteins: *ABCB1, ABCC1, ABCC2, ABCC3, ABCC4, ABCG2, SLC19A1, SLC22A6, SLC22A8, SLCO1A2, SLCO1B1, and SLCO1B3*.

A region ranging from 10-kb upstream of the translation initiation site to 10-kb downstream of the translation stop site of each gene was selected. SNPs were selected based on the following criteria: (i) In order to cover the whole genes with an affordable number of SNPs, we selected tagSNPs, that are representative SNPs in a region of the genome with high Linkage Disequilibrium (the non-random association of alleles at two or more loci) and that can capture the genetic variation in a population without genotyping every single SNP. TagSNPs were defined using Haploview software v.4.2 (http://www.broadinstitute.org/haploview/haploview) with an r2 threshold of 0.8 and a minimum minor allele frequency (MAF) of 0.10. In 11 genes, all tag-SNPs were selected for genotyping. In the *ABCC4* gene, due to its large size, a subset of 71 of the 110 defined tag-SNPs was selected. (ii) SNPs with potentially functional effects (amino acid changes, alternative splicing, promoter regions, putative transcription factor binding sites, CpG sites or miRNAs targets) identified using bioinformatics tools (F-SNP, Fast-SNP, polymirTS, Patrocles). (iii) SNPs previously reported to be associated with toxicity in the literature.

This preliminary list of SNPs was filtered using suitability for the Illumina genotyping platform as criteria (selecting from each linkage block those SNPs with an assay score >0.6, associated with a high success rate).

A final 384 SNPs relevant to this study was included in an oligonucleotide pool assay for analysis using the Illumina Veracode technology (Illumina Inc., San Diego, CA) (see Table, Supplemental Digital Content 1, which contains the list of SNPs included and the reasons for selection).

Genotype analyses

Genomic DNA was extracted from peripheral blood or bone marrow slides in remission using the phenol-chloroform method as previously described [18]. DNA was quantified using PicoGreen (Invitrogen Corp., Carlsbad, CA).

For each sample, 400 ng of DNA were genotyped using the GoldenGate Genotyping Assay with Veracode technology according to the published Illumina protocol. Data were analyzed with GenomeStudio software for genotype clustering and calling. Duplicate samples and CEPH trios (Coriell Cell Repository, Camden, NJ) were genotyped across the plates. SNPs showing Mendelian allele-transmission errors or showing discordant genotypes were excluded from the analysis.

Statistical analysis

The association between MTX plasma levels and genetic polymorphisms was evaluated by the χ2 or Fisher's exact test, as well as the Hardy-Weinberg equilibrium. The effect sizes of the associations were estimated by the OR's from univariate logistic regression. The most significant test among dominant and recessive genetic models was used to determine the statistical significance of each SNP. The results were adjusted for multiple comparisons by the False Discovery Rate (FDR) [19]. We reasoned that gene-based correction was sufficiently conservative because of the a priori hypotheses for these genes. Multivariate logistic regressions were also performed to account for the possible confounding effect of sex, age and MTX dose. In all cases the significance level was set at 5%. Analyses were performed by using R v2.11 software. Haploview v.4.2 was used to determine haplotype block structure and to infer haplotype frequencies between individuals with and without toxicity. For haplotype and correction analysis *SLC22A6* and *SLC22A8*, which are located in the same region, were considered as a single entity.

Results

Patients' baseline characteristics

In this study, we have analyzed 151 B-ALL patients, whose characteristics are reported in Table 1. Clinical data regarding MTX plasma concentration 72 h after infusion were available for 143 patients, all of them receiving 3 MTX courses. There were 51 patients (35.7%) with high MTX plasma levels (>0.2 µM). Clinical data for other therapy-related toxicities were available for 130 patients. Among all patients who developed any of these toxicities (n = 66, 41.7%), the prevalence of different types of toxicity were as follows: hepatic (n = 36, 28.1%), vomiting (n = 28, 21.9%), mucositis (n=12, 9.4%), renal (n = 12, 9.4%), hyperbilirubinemia (n = 12, 9.4%) and diarrhea (n = 7, 5.5%).

Genotyping Results

Successful genotyping was obtained in 137 DNA samples (90.7%). During the genotyping process, 41 SNPs out of 384 failed (no PCR amplification, insufficient intensity for cluster separation, or poor or no cluster definition) and 343 were genotyped satisfactorily (89.3%). The average genotyping rate for all SNPs was 96.7%.

Polymorphisms in association with toxicity

In order to investigate if genetic variation may influence MTX toxicity, we tested the association between the 343 genotyped polymorphisms and MTX plasma concentration 72 h after intravenous infusion, as an objective and quantifiable toxicity marker, as other toxicity parameters, such as mucositis or vomiting, are very difficult to record even in prospective studies.

Significant association with MTX plasma levels (p<0.05) was found for 21 polymorphisms from 7 genes: 6 SNPs in *ABCC4* , 4 SNPs in *ABCC2*, 3 SNPs in *SLC22A6*, 3 SNPs in *SLC19A1*, 2 SNPs in *ABCG2*, 1 SNP in *ABCC1* and 2 SNPs in *SLCO1B1*, including rs11045879, which had been previously described by our group. Most SNPs remained associated with MTX plasma levels when we accounted for the possible confounding effect of sex, age and MTX dose (Table 2).

After FDR correction, rs9516519 in *ABCC4* and rs3740065 in *ABCC2* continued to be significantly associated with MTX plasma levels (corrected P-value 0.019 and 0.034 respectively). Nucleotide T in rs9516519 (*ABCC4*), and nucleotide C in rs3740065 (*ABCC2*) were associated with an increased risk of MTX toxicity.

We also analyzed other toxicity parameters (hepatic toxicity, hyperbilirubinemia, vomiting, diarrhea, mucositis, renal toxicity and global toxicity). We also found significant associations between some of these SNPs and renal toxicity, hyperbilirubinemia, mucositis, diarrhea or global toxicity (see Table, Supplemental Digital Content 2).

Haplotypes in association with toxicity

To test the association between haplotypes and MTX plasma levels, we first determined the linkage disequilibrium (LD) block structure for each gene (block definition was based on Gabriel et al., 2002). *ABCB1* was defined by 5 blocks that showed 21 haplotypes with frequencies higher than 1%; *ABCC1* was defined by 7 blocks that showed 27 haplotypes; *ABCC2* was defined by 3 blocks that showed 12 haplotypes (Figure 2); *ABCC3* was defined by 6 blocks that showed 20 haplotypes; *ABCC4* was defined by 18 blocks that showed 61 haplotypes (Figure 3); *ABCG2* was defined by 3 blocks that showed 13 haplotypes; SLC19A1 2 blocks that showed 10

haplotypes; *SLC22A6-SLC22A8* cluster was defined by 3 blocks that showed 18 haplotypes; *SLCO1A2* 6 blocks that showed 24 haplotypes; *SLCO1B1* 6 blocks that showed 28 haplotypes; *SLCO1B3* was defined by 4 blocks that showed 15 haplotypes.

Significant results of the association analyses comparing the frequency of each haplotype between normal MTX clearers (<0.2μM) and slow clearers (>0.2μM) are shown in Table 3. Significant associations were found for 15 haplotypes (4 in *ABCC2,* 6 in *ABCC4*, 2 in *SLC22A6-SLC22A8*, 1 in *SLCO1B1*, 1 in *SLCO1A2* and 1 in *ABCG2*). After p correction, haplotype GCGGG in *ABCC2* remained statistically significant (p= 0.0360). This haplotype was associated with increased MTX plasma levels and included polymorphisms rs3740066, rs3740065 and rs12826, which were also associated in the single SNP analysis.

Discussion

In the present study, we evaluated the correlation of 384 polymorphisms in 12 key genes involved in the MTX transport pathway with toxicity in a group of 151 children diagnosed with B-ALL and treated according to the standardized LAL/SHOP protocol. Among the strengths of our retrospective study were a good sample size compared with other studies, a homogeneous diagnostic of B-ALL, a standardized treatment protocol followed by all patients, and objective and well-recorded toxicity data.

Recently, Treviño and collaborators carried out a GWAS study in which they found an association between polymorphisms in a MTX transporter, *SLCO1B1*, and MTX toxicity [17]. This association was confirmed by our group [16]. However, to date there have been no in depth studies of polymorphisms in the genes involved in MTX transport and toxicity. In the present study, we have assessed the most important genes involved in MTX transport in order

to detect novel markers that could play a role in the interindividual differences observed in MTX toxicity in pediatric ALL patients.

We have found significant association with MTX toxicity for 21 polymorphisms ($p < 0.05$) from 7 genes. In our previous work, we found an association between rs11045879 in *SLCO1B1* and MTX plasma levels [16]. In the present study, this association was confirmed in a larger population, in both the univariate and multivariate analysis (adjusting for age, sex and MTX dose). However, it should be noted that in this larger study, the most significant SNPs are located in ABCC2 and ABCC4. In fact, from those 21 significant polymorphisms, 6 were located in *ABCC4* and 4 in *ABCC2*, which represents half of the significant SNPs. When we applied the FDR correction, 2 polymorphisms (rs9516519 and rs3740065) in those 2 genes (*ABCC4* and *ABCC2*) remained statistically significant. When haplotypes were analyzed, we found 15 significant and, after p correction, haplotype GCGGG in *ABCC2* remained statistically significant ($p = 0.0360$). All these results point to a relevant role of *ABCC4* and *ABCC2* polymorphisms in MTX toxicity in pediatric ALL.

Although we chose MTX plasma levels due to its direct linkage to MTX and because it is an objective and quantifiable toxicity parameter, we also analyzed other toxicity parameters, such as renal toxicity, hepatic toxicity or mucositis. In this case, the associations were not so clear. This may be due to the reduced number of patients in some of the categories. In fact, 5 SNPs of our subset of significant polymorphisms (rs3740065, rs2619312, rs1678392, rs2622621, rs4149035) were slightly associated with renal toxicity. As renal toxicity is not very frequent (9.4%), with a larger population this association might have been more evident. On the other hand, there could also be a masking effect due to the other drugs that are given that could also be the cause of these toxicities and to the clinical monitoring and increased hydration and

alkalinization in patients with slower MTX elimination. That is why these criteria are not as suitable for use as MTX toxicity parameters as MTX plasma levels.

The SNP rs9516519 in *ABCC4*, which showed the stronger association with MTX plasma levels in our study (p = 3 x 10^{-4}), is located in a putative microRNA mir-367 binding site. The G allele, which is associated with a decrease in toxicity, disrupts the putative binding site. Consequently, the loss of a miRNA binding site and increased *ABCC4* function could explain the consequent decrease in MTX toxicity. From the other 5 SNPs in *ABCC4* with a milder association with MTX toxicity, rs9302061 is an upstream tag-SNP and rs2619312, rs1678392, rs10219913 and rs7317112 are located in putative intronic enhancers and CpG sites. These polymorphisms could result in changes in the methylation pattern, which could in turn affect *ABCC4* expression [20].

As far as we know, none of the associated polymorphisms has previously been included in pharmacogenetic studies. Even in the Affymetrix 500K platform, used in a GWAS study [17], these polymorphisms are not well represented. This could explain the fact that there have been no matches until now. However, previous studies have reported associations between *ABCC4* polymorphisms and the toxicity of various drugs, such as cyclophosphamide [21], bisphosphonate [22] or thiopurines [23]. This gives strength to the idea that polymorphisms in this gene can affect its ability to eliminate its substrates. In the only study that analyzed *ABCC4* polymorphisms in pediatric ALL to date, Ansari et al. studied only 4 *ABCC4* polymorphisms and found an association between the TC genotype in the upstream polymorphism rs868853 and decreased MTX plasma levels [24]. Although, we did not replicate this association, the cumulative evidence contributes to the idea that polymorphisms in *ABCC4* can act as novel predictors of MTX toxicity.

ABCC4 encodes multidrug resistance protein 4 (MRP4), a member of the ATP-binding cassette family of membrane transporters involved in the efflux of endogenous and xenobiotic molecules [25]. Among other substances, MRP4 is able to transport folates and MTX. Due to its ability to pump MTX out, MRP4 has been described as a resistance factor for this drug in *in vitro* experiments with *ABCC4*-transfected cells [26-28]. Consequently, we might expect that higher expression of *ABCC4* could result in increased resistance to MTX and reduced toxicity.

SNP rs3740065 in *ABCC2*, which was associated with MTX toxicity in our study (p=2x10^{-3}), is located in a putative intronic enhancer. The C allele, which increases MTX toxicity, creates a putative cap signal for transcription initiation in intron 29. This polymorphism has been previously associated with gastrointestinal MTX toxicity in rheumatoid arthritis patients [29]. The rs717620 polymorphism is located in the 5'UTR region and is also associated with MTX toxicity in our study. Rau et al, the only authors that have studied the association between 4 *ABCC2* polymorphisms and toxicity in a small population of ALL patients, also found an association between rs717620 polymorphism and MTX clearance [30]. Other studies have also reported association between this polymorphism and toxicity produced by other drugs [31-35]. Regarding the other SNPs in *ABCC2* associated with MTX toxicity in our study, rs3740066 is a synonymous SNP and has already been associated with toxicity by other drugs [34, 36-38]. Furthermore, rs12826 is a downstream regulatory polymorphism that, as far as we know, had not previously been studied.

In our study we also found an association between the GCGGG haplotype (rs3740066; rs3740065; rs12826; rs12762549; rs11190298) in *ABCC2* and MTX toxicity. In this risk

haplotype, the risk alleles of rs3740066, rs3740065 and rs12826, which were previously associated in the individual analysis, were included. Consequently, the involvement of these SNPs in MTX toxicity risk was strengthened by the haplotype association analysis.

ABCC2 encodes multidrug resistance protein 2 (MRP2), another member of the ATP-binding cassette family. MRP2 is primarily expressed in the body at critical sites of uptake and elimination, including liver canalicular membranes and kidney proximal tubules. The apical subcellular localization of Mrp2 at these sites implicates the pump in hepatobiliary and urinary elimination. The substrate selectivity of MRP2 includes a range of anticancer agents, including MTX [39-40]. A dose-dependent role for Mrp2 in the disposition of MTX was suggested by experiments showing increased drug levels in plasma and in the contents of the small intestine when MTX was administered to Mrp2$-/-$ mice at high, but not low, concentrations. This indicates that Mrp2 is involved in the elimination of MTX as a result of its function in liver canaliculi and/or intestine [26].

In conclusion, this study identified two significant polymorphisms and one haplotype in two MTX transporter genes, *ABCC4* and *ABCC2*, which are associated with MTX plasma levels in pediatric ALL patients. We have provided additional insight into the possible genetic modulation of treatment responses in childhood ALL. Further functional analysis and replication in an independent cohort are needed to support the validity of this pilot study. Identification of these polymorphisms in children with ALL could be a useful tool for monitoring patients at risk of low-MTX clearance in order to avoid MTX-related toxicity.

Acknowledgments

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Table 1. Characteristics of the study population.

No. of patients, n	151
Mean age at diagnosis ± SD, years	5.32 ± 3.47
Sex, n (%)	
Female	63 (41.7)
Male	88 (58.3)
Risk group, n (%)	
Standard	54 (38.6)
High	61 (43.6)
Very high	25 (17.8)
Treatment protocol, n (%)	
LAL-SHOP 94/99	58 (38.4)
LAL-SHOP 2005	93 (61.6)
MTX dose in consolidation, n (%)	
$3g/m^2$	66 (43.7)
$5g/m^2$	85 (56.3)
Toxicity during consolidation therapy, n (%)	
Any toxicity	66 (50.8)
Hepatic	36 (28.1)
Vomiting	28 (21.9)
Diarrhea	7 (5.5)
Mucositis	12 (9.4)
Hyperbilirubinemia	12 (9.4)
Renal	12 (9.4)
MTX concentration in plasma, n (%)	
Higher than 0.2uM at 72h	51 (35.7)

SD: standard deviation.

Table 2. Genetic polymorphisms and methotrexate plasma levels.

Gene	Polymorphism	Chromosome position	Location	Genotype	MTX concentration in plasma at 72h					
					< 0.2 µM n (%)	>0.2 µM n (%)	OR (95% CI)	p	p adjusted for age sex and dose	p after FDR correction
ABCC4	rs9516519	13: 95672457	3'UTR	TT	52 (55.3)	42 (44.7)	1.00			
				GT/GG	30 (88.2)	4 (11.8)	0.17 (0.05-0.51)	0.00026	0.00021	0.01878
ABCC2	rs3740065	10: 101605693	intron 29	TT	69 (71.1)	28 (28.9)	1.00			
				TC/CC	12 (40)	18 (60)	3.7 (1.58-8.67)	0.00226	0.00358	0.03395
SLC22A6	rs11231294	11: 62755519	upstream	TT	35 (53.8)	30 (46.2)	1.00			
				TC/CC	47 (75.8)	15 (24.2)	0.37 (0.17-0.80)	0.00917	0.02783	N.S.
SLC22A6	rs4149172	11: 62750858	intron 3	AA	33 (34.0)	64 (66.0)	1.00			
				AG/GG	48 (57.8)	35 (42.2)	0.38 (0.18-0.81)	0.01036	0.02762	N.S.
ABCC4	rs2619312	13: 95723039	intron 26	TT	49 (57.0)	37 (43.0)	1.00			
				TC/CC	34 (79.1)	9 (20.9)	0.35 (0.15-0.82)	0.011367	0.00445	N.S.
SLC19A1	rs1051266	21: 46957794	exon 2	AA/AG	53 (59.6)	36 (40.4)	1.00			
				GG	28 (82.4)	6 (17.6)	0.32 (0.12-0.84)	0.01328	0.03916	N.S.
ABCC4	rs1678392	13: 95722180	intron 25	GG	51 (57.3)	38 (42.7)	1.00			
				GA/AA	31 (79.5)	8 (20.5)	0.35 (0.14-0.84)	0.01330	0.00375	N.S.
ABCG2	rs2725252	4: 89061910	intron 1	GG/GT	58 (60.4)	38 (39.6)	1.00			
				TT	22 (84.6)	4 (15.4)	0.28 (0.09-0.87)	0.015306	N.S.	N.S.
ABCG2	rs2622621	4: 89030920	intron 9	CC	54 (73.0)	20 (27.0)	1.00			
				CG/GG	27 (51.9)	25 (48.1)	2.50 (1.18-5.28)	0.01540	N.S.	N.S.
SLC19A1	rs3788200	21: 46956571	intron 2	GG	28 (82.4)	6 (17.6)	1.00			
				GA/AA	53 (60.2)	35 (39.8)	3.08 (1.16-8.21)	0.01609	0.04449	N.S.
ABCC2	rs3740066	10: 101604207	exon 28	GG	21 (50.0)	21 (50.0)	1.00			

				GA/AA	58 (71.6)	23 (28.4)	0.40 (0.18-0.86)	0.01864	0.00903	N.S.
ABCC2	rs12826	10: 101612320	3'UTR	GG	21 (50.0)	21 (50.0)	1.00			
				GA/AA	58 (71.6)	23 (28.4)	0.40 (0.18-0.86)	0.01864	0.00996	N.S.
ABCC2	rs717620	10: 101542578	5'UTR	GG	39 (54.9)	32 (45.1)	1.00			
				GA/AA	41 (74.5)	14 (25.5)	0.42 (0.19-0.90)	0.02197	N.S.	N.S.
ABCC4	rs7317112	13: 95923523	intron 1	AA/AG	77 (67.5)	37 (32.5)	1.00			
				GG	5 (35.7)	9 (64.3)	3.75 (1.17-11.97)	0.02215	N.S.	N.S.
SLC19A1	rs1131596	21: 46957916	5'UTR	CC/CT	47 (59.5)	32 (40.1)	1.00			
				TT	20 (83.3)	4 (16.7)	0.29 (0.09-0.94)	0.02486	0.04668	N.S.
ABCC4	rs9302061	13: 95966704	Upstream	TT/TC	44 (62.0)	27 (38.0)	1.00			
				CC	11 (91.7)	1 (8.3)	0.15 (0.02-1.21)	0.02660	0.00284	N.S.
SLC22A6	rs10897310	11: 62741176	downstream	TT	25 (52.1)	23 (47.9)	1.00			
				TC/CC	51 (70.8)	21 (29.2)	0.45 (0.21-0.96)	0.03733	N.S.	N.S.
ABCC4	rs10219913	13: 95700935	intron 28	TT	67 (69.1)	30 (30.9)	1.00			
				TC/CC	15 (48.4)	16 (51.6)	2.38 (1.04-5.44)	0.03933	0.03885	N.S.
SLCO1B1	rs4149035	12: 21318265	intron 2	CC	26 (54.2)	22 (45.8)	1.00			
				CT/TT	57 (72.2)	22 (27.8)	0.46 (0.22-0.97)	0.03995	N.S.	N.S.
SLCO1B1	rs11045879	12: 21382619	intron 14	TT/TC	83 (65.9)	43 (34.1)	1.00			
				CC	0 (0.0)	3 (100)	NE	0.04343	0.01129	N.S.
ABCC1	rs2230671	16: 16228242	exon 28	GG	39 (55.7)	31 (44.3)	1.00			
				GA/AA	38 (73.1)	14 (26.9)	0.46 (0.21-1.00)	0.04738	N.S.	N.S.

N.E. Not Estimable. N.S. non significant (p>0.05)

Table 3. Haplotypes and MTX plasma levels.

Gene	SNPs	Haplotype	< 0.2 μM (freq)	>0.2 μM (freq)	p-Value
ABCC2	rs1885301; rs717620; rs2756105; rs4148385; rs2145853	AATAA	0.299	0.163	0.0181
	rs1885301; rs717620; rs2756105; rs4148385; rs2145853	AGTAA	0.206	0.337	0.0235
	rs3740066; rs3740065; rs12826; rs12762549; rs11190298	ATAGG	0.475	0.318	0.0171
	rs3740066; rs3740065; rs12826; rs12762549; rs11190298	GCGGG	0.076	0.193	0.0063*
ABCC4	rs1059751; rs3742106	TA	0.170	0.076	0.0349
	rs10219913; rs1189445	TG	0.319	0.201	0.0428
	rs10219913; rs1189445	CG	0.092	0.207	0.0097
	rs1189457; rs1678392; rs2619312	GAC	0.198	0.087	0.0193
	rs9524849; rs4148455; rs4148454; rs4283094; rs4148446; rs4148436	AGAGGT	0.065	0.007	0.0298
	rs870004; rs7317112	GG	0.118	0.240	0.0109
SLC22A6/SLC22A8	rs10897310; rs3017670; rs6591722; rs4149172; rs11231294	CGTGC	0.319	0.191	0.0268
	rs10792367; rs2276299; rs4149182; rs2187383	CAGC	0.013	0.072	0.0125
SLCO1B1	rs11045813; rs2291073; rs964614; rs11045818; rs11045819; rs4149050;	GGTGCCTTCGAGGTT	0.000	0.034	0.0174

	rs4149056; rs2291075;				
	rs2291076; rs11045821;				
	rs12812279; rs4149058;				
	rs11045823; rs2900476;				
	rs2100996				
SLCO1A2	rs11045994; rs2045940; rs2045939; rs2045938	TGCT	0.000	0.034	0.0189
ABCG2	rs2622621; rs13120400; rs2725261	GTA	0.210	0.322	0.0464

* Statistically significant (p<0.05) after FDR correction.

Figure 1. MTX transport pathway. MTX transporters are encircled.

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Figure 2. Gene Map and LD Plot of *ABCC4* and flanking regions.

White: $r^2 = 0$, shades of grey: $0 < r^2 < 1$, black: $r^2 = 1$. Numbers in squares are D' values. Block definition is based on the Gabriel et al. method. SNPs significantly associated with MTX plasma levels are encircled and significant haplotypes are squared.

Figure 3. Gene Map and LD Plot of *ABCC2* and flanking regions.

White: $r^2 = 0$, shades of grey: $0 < r^2 < 1$, black: $r^2 = 1$. Numbers in squares are D' values. Block

definition is based on the Gabriel et al. method. SNPs significantly associated with MTX plasma

levels are encircled and significant haplotypes are squared.

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