

# Genetic polymorphisms associated with adverse events and elimination of methotrexate in childhood acute lymphoblastic leukemia and malignant lymphoma

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**Abstract** Methotrexate is administered in high doses to treat childhood acute lymphoblastic leukemia and malignant lymphoma. Hepatotoxicity and bone marrow suppression often limit its use, however. The objective of this study was to determine the genetic polymorphisms associated with the hepatotoxicity and elimination of methotrexate. Genetic polymorphisms of glutathione S-transferase (GST) genes including *GSTT1* positive/null, *GSTMI* positive/null, and *GSTP1* A313G, and genes for reduced folate carrier 1 G80A (*RFC1* G80A), methylenetetrahydrofolate reductase C677T (*MTHFR* C677T), and breast cancer resistant protein C421A (*BCRP* C421A) were determined for 26 patients by the polymerase chain reaction (PCR) method or by direct sequencing. A high frequency of hepatotoxicity ( $P = 0.035$ ) was observed for patients with *GSTMI* positive and *RFC1* AA<sup>80</sup>, and serum concentrations of methotrexate 48 h after the start of infusion were higher for patients with the TT<sup>677</sup>

genotype of *MTHFR* ( $P = 0.028$ ). In conclusion, *GSTMI* positive/null and *RFC1* G80A polymorphisms could be predictors for hepatotoxicity, and the *MTHFR* C677T polymorphism is associated with elimination of methotrexate.

**Keywords** Methotrexate · Polymorphism · Acute lymphoblastic leukemia · Hepatotoxicity

## Introduction

Childhood acute lymphoblastic leukemia (ALL) is characterized by uncontrolled proliferation and maturation arrest of lymphoid progenitor cells in the bone marrow. Marked advances in chemotherapy for childhood ALL have resulted in an expected cure rate of 80% (Pui et al. 2003; Aplenc and Lange 2004). Malignant lymphoma (ML) is also treated by chemotherapy with a high cure rate, although outcome is believed to depend on stage, histological type, etc. (Murphy et al. 1989; Anderson et al. 1993).

High-dose treatment with methotrexate often causes hepatotoxicity and bone marrow suppression, however (Schmiegelow et al. 1989; Schmiegelow and Pulczynska 1990), necessitating dose reduction or cessation of treatment, one reason for a relapse of ALL and ML. For appropriate use of methotrexate it would therefore be useful to identify a predictor and/or the mechanism of the adverse effects of methotrexate. In this work we focused on genetic polymorphisms of glutathione S-transferases (GSTs) including *GSTT1* positive/null, *GSTMI* positive/null, and *GSTP1* A313G, reduced folate carrier 1 G80A (*RFC1* G80A), methylenetetrahydrofolate reductase C677T (*MTHFR*

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C677T), and breast cancer resistant protein C421A (*BCRP* C421A).

Information on the genetic polymorphisms associated with the toxicity and efficacy of drugs has recently been accumulating (Donnelly 2004). It has been reported that the idiosyncratic severe hepatotoxicity of troglitazone, which was used to treat type-2 diabetes mellitus, is associated with genetic polymorphisms of *GSTM1* and *GSTT1* (Watanabe et al. 2003). Patients without *GSTM1* and *GSTT1* were more at risk of hepatotoxicity of troglitazone. GSTs are thought to detoxify by conjugation of xenobiotics with glutathione. Patients with the null type of *GSTM1* were at increased risk of hepatotoxicity of antituberculosis drugs (Roy et al. 2001). Null types of *GSTT1* and *GSTM1* have also been associated with an increase in the frequency of hepatotoxicity of tacrine, an anticholinesterase inhibitor (Simon et al. 2000). In contrast, lack of *GSTP1* has been related to the toxicity of acetaminophen in mice (Henderson et al. 2000). The genetic polymorphism at A313G of *GSTP1*, resulting in amino acid substitution, has been reported to be associated with enzymatic activity (Watson et al. 1998). It is generally believed that polymorphisms of GSTs could be factors affecting drug-induced toxicity.

Reduced folate carrier 1, the function of which is to transport reduced folate, is expressed ubiquitously throughout the body. Methotrexate is a substrate for RFC1 and its distribution to tissue is thought to be mediated by RFC1. RFC1 expression levels (Levy et al. 2003) and genetic polymorphisms of *RFC1* G80A (Laverdiere et al. 2002) have been reported to be associated with ALL outcome, and the latter has also been associated with plasma methotrexate levels in patients with ALL. Here we have evaluated the association of the polymorphism G80A in *RFC1* with hepatotoxicity after high-dose treatment with methotrexate.

*MTHFR* is a key enzyme in the metabolism of folate. It has been reported that genetic polymorphism at C677T of *MTHFR* is associated with the toxicity of methotrexate in patients with bone marrow transplants (Ulrich et al. 2001), rheumatoid arthritis (Urano et al. 2002), and ovarian cancer (Toffoli et al. 2003).

Breast cancer resistant protein (*BCRP*, *ABCG2*) is an ABC transporter with an ATP binding cassette. It has been reported to remove substrates from cells to confer resistance to anticancer agents including methotrexate (Doyle and Ross 2003; Abbott 2003). It is also believed that *BCRP* in the bile canalicular membrane of the liver is a key determinant of the disposition to secrete substrates of a drug into bile. Genetic polymorphism at C431A of *BCRP* has been reported to be

associated with expression and function in vitro and in vivo (Imai et al. 2002; Mizuarai et al. 2004; Kobayashi et al. 2005).

Here we have investigated the effects of genetic polymorphisms of positive/null *GSTT1* and positive/null *GSTM1*, and *GSTP1* A313G, *RFC1* G80A, *MTHFR* C677T, and *BCRP* C421A on the hepatotoxicity and elimination of methotrexate in patients with childhood ALL or ML after high-dose treatment with the drug.

## Materials and methods

### Subjects

Blood samples were obtained from 26 patients with ALL ( $n = 20$ ) or ML ( $n = 6$ ) who were treated at Kobe University Hospital, Japan ( $n = 26$ , 16 males and 10 females). The patients were administered a high dose of methotrexate ( $3 \text{ g m}^{-2}$ ) followed by leucovorin rescue treatment in the event of complete remission. If the methotrexate concentration 48 h after starting the administration was higher than  $1 \mu\text{mol L}^{-1}$ , additional leucovorin rescue was performed. The age range was 10 months to 15 years, average  $6.7 \pm 4.7$ . Informed consent was obtained from all the patients or their guardians before the study. The study procedure was approved by the institutional review board of Kobe University Hospital, Kobe University, Japan.

### Genotyping for *GSTT1*, *GSTM1*, *GSTP1*, *RFC1*, *MTHFR*, and *BCRP*

Genomic DNA was extracted from blood samples by use of a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and from buccal mucosa using a Smi-test EX R&D DNA extraction kit (Genome Science Laboratories, Fukushima, Japan), according to the manufacturer's directions. Table 1 lists the primer pairs used in this study. Each PCR was performed with 30 ng DNA, ExTaq Buffer,  $0.5 \text{ mmol L}^{-1}$  dNTP mixture, 1.25 U ExTaq polymerase (Takara Bio, Shiga, Japan), and  $0.4 \mu\text{mol L}^{-1}$  each of the forward and reverse primers in a  $25 \mu\text{L}$  reaction volume. PCR cycling was programmed as shown in Table 1. For *GSTT1* and *GSTM1*, PCR products were electrophoresed in 3% agarose gel (Takara Bio) and positive/null genotypes were determined by the position of bands. For determination of genotypes of *GSTP1*, *RFC1*, *MTHFR*, and *BCRP*, PCR products were used for direct sequencing. They were treated with Applied Biosystems Version 1.1 BigDye terminator chemistry. The reaction mix-

**Table 1** Primers and polymerase chain reaction (PCR) conditions used to determine genetic polymorphisms

	Primer lists	Accession no.	PCR conditions
<i>RFC1</i> G80A	Forward	NT_011515	95°C for 15 s, 61°C for 30 s and 72°C for 15 s) ( 35 cycles and 72°C for 5 min
	Reverse	NT_011515	
<i>GSTM1</i> positive/null	Forward	NT_019273	94°C for 30 s, 58°C for 30 s and 72°C for 30 s) ( 35 cycles and 72°C for 5 min
	Reverse	NT_019273	
<i>GSTT1</i> positive/null	Forward	NT_011520	94°C for 30 s, 58°C for 30 s and 72°C for 30 s) ( 35 cycles and 72°C for 5 min
	Reverse	NT_011520	
<i>GSTP1</i> A313G	Forward	NT_033903	94°C for 30 s, 58°C for 30 s and 72°C for 30 s) ( 35 cycles and 72°C for 5 min
	Reverse	NT_033903	
<i>MTHFR</i> C677T	Forward	NT_021937	95°C for 30 s and 72°C for 1 min) ( 35 cycles and 72°C for 7 min
	Reverse	NT_021937	
<i>BCRP</i> C421A	Forward	NT_016354	94°C for 30 s, 58°C for 30 sec and 72°C for 30 s) ( 35 cycles and 72°C for 5 min
	Reverse	NT_016354	

tures were purified with a CentriSep column (Prometec Separations, Adelphia, NJ, USA). The products were electrophoresed for sequencing on an Applied Biosystems 3100 Avant Genetic Analyzer (Applied Biosystems). All sequence chromatograms were read in both the sense and antisense directions.

### Assessment of hepatotoxicity

Laboratory test values were monitored routinely and alanine aminotransferase (ALT) activity was used to assess hepatotoxicity. Toxicity was graded on the basis of National Cancer Institute Common Toxicity Criteria (Ver 2.0) using maximum ALT levels within one week of administration of methotrexate. Relationships between genetic polymorphisms and hepatotoxicity of methotrexate were investigated using the data for 18 patients whose ALT levels were within the normal range ( $\geq 34$ ) before methotrexate dosing.

### Serum concentration of methotrexate

Serum concentrations of methotrexate were measured with a TDx analyzer (Abbott Japan, Tokyo, Japan).

### Statistical analysis

Statistical analysis of the association of genetic polymorphisms with hepatotoxicity or serum concentrations were performed with Fisher's extended exact test.

## Results

HD-MTX resulted in grade 2/3 hepatotoxicity in five of eighteen patients, three of eleven for males and two of seven for females. The mean ages of patients without and with MTX-induced hepatotoxicity were  $5.5 \pm 4.5$  and  $8.0 \pm 4.8$ , respectively. These results suggest that gender and age affect susceptibility to MTX-induced hepatotoxicity, although larger studies are needed to confirm this.

Table 2 shows frequencies for the genotypes of *GSTM1*, *GSTT1*, *GSTP1*, *RFC1*, *MTHFR*, and *BCRP*. Table 3 shows the association of genetic polymorphisms of these genes with hepatotoxicity. No significant association was found on the basis of the genotypes of one gene. Table 4 shows the effect of *RFC1* genotypes on hepatotoxicity in patients carrying *GSTM1*, indicating that patients with AA<sup>80</sup> are likely to suffer from hepatotoxicity ( $P < 0.05$ ; Fisher's extended exact test).

**Table 2** Frequencies of genetic polymorphisms in pediatric patients with ALL or ML

Gene	Genotype	Number (%)
<i>GSTT1</i>	Positive	11 (42)
	Null	15 (58)
<i>GSTM1</i>	Positive	20 (77)
	Null	6 (23)
<i>GSTP1</i> (A313G)	AA	16 (62)
	AG	7 (27)
	GG	3 (11)
<i>RFC1</i> (G80A)	GG	4 (15)
	GA	14 (54)
	AA	8 (31)
<i>MTHFR</i> (C677T)	CC	6 (23)
	CT	11 (42)
	TT	9 (35)

**Table 3** Hepatotoxicity and genetic polymorphisms of *GSTM1*, *GSTT1*, *GSTP1*, *RFC1*, *MTHFR*, and *BCRP*

		Hepatotoxicity		<i>P</i>
		Grade 0/1	Grade 2/3	
<i>GSTM1</i>	Positive	8	5	0.249
	Null	5	0	
<i>GSTT1</i>	Positive	6	1	0.596
	Null	7	4	
<i>GSTP1</i> (A313)	AA	7	5	0.114
	AG/GG	6	0	
	A allele	19	10	
<i>RFC1</i> (G80A)	G allele	7	0	0.155
	GG/GA	10	2	
	AA	4	2	
<i>MTHFR</i> (C677T)	G allele	12	2	0.441
	A allele	16	6	
	CC/CT	10	2	
<i>BCRP</i> (C421A)	TT	3	3	0.268
	C allele	16	2	
	T allele	10	8	
	CC	5	4	0.294
	CA	8	1	
	C allele	18	9	
	A allele	8	1	0.392

**Table 4** Hepatotoxicity and *RFC1* polymorphism in *GSTM1*-positive patients

		Hepatotoxicity		<i>P</i>
		Grade 0/1	Grade 2/3	
<i>RFC1</i> (G80A)	GG/GA	8	2	0.035
	AA	0	3	
	G allele	10	2	
	A allele	6	8	

To investigate the genetic factors affecting elimination of methotrexate, association of genetic polymorphisms with the serum concentration of methotrexate 48 h after intravenous infusion at a dose of 3 g m<sup>-2</sup> was evaluated. Although there was no association for *GSTM1*, *GSTT1*, *GSTP1*, *RFC1*, or *BCRP*, significantly more of patients whose concentration remained >1 μmol L<sup>-1</sup> at 48 h had the TT<sup>677</sup> genotype (*P* < 0.05; Fisher’s exact test) (Table 5).

**Discussion**

Acute lymphoblastic leukemia is the most common malignancy in children. Recent advances in chemotherapy have resulted in a cure rate of approximately 80% (Pui et al. 2003; Aplenc and Lange 2004). ML is also treated by chemotherapy, with a high cure rate (Murphy et al. 1989; Anderson et al. 1993). Treatment is, however, often limited by severe toxicity, including hepatotoxicity and myeloid suppression (Schmiegelow et al. 1989; Schmiegelow and Pulczynska 1990). It is therefore important to predict the occurrence of adverse events. Here, we investigated genetic polymorphisms to predict the hepatotoxicity of methotrexate.

We showed that the genetic polymorphism G80A in *RFC1* was associated with hepatotoxicity in patients carrying *GSTM1*. These results suggested that *RFC1* is a key enzyme determining the residual amount of methotrexate in the liver. It is thought that the residual

**Table 5** MTX concentration at 48 h and genetic polymorphisms

		MTX concentration		<i>P</i>
		≤1 μmol L <sup>-1</sup>	>1 μmol L <sup>-1</sup>	
<i>GSTM1</i>	Positive	13	7	0.146
	Null	6	0	
<i>GSTT1</i>	Positive	7	4	0.407
	Null	12	3	
<i>GSTP1</i> (A313G)	AA	13	3	0.369
	AG/GG	6	4	
	A allele	30	9	
<i>RFC1</i> (G80A)	G allele	8	5	0.300
	GG/GA	12	6	
	AA	7	1	
<i>MTHFR</i> (C677T)	G allele	14	8	0.220
	A allele	24	6	
	CC/CT	15	2	
	TT	4	5	0.028
	C allele	21	2	
	T allele	17	12	
<i>BCRP</i> (C421A)	CC	11	2	0.378
	CA	8	5	
	C allele	30	9	
	A allele	8	5	

amount of methotrexate should be higher in patients with an A allele at position 80 in *RFC1*, which might reduce the activity of RFC1, resulting in a greater risk of methotrexate-induced hepatotoxicity. GSTs are believed to be responsible for reducing the toxicity of xenobiotics. Patients with null types of *GSTM1* and/or *GSTT1* are reportedly at higher risk of hepatotoxicity of troglitazone (Watanabe et al. 2003), antituberculosis drugs (Roy et al. 2001), and tacrine, an anticholinesterase inhibitor (Simon et al. 2000), suggesting that a lack of or reduction of GSTs increases the risk of drug induced-hepatotoxicity. Our current results also suggest that carrying *GSTM1* results in a greater risk of methotrexate-induced hepatotoxicity. No reasonable explanation of this is yet available, but *GSTP* has been reported to increase the risk of hepatotoxicity from acetaminophen by inhibition of Jun N-terminal kinase (Adler et al. 1999). In our study hepatotoxicity tended to be more frequent in patients with the T allele at 677, although this was not statistically significant. Toffoli et al. (2003) reported association of *MTHFR* C677T with severe toxicity of MTX, including neutropenia, anemia, and mucositis in ovarian cancer patients, but they did not report data for hepatotoxicity. Chiusolo et al. (2002) have also shown that the *MTHFR* C677T genotype is associated with hematopoietic and hepatic toxicity. On the basis of their results and ours, the *MTHFR* genotype might lead to susceptibility to MTX hepatotoxicity, although more studies should be conducted.

We also found a significant association of C677T in *MTHFR* with the residual concentration of methotrexate at 48 h. Because elimination of methotrexate at the terminal phase is thought to be associated with the toxicity of methotrexate (Stoller et al. 1977), additional leucovorin rescue is usually performed when the concentration of methotrexate was higher than  $1 \mu\text{mol L}^{-1}$ . Our current findings suggest that genotyping of *MTHFR* could enable us to predict the need for additional leucovorin rescue, although further clinical study is necessary. *MTHFR* is not thought to be responsible for the metabolism or transport of methotrexate. The mechanism has not been clarified, but Toffoli et al. (2003) showed that plasma concentrations of homocysteine after methotrexate dosing were higher in patients with the TT genotype at *MTHFR* C677T. Homocysteine concentration might be useful for discussion of the association of this genotype with MTX concentration.

Laverdière et al. (2002) reported higher concentrations of MTX in patients with AA at G80A of *RFC1*, leading poor prognosis. We did not find association of this genotype with MTX concentration. The reason for

the discrepancy is unclear, although it is possibly because of sample size.

In conclusion, we have shown that genetic polymorphisms of *RFC1* G80A and *GSTM1* positive/null are risk factors for methotrexate hepatotoxicity and polymorphism of *MTHFR* C677T is associated with the serum concentration of methotrexate. These results are useful for treatment of ALL by use of methotrexate.

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